THE ROLE OF MULTIPLE ENZYME ACTIVATION IN METABOLIC FLUX CONTROL

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INTRODUCTION
For many decades the predominant view about metabolic control has been that:

1. a metabolic pathway is controlled by a rate-limiting step, generally situated near the start of the pathway;
2. the rate-limiting step is an enzyme catalyzing a non-equilibrium reaction, its maximal activity is low compared to the activity of the other enzymes in the pathway, and it is subject to regulation by metabolites other than its substrates;
3. feedback inhibition is a common regulatory feature of rate-limiting steps;
4. large changes in metabolic flux are brought about by effectors (often allosteric) or covalent modification acting on rate-limiting steps; and
5. pathway steps that are not rate-limiting, often considered to be ‘near-equilibrium’, are of sufficiently high activity to automatically deliver the required flux by responding to the changes in pathway metabolites generated by change in activity of the rate-limiting step.

The concept of the rate-limiting step was originally proposed by Blackman (1), and has had the support of many influential figures in biochemistry. For example, Krebs endorsed it with his concept of ‘pace-maker’ enzymes (2), which he saw as the target sites for hormone and drug action on metabolism. With the discovery of feedback inhibition of enzymes (3,4) and cooperativity of enzyme action (5), a model evolved of pathway control exerted by rate limiting enzymes which could be identified by the criteria defined above. However, since the early 1970s, an alternative view of metabolic control has been gaining increased support, namely that control can be distributed amongst a number of enzymes in a pathway, and that the degree of control exerted by each enzyme can be quantified. Furthermore, this view recognizes that the distribution of con-
control between pathway enzymes can vary, depending on the physiological state. The method of study is also different from the traditional enzymology entailed in the study of putative rate-limiting steps. First, acknowledging that several steps in a pathway can potentially contribute to flux control requires that the properties of pathways in their entirety, rather than the properties of individual steps, are the primary focus of study. Second, because of the number and complexity of interactions that have to be considered when studying even a simple pathway, interpretation and analysis are inevitably quantitative and often heavily mathematical.

There are two main approaches to the study of control of metabolic systems. Savageau developed a comprehensive theory for the study of many aspects of metabolism and its control, which he called 'Biochemical Systems Theory' (BST) (6). However, it is mathematically complex and contains some non-intuitive aspects that make its application difficult. Consequently, it has been used by only a small number of workers. The other major systems approach, 'Metabolic Control Analysis' (MCA), has evolved from ideas developed simultaneously by two different groups (7–9). Its scope is narrower than that of BST, being restricted to the study of control and regulation, without the capacity for simulation of metabolism that is an integral part of BST. It has developed over more than two decades, with important contributions being made by many workers. As a result, it is less homogeneous than BST. It is, however, mathematically much more simple than BST, and has consequently become the major systems approach used in the study of metabolic control. The ideas we present here are based on the formal theory of MCA, though couched predominantly in non-mathematical form. The important features of MCA are that:

1. The control exerted by each and every enzyme in a pathway over the pathway flux can be quantified as a flux control coefficient. If a small change of x% in an enzyme’s activity leads to a y% change in flux, the flux control coefficient is $x/y$. Similar coefficients define the control exerted by each enzyme over the concentrations of metabolic intermediates, and over the temporal behaviour of pathways.

2. Control over a pathway flux can be potentially distributed over all steps in the pathway, characterized by the values of coefficients that map onto a scale where 0 represents an enzyme with no control and 1 represents a rate-limiting enzyme. Control over a pathway flux can be exerted by steps outside the pathway, and this control can be negative. e.g., enzymes of nucleotide salvage can have negative flux control coefficients over the flux of de novo nucleotide synthesis, as their activation could decrease the de novo synthesis flux. The sum of flux control coefficients of all enzymes over any flux is 1.
3. The distribution of control can vary with physiological state.
4. Control coefficients can be measured or calculated using a number of experimental and theoretical approaches (reviewed in (10, 11)).

There has, at least until recently, been relatively little useful communication between the two camps studying metabolic control. On the one hand, the terminology and mathematics employed by the system-oriented camp has not been readily understood by many biochemists. Furthermore, although proponents of the systems approach have urged the replacement of the concept of control by rate-limiting steps with that of some form of control distributed over multiple steps, there have been few concrete suggestions as to how this distributed control may be implemented physiologically. On the other hand, there has been an element of resistance to the abandonment (or at least modification) of the concept of control by rate-limiting steps, that appears to be unreasonable in the face of mounting evidence of its shortcomings.

Our intentions in this paper are three-fold, but interrelated. First, we present several lines of data that, we propose, are powerful evidence against the control of metabolic flux by a single rate-limiting step at the start of each pathway. This argument applies with particular force to relatively large flux changes (either chronic or acute) in response to external stimuli, rather than to minor adjustments around a central state. Second, we propose models of metabolic control that are consistent with the observations we have described, and discuss the implications of these models, both for the understanding and further study of the control of metabolism. Third, we describe how a systems approach to the study of metabolic control can be used to interpret the changes occurring in metabolic pathways responding to external stimuli, particularly in those instances where the response involves the simultaneous change in activity of multiple enzymes.

There are three types of experiments that we shall examine in pursuit of these conclusions, each of which is discussed at length in a following section:

- Long-term responses of metabolic pathways to external stimuli, such as hormones or mitogens, frequently entail activation of several enzymes in addition to the postulated rate-limiting enzyme. Similarly, tumor cells often overexpress multiple enzymes in a pathway when compared to the parental cell. These observations are inconsistent with the cellular requirement for protein economy if flux control could be satisfactorily achieved by activation of a single step in any given pathway.
- Genetic engineering of microorganisms and plants to overexpress postulated rate-limiting enzymes has usually resulted in little increase in the
relevant pathway flux, whereas flux increase has been achieved by over-expression of multiple pathway enzymes.

- The remarkable homeostasis of metabolic intermediates, shown in several pathways undergoing large flux changes in response to various stimuli, is difficult to explain satisfactorily by the activation of a rate-limiting step near the start of a pathway, as this would tend to increase concentrations of downstream metabolites. Furthermore, the homeostatic behavior of a pathway is dependent upon several enzymes in the pathway, their interactions with cofactors such as ATP and ADP, and the utilization of pathway products, including ATP. These facts imply the involvement of multiple steps in flux control, including some outside the pathway.

**Expression of Multiple Pathway Enzymes in Response to Stimuli**

Of the lines of evidence we discuss here, this—the increase in activity of multiple pathway enzymes in response to a long-term stimulus—is the most widely documented, and has been observed over many years. In Table 1 we present a number of examples covering pathways of central metabolism, as well as an example from a signal transduction pathway. We have used the term 'stimulus' in a very loose sense, to convey the concept of a metabolic state identifiably different from a basal state, in which the pathway flux is increased compared to that basal state. Thus, as well as effects such as electrical stimulation of muscle and antigenic stimulation of lymphatic cells, we have included genetic effects that cause phenotypic differences from the parent tissue. For example, the mouse obese (ob) gene, seemingly specifically expressed in adipose tissue, appears to code for a secreted protein with signalling functions. Recessive mutations in this gene cause profound obesity and type II diabetes (12). Known metabolic alterations include the increased expression of many enzymes of carbohydrate and fat metabolism in liver and adipose tissue (13). Similarly, hepatomas, which display increased rates of growth compared to hepatocytes, with consequent need for greater nucleotide and nucleic acid synthesis, have elevated levels of many enzymes, including many involved in nucleotide and nucleic acid synthesis (14) and signal transduction (15). The examples are by no means exhaustive, and have been chosen primarily to illustrate the many areas of metabolism in which the phenomenon of multiple activation can be observed. Sreere (16) has also cited a number of studies from several areas of metabolism, including the TCA cycle, electron transport and fatty acid synthesis, that have reported
expression of enzymes in relatively constant proportion in response to various external stimuli.

Multiple enzyme activation can entail the activation of putative rate-limiting steps in a number of linked pathways, and this is a well-known phenomenon. For example, stimulation of triacylglycerol synthesis by insulin in adipose tissue involves activation of glucose transport, pyruvate dehydrogenase (E.C. 1.2.4.1), acetyl-CoA carboxylase (E.C. 6.3.4.15), ATP-citrate lyase (E.C. 4.1.3.8) and glycerol phosphate acyl transferase (E.C. 2.3.1.15) (23). Each of these steps would, essentially, be considered to be in a different 'pathway', that can be independently regulated, depending upon the particular physiological state, and the steps listed have properties of rate-limiting enzymes as listed in the introduction. However, the examples in Table 1 also illustrate a different phenomenon: the activation of multiple steps within a pathway, including 'near-equilibrium' enzymes (see point 5 in the Introduction) that, according to traditional theory of metabolic control, need not be activated to achieve a flux increase. Specific instances listed in Table 1 include PGI, TPI and LDH in activated lymphocytes or thymocytes, though in all of the studies cited in the Table there are examples of enzymes that are normally not considered to have any control over flux, whose activities are increased in response to stimulation. In the case of enzymes of very high activity, such as PGI and TPI, it is likely that they exert very little flux control. It is, nevertheless, possible to postulate reasons for their increased expression.

We have previously proposed (24) that one reason for the (near-) simultaneous activation of several steps may be to maintain metabolite homeostasis. Activation of every step in a pathway by an equal proportion will generate the best homeostasis. Thus, an increase in the activity of every enzyme by x% would increase the pathway flux by x%, whilst intermediate metabolite levels would be unchanged. Consequently, if the maintenance of pathway metabolite homeostasis is important, even the activation of already very active enzymes could be expected. However, in several pathways, it has been observed that there is an inverse logarithmic relationship between the unstimulated activity and the increase in activity upon stimulation, particularly in pathways in which there is a range of starting activities of several orders of magnitude. This is particularly evident in the activation in hepatomas in pathways leading to DNA synthesis, and in the growth-factor mediated activation of phospholipase C (15), but has also been observed in a number of other pathways (D. A. Fell, unpublished work). This might reflect a balance between the conflicting needs for maintaining metabolite homeostasis on the one hand and the restriction imposed by cellular protein economy on the other.

A second reason for increasing the activity of very rapid enzymes may be that, even though they exert very little flux control in the basal state,
their activation may become important in achieving large flux changes. We shall return to this point in the following section, in relation to the activation of tryptophan synthesis in yeast.

In addition to these examples from eukaryotic metabolism are the well-known examples of bacterial operons, in which several enzymes are under the control of a single promoter, and are consequently always expressed in equal amounts in response to a relevant signal. Here, too, there is evidence for overexpression of enzymes with low flux control. Dykhuizen et al. (25) estimated the control on β-galactosidase (E.C. 3.2.1.23) to be more than an order of magnitude less than that of lactose permease, for the use of lactose by Escherichia coli, even though both are expressed nearly equally in response to lactose because of their presence on the lac operon.

EFFECTS OF GENETIC ENGINEERING OF METABOLIC PATHWAYS

Some of the most direct evidence for the importance of multiple enzyme activation in metabolic control has come from attempts to genetically modify organisms to increase fluxes through specific pathways. Glycolysis has been a favored target for modification because of the widely-held belief that it is controlled almost exclusively by PFK, so that overexpression of PFK should generate sizeable flux changes. However, overexpression of PFK in yeast has been reported to have little (26), or no (27, 28) effect on glycolysis. Similarly in potato tuber no increase in aerobic respiration was achieved by overexpression of PFK up to 36-fold (29).

Although these results were surprising to many, they are totally in accord with predictions made over 20 years ago by Kacser and Burns in their original paper on MCA (7, 9), in which they proved that feedback inhibition serves to move flux control from the inhibited enzyme to those responsible for the further metabolism of the inhibiting metabolite. In plants and many bacteria, the main inhibitor of PFK is PEP, so a degree of control is likely to reside in PK and/or in the aerobic metabolism of pyruvate (30). In mammals, citrate and ATP are important inhibitors, so control may reside in citrate and/or ATP metabolism. Although it is possible to describe, qualitatively, likely features of flux control for a given pathway structure, it is not feasible either to quantify that control without a formal systems approach, or to consider systems with complex and interlocking allosteric effectors.

One report of the successful achievement of flux increase has been in the pathway of tryptophan synthesis of yeast, a five-step pathway whose substrate is chorismate (31). The first enzyme in this pathway, anthranilate synthase (E.C. 4.1.3.27), is subject to powerful feedback inhibition by tryptophan (32), and would consequently have been considered to be a
good candidate for the regulatory step of the pathway. Overexpression of any of the enzymes singly, even up to 50-fold, had a negligible effect on the flux. To achieve a significant increase in tryptophan synthesis required considerable up-regulation of either four or all five steps. Furthermore, the selection of the four steps was critical: overexpression of all except phosphoribosyl anthranilate isomerase (E.C. 5.3.1.24) increased the flux 7-fold, whereas overexpression of all except tryptophan synthase (E.C. 4.1.2.20) only doubled the flux. Overexpression of all five steps achieved an 8-fold flux increase although the individual enzymes were expressed between 20- and 27-fold. Thus the most important results are that: (i) the flux increase obtained when all enzymes are changed together is far greater than the product of increases caused by changing each enzyme individually, and; (ii) the proportional increase in flux is far less than the lowest increase in enzyme activity, because nothing has been done to increase the supply of chorismate and other substrates used by the pathway, or to increase the removal of products.

In a preliminary set of experiments, the control exerted by each step over the synthesis flux had been calculated (31), and four of the steps had been shown to have negligible flux control coefficients, including phosphoribosyl anthranilate isomerase and tryptophan synthase. Nevertheless, the degree of expression of these enzymes with low flux control played a critical role in the increase of tryptophan synthetic rate, as shown by the different results of the up-modulation experiments with phosphoribosyl anthranilate isomerase and tryptophan synthase.

Another feature of significance in the experiments on tryptophan synthesis described above was that the plasmid introduced into several of the engineered strains carried a mutant allele of anthranilate synthase, catalytically normally active, but feedback-resistant. The introduction of this allele had no significant effect on the flux to tryptophan. This result is contrary to the expectation of the classical view, in which feedback inhibition of rate-limiting enzymes is an integral feature of flux control. However, it is consistent with the predictions of Hofmeyr and Cornish-Bowden (33). They used MCA and computer simulation of a hypothetical biochemical pathway, and showed that the effect of inhibition on stabilizing the concentrations of metabolites within the feedback loop is quantitatively greater than the effect on flux. Furthermore, this homeostatic effect is greatest when the inhibition is co-operative, as is frequently observed in steps subject to feedback inhibition. Coupled with the observation of Kacser and Burns (7, 9) already mentioned, that feedback inhibition actually reduces the flux control of the inhibited enzyme this is further theoretical evidence that the assumed role of feedback inhibition in flux control by rate-limiting enzymes is incorrect.
<table>
<thead>
<tr>
<th>Pathway</th>
<th>Tissue</th>
<th>Stimulus</th>
<th>Flux increase (if determined)*</th>
<th>Enzymes activated</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycolysis</td>
<td>rat thymocyte</td>
<td>con-A</td>
<td>20-fold‡</td>
<td>HK, PGK, aldolase, PGI, PK, LDH§</td>
<td>(17)</td>
</tr>
<tr>
<td></td>
<td>human lymphocyte</td>
<td>phytohaemagg</td>
<td></td>
<td>PGI, TPI, LDH§</td>
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</tr>
<tr>
<td></td>
<td>human lymphocyte</td>
<td>lutinin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>phorbol</td>
<td>8-fold</td>
<td>HK, aldolase, PFK, GAPDH, enolase, PK, LDH§</td>
<td>(19)</td>
</tr>
<tr>
<td></td>
<td>mouse lung macrophage L8 skeletal muscle cells</td>
<td>myristate acetate and con-A</td>
<td></td>
<td>all</td>
<td>(20)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hypoxia</td>
<td></td>
<td>all except HK</td>
<td>(20)</td>
</tr>
<tr>
<td></td>
<td>rabbit fast-twitch skeletal muscle (tibialis anterior)</td>
<td>electrical stimulation</td>
<td></td>
<td>three TCA cycle enzymes, three aminotransferases and four other enzymes of oxidative metabolism§</td>
<td>(21)</td>
</tr>
<tr>
<td>Oxidative energy metabolism</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urea cycle</td>
<td>rat liver</td>
<td>dietary protein (15-60% casein)</td>
<td>4-fold</td>
<td>all four enzymes of urea cycle; CPS; G6PDH; asp- and ala transaminases many enzymes</td>
<td>(22)</td>
</tr>
<tr>
<td>Nucleotide and nucleic acid synthesis</td>
<td>rat liver/hepatoma</td>
<td>proliferation rate</td>
<td></td>
<td></td>
<td>(14, 15)</td>
</tr>
<tr>
<td>Triglyceride synthesis</td>
<td>mouse liver and adipose Obese mutation tissue</td>
<td>3-fold</td>
<td>many enzymes of carbohydrate and fatty acid metabolism in liver; GK and G6PDH in adipose tissue phospholipase C, PI kinase, PIP kinase §</td>
<td></td>
<td></td>
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<tr>
<td>Inositol polyphosphate metabolism</td>
<td>rat liver/hepatoma proliferation rate</td>
<td></td>
<td>(15)</td>
<td></td>
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</tbody>
</table>
HOMEOSTASIS OF PATHWAY METABOLITES

For a change in activity of any enzyme to cause a change in a metabolic flux, there has to be a consequent change in concentration of one or more pathway metabolites. This is the mechanism by which the signal (change in enzyme activity) is propagated to cause a metabolic effect (change in flux). A method frequently used to locate the position of a rate-limiting step has been to search for a crossover, at which the product(s) of the putative rate-limiting enzyme increase, whilst its substrate(s) decrease when the flux increases, and vice versa for a flux decrease (although it is not applicable in all situations (34, 35)). Thus, making the assumption that glycolytic flux is controlled by PFK, the signals from PFK activation that could generate a flux increase are: increases in fructose 1,6-bisphosphate (F1,6BP) and ADP, and a decrease in ATP (see Fig. 1). Increase in F1,6BP would directly cause an increase in the flux through aldolase, which would then be transmitted to the remainder of the glycolytic chain. In a number of tissues, this would be supplemented by feedforward activation of PK by F1,6BP. Changes in ATP and ADP would directly increase the rates of PGK and PK. The rates of GAPDH, TPI and aldolase would be increased by the consequent decrease in product inhibition as the rate of consumption of 1,3BPG increases. Flux through enolase and phosphoglycerate mutase (PGM, E.C. 5.4.2.1) would increase by a combination of increased substrate concentration and decreased product inhibition. During the activation of glycolysis, an increase in F1,6BP can be observed in many tissues, and there is little doubt that control of PFK activity plays a part in glycolytic flux control. However, it is not necessarily the only enzyme involved, as we have discussed in the preceding sections.

One means of gaining insight into the enzymatic changes occurring during physiological flux change is to study the behavior of the pathway metabolites. Figure 2 shows examples of metabolite concentration changes following a stimulation of glycolysis in a number of different tissues. The relative concentrations of the glycolytic metabolites in the stimulated state

![Diagram of glycolysis reactions](image)

FIG. 1. Reactions of glycolysis from hexose monophosphates to pyruvate. ATPase represents all cellular reactions hydrolyzing ATP.
FIG. 2. Changes in steady-state concentrations of glycolytic intermediates following stimulation of glycolysis. The four sets of data (left to right) are from: 1. horizontal bars: *Saccharomyces cerevisiae* transferred from aerobic to anaerobic conditions (38); 2. vertical bars: rat thymocytes stimulated with phytohaemagglutinin (39); 3. hatched shading, chick embryo cells transformed with Rous sarcoma virus (40); 4. no shading: blowfly flight muscle, undergoing a rest to flight transition (41). Not all intermediates were measured in each study: missing bars correspond to this missing data. In (39), triose phosphates were measured as a single entity, and are depicted (set 2) as DHAP.
compared to the unstimulated state are plotted in Fig. 2. To extract useful information concerning flux control from the metabolite changes, it is necessary to take into account the magnitudes of the flux increases upon stimulation, because of the relationship between enzyme activities, metabolite concentrations and flux described above. The relationships can be expressed as flux/metabolite ‘co-responses’, that are simply the ratio of the fractional change in flux divided by the fractional change in metabolite concentration:

\[ O_{\text{flux,metabolite}} = \frac{\Delta \text{flux}/\text{flux}}{\Delta \text{metabolite}/\text{metabolite}} \]

Thus, for each metabolite in the glycolytic pathway there is a corresponding flux-metabolite co-response. We have drawn the concept from the work of Hofmeyr and colleagues (36), who defined a ‘co-response coefficient’ in terms of the changes brought about by the activation of a single enzyme:

\[ O_{\text{flux,metabolite}}^{\text{enzyme}} = \frac{\partial \text{flux}/\text{flux}}{\partial \text{metabolite}/\text{metabolite}} \]

where enzyme is the enzyme whose change in activity has caused the corresponding changes in the metabolite and flux. The co-response coefficient is based on the formal theory of MCA, so is defined in terms of a partial derivative. We have used the less precise symbol, \( \Delta \), in co-response ratios to signify a measurable physiological response, rather than the \( \partial \) required in calculus. Similarly, in view of the potential role of multiple enzyme activation in physiological flux control, the co-response ratio cannot be assigned to a particular enzyme responsible for metabolic changes. The combination of co-response coefficients and ratios provides a powerful tool for quantitatively analyzing the response of pathways to stimuli: knowing the thermodynamics and kinetics of a pathway, co-response coefficients can be calculated for the activation of one or more pathway steps, and compared to observed co-response ratio(s). We have previously used this technique to study the co-responses in linear sequences of enzymes (37) and in a model of glycolysis similar to that in Fig. 1 (S. Thomas and D. A. Fell, manuscript submitted for publication). Here, we use co-response analysis to interpret the data presented in Fig. 2, and show that the co-responses are not compatible with activation of PFK as the sole means of flux control in these cases.

Two of the datasets in Fig. 2 show notable similarity in the behavior of the metabolites downstream of PFK: Lagunas and Gancedo (38) studied the Pasteur effect in Saccharomyces cerevisiae by transferring aerobically respiring yeast into anaerobic conditions, whereas Culvenor and
Weidemann (39) studied the alterations in glycolysis accompanying phytohaemagglutinin stimulation of rat thymocytes. In each case, there was a large increase in F1,6BP, but decreases in triose phosphates and 3-phosphoglyceric acid (3PGA), and finally an increase in pyruvate (though this is only slight in the yeast). Culvenor and Weidemann also measured 2-phosphoglyceric acid (2PGA) and phosphoenolpyruvate (PEP), recording a very large decrease and a slight increase, respectively. Lagunas and Gancedo interpreted their data as illustrating that PFK activation was the sole regulatory effect. Their argument was based on the fact that no crossover was observed, other than at PFK. However, the negative flux/metabolite co-responses shown by dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP) are not compatible with the increase in flux, even allowing for feedforward activation of PK by F1,6BP. Culvenor and Weidemann recognized that aldolase in rat thymocytes has a low catalytic capacity, and recorded that the disequilibrium ratio (i.e. mass-action ratio/equilibrium constant) was low, but did not consider it to exert any flux control.

The third set of data in Fig. 2 illustrates the results of transformation of chick-embryo cells by Rous sarcoma virus (40). There are true crossovers at HK (internal glucose decreased, not shown in Fig. 2) and PK, though the increase in F1,6BP suggests activation of PFK as well. The conclusion drawn by Singh et al. (40) that glucose transport, HK, PFK and PK are all activated seems perfectly acceptable, though the data do not rule out the activation of other enzymes. However, the difference between the large increase in triose phosphates they recorded, compared to the decreases recorded by Lagunas and Gancedo and by Culvenor and Weidemann, does appear to indicate a significant difference between the response of the transformed chick-embryo cells on the one hand, and the yeast and thymocytes on the other.

A contrasting pattern of metabolite concentrations is shown in insect flight muscle undergoing a rest to flight transition, in which glycolysis can be increased by 100-fold. The data presented in Fig. 2 are from the blowfly Phormia regina (41), although the pattern in the locust Locusta migratoria (42) is almost identical. Steady-state concentrations of all glycolytic intermediates in flight either are unchanged or, including F1,6BP, are lower than in the resting state. In reaching the steady-state, however, which is attained some 10 min or so after initiation of flight, the concentrations of F1,6BP, DHAP and 3PGA undergo transient increases before falling to their steady-state values (41,42). These transient changes are consistent with activation of PFK in the early stages. However, the low steady-state concentrations are not consistent with PFK activation as the sole means of achieving the observed flux increase. This raises the question of what is the mechanism that stimulates the rate of reaction at those
steps where the substrate and product levels have changed so little? Feedforward activation of PK by F1,6BP has not been observed in insect flight muscle (43), so this is an unlikely explanation. Furthermore, we have shown that, although feedforward activation can be responsible for negative flux-metabolite co-responses of PEP, 2PGA, 3PGA and 1,3-bisphosphoglycerate (1,3BPG), it can not cause negative co-responses of GAP, DHAP or F1,6BP (S. Thomas and D. A. Fell, unpublished work). In contrast, activation of myofibrillar ATPase can generate negative co-responses, a corollary of this being that ATPase itself is invested with a degree of glycolytic flux control that can be considerable (S. Thomas and D.A. Fell, manuscript submitted for publication).

There is no question that the accurate determination of metabolite amounts can be difficult, particularly if metabolites are distributed between several intracellular compartments, and if the distribution between those compartments, not only the total cellular amounts, is required. Nevertheless, if data of sufficient quality can be collected, it can be an useful aid in determining how and where flux control is exerted. Metabolite homeostasis in the face of large flux changes can be quite remarkable. The examples from insect flight muscle (41,42) are extreme, but the fact that transient changes were recorded proves that the homeostasis is not an artefact due to the inability to sample metabolites with sufficient accuracy. There are many other examples in which large flux increases are achieved, either with much smaller proportional metabolite concentration increases (i.e. $O_{\text{flux,metabolite}}$ is large), or with decreases (i.e. $O_{\text{flux,metabolite}}$ is negative). Some examples are listed in Table 2.

Although near-equilibrium sections of pathway can, in principle, generate large, positive flux/metabolite co-responses, the magnitude of the co-response diminishes very rapidly as the reactions move away from equilibrium. Furthermore, the flux/metabolite co-response of any given metabolite depends, not only on the disequilibrium ratio of the reaction that consumes it, but on the overall disequilibrium ratio of the sequence of reactions downstream of it*. Observed disequilibrium ratios, for example in glycolysis in working rat heart (47) do not appear to be sufficiently high (i.e. close to 1) to account for the observed co-responses.

There are, however, other means of generating large flux/metabolite co-response that could explain the data in Table 2. Activation of multiple steps in a pathway, as described earlier, can generate large and/or negative co-responses, depending upon the magnitudes of the relative activity increases of the different enzymes. For example, the last entry in Table 2,

\[ * \text{Disequilibrium ratio, } \rho = \frac{\Gamma}{K_{\text{eq}}}, \text{ where the mass-action ratio, } \Gamma = \frac{\Sigma \text{products}}{\Sigma \text{reactants}}, \text{ and } K_{\text{eq}} \text{ is the equilibrium constant for the reaction. If } \rho = 1 \text{ the reaction is at equilibrium; consequently there is no net flux} \]


<table>
<thead>
<tr>
<th>Flux</th>
<th>Tissue</th>
<th>Stimulus</th>
<th>Metabolite</th>
<th>( Q_{\text{flux,metabolite}} )</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>glycolysis</td>
<td>blowfly flight muscle</td>
<td>rest to flight transition</td>
<td>F1, 6BP, DHAP, GAP, PGA3, PGA2, PEP</td>
<td>all either v. large positive (&gt; 50) or negative (&lt; -50)</td>
<td>(41)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>citrate, ( \alpha )-ketoglutarate,</td>
<td>all either v. large positive (≥50) or negative (≤ -50)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>malate, oxaloacetate</td>
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<tr>
<td>glycolysis</td>
<td>locust flight muscle</td>
<td>rest to flight transition</td>
<td>F1, 6BP, DHAP, GAP, PGA2, PEP</td>
<td>all either v. large positive (≥50) or negative (≤ -50)</td>
<td>(42)</td>
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<td></td>
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<td>citrate, ( \alpha )-ketoglutarate,</td>
<td>all either v. large positive (≥50) or negative (≤ -50)</td>
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<td>mouse brain</td>
<td>anoxia</td>
<td>F1, 6BP; DHAP; GAP; 1.3BPG; 3PGA; 2PGA</td>
<td>1.3 to 3.9</td>
<td>(44)</td>
</tr>
<tr>
<td>TCA cycle</td>
<td>rest to flight transition</td>
<td>citrate, ( \alpha )-ketoglutarate, malate, oxaloacetate</td>
<td>all either v. large positive (≥50) or negative (≤ -50)</td>
<td>1.2 to 1.68</td>
<td>(45)</td>
</tr>
<tr>
<td>glycolysis</td>
<td>frog sartorius</td>
<td>electrical stimulation</td>
<td>F1,6BP</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rat skeletal muscle</td>
<td>injection</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rat heart</td>
<td>perfusion with insulin</td>
<td>F1,6BP, 2PGA, DHAP; GAP, 1.3BPG; 3PGA</td>
<td>-0.54, -0.98, 0.15, 4.3, 21</td>
<td>(47)</td>
</tr>
<tr>
<td>glycolysis</td>
<td>heptomas</td>
<td>work ( \text{DNA} )</td>
<td>F1,6BP</td>
<td>8.78 to 3.79</td>
<td>(48)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>proliferation rate</td>
<td>dATP, dGTP, dCTP, dTTP</td>
<td>all large, positive (from 2 to 7)</td>
<td>(49)</td>
</tr>
</tbody>
</table>

Showing that the increased rate of DNA synthesis in hepatomas of increasing proliferative rate is accompanied by much smaller relative increases in the deoxyribonucleoside triphosphate (dNTP) pools, is consistent with the observations listed in Table 1 (14, 15) that the same hepatomas have increased activities of enzymes synthesizing the dNTPs and of DNA polymerase (E.C. 2.7.7.7). The same observations imply that the intermediates of dNTP synthesis should also show large co-response ratios.

In addition, it has been shown that large flux changes with good metabolite homeostasis can be achieved by increasing the demand for a pathway
product (33). The concept of pathway control by demand for products has been postulated previously. For example, a role for ATP hydrolysis in the control of glycolysis has been studied in cell-free systems (50, 51) and in Ehrlich ascites tumor cells (52). However, the conclusion that the internal behavior of a pathway can be influenced by events outside that pathway has crucial implications for the study of metabolic control, particularly for the interpretation of data such as that presented in Fig. 2 and Table 2.

**DISCUSSION**

In the 90 years since the proposal of the rate-limiting step as a theory of metabolic flux control (1), our understanding has improved enormously in some areas of the subject, whilst in others the improvement has been modest. Whereas the discoveries of the mechanisms, such as non-covalent binding of effectors, reversible covalent modification and change of enzyme expression, by which organisms control metabolism have been amongst the most significant in biochemistry, the explanations of how these features interact to function as a metabolic unit have been less satisfactory. The concept that metabolism is controlled by a rate-limiting step near the start of each pathway is appealing - and this is a major factor in its continued longevity - but many of the arguments in its support have been shown to be theoretically flawed (7, 9, 11, 33). Furthermore, additional data have been presented (16, 24, 37), highlighting observations that cannot be satisfactorily explained by this theory.

One of the crucial facts about the control of metabolic flux is that it is not a symmetrical phenomenon. Any enzyme will become rate-limiting if it is inhibited sufficiently as, in the limit of complete inhibition, pathway flux will become zero. On the other hand, the activation of an enzyme causes an inevitable decrease in the flux control it possesses itself, and so in the flux increase that can be achieved by its further activation. Applying the finite change theory of MCA (53), the maximum flux achievable in a linear pathway by activating a step that possesses, initially, 90% of the flux control (such that a 1% change in enzyme activity causes a 0.9% change in flux) can be shown to asymptotically approach 10-fold the initial flux. For example, activating the enzyme 10-fold will only cause a 5.1-fold flux increase. If the enzyme starts with only 50% of the control, the maximum flux that can be achieved will be double the starting flux. In this case, a 10-fold increase in activity will increase the flux by only 80%. Faced with these theoretical limitations, it is easy to appreciate why multiple enzyme activation appears to be so physiologically important.

Even if one assumes that, within a given pathway (in the textbook sense, e.g. glycolysis), flux control could be invested largely in one step,
for a given route through metabolism, it must be necessary to activate at least one step in each 'pathway', as we have already described with respect to insulin activation of triglyceride synthesis in adipose tissue (23). However, the physiological requirements, at least in long-term flux change, appear to include activation of a number of 'near-equlibrium' steps that have generally been considered to exert negligible flux control. The reason for this requirement—whether because these steps do exert some degree of flux control, or for a homeostatic, or other, reason—remains to be determined, and must be seen as a major barrier to our future understanding of physiological flux control.

The application of quantitative systems methods has brought into focus problems that exist with, but are not readily apparent in, traditional qualitative explanations. In addition, the likely involvement of multiple pathway steps in metabolic flux control emphasizes the importance of using such methods in its study, to attempt to quantify the contribution to the overall response of a pathway of each step whose activity is altered. It also raises the question of where control is implemented. If physiological response to a stimulus entails the activation of several steps in a pathway, then it is necessary to know how control is distributed over the steps involved in the activation and inactivation of these steps. MCA has predominantly been concerned with the reactions of primary and intermediary metabolism, for the study of pathways in which all elements (i.e. metabolic intermediates) are interconnected by mass transfer (i.e. enzyme-catalyzed reactions or transport between compartments). The pathways in Schemes 1 and 2 are of this type. However, a modification—modular MCA—has been developed, that allows the study of signal transduction, enzyme activation and related aspects of metabolic control. This is achieved by separating the pathway under consideration into modules. Within a module, intermediates are inter-converted by mass action, but between modules, information is transferred by signalling molecules without (or with negligible) mass transfer, e.g. (54).

The pathway in Fig. 3 illustrates the use of modular MCA. Module 4 represents a pathway in which steps 1 and 3 are activated by a stimulus. The reactions of modules 2 and 3 represent the activation and inactivation of steps 1 and 3 respectively. For example, if the activation entails increased expression, the two steps in each of modules 2 and 3 represent enzyme synthesis and degradation, respectively. Alternatively, if activation entails reversible phosphorylation, they represent the phosphorylation and dephosphorylation steps. The signals transmitted from modules 2 and 3 to module 4 are a consequence of the altered activities of enzymes E1 and E2. Module 1 represents the synthesis and degradation of the stimulus. Its action is shown to be on the activation steps of modules 2 and 3. For example via phosphorylation and activation of a transcription factor.
Within this scheme, modules can be expanded or coalesced. Thus, enzyme synthesis can be expanded to contain a module at the levels of RNA, with transcription and RNA hydrolysis. Module 1 can be expanded to contain the steps involved in signal transduction. Each module can be of any complexity. If flux through module 4 is of interest, then the distribution of the control over this flux can be calculated, not only between the steps in module 4 itself, but also between the activation and inactivation steps of modules 1, 2 and 3. This control is a consequence of the effect of the steps in these three modules on the concentration of the active forms of E1 and E3. Finally, as well as expanding or coalescing modules, any intermediate in any module can, in principle, affect the rate of any step in any module. For example, in many apparently hierarchical systems, there is feedback regulation from within modules lower in the hierarchy on steps in higher modules, such as the reduction by activated protein kinase C of the affinity of the EGF receptor (EGFR) for EGF (55). In Fig. 3, this effect would be shown as an inhibitory signal from, for example, E1 in module 2 onto act in module 1.

Experimental discoveries concerning metabolic control are currently being made at a rapid rate, particularly, but not exclusively, in the areas concerning signal transduction. Each discovery adds to the view of a signal transduction and metabolic control network of astonishing complexity. The discoveries in mechanistic insight parallel the period during the 1950s and 1960s, when great advances were made in understanding the mechanisms of control of enzyme activity, though our understanding of
why metabolic systems behave as they do is still relatively poor. Nevertheless, the tools to study these questions are available: advances in the theory of metabolic control and in affordable computing power to analyze metabolic systems have kept pace with experimental developments. Continued and improved co-ordination between experimental and theoretical investigations should see important advances in this area in the coming years.

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