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REVIEW ARTICLE

Metabolic Control Analysis: a survey of its theoretical and experimental development

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INTRODUCTION

As the details of the chemical transformations in metabolism have become increasingly clear, and the enzymes catalysing many of the reactions have been characterized, it is understandable that biochemists should want to explain at the molecular level the metabolic homeostasis observed at the physiological level. How are the rates of synthesis and degradation of metabolites kept in close balance over a very wide range of external conditions without catastrophic rises or falls in the metabolite concentrations? The discoveries of feedback inhibition, co-operativity and covalent modification in enzymes, and of mechanisms for the control of enzyme synthesis and degradation, have disclosed a repertoire of molecular effects that potentially alter the fluxes in metabolic pathways. With such a range of effects to choose from, it is not surprising that disputes arise over explanations for the changes in flux through particular pathways under given circumstances. Since the explanations are usually verbal and qualitative, discrimination between different explanations, or assessment of their adequacy, is difficult. More recently, several groups have attempted theoretical analysis of the potential of these different molecular mechanisms to contribute to the control of metabolic flux. Since these theories can be given a mathematical formulation, they can be used in combination with appropriate experimental measurements to provide quantitative explanations and, potentially, predictions. The theories have often been controversial. Matters at issue have included the extent to which it is feasible to perform experiments to obtain the necessary data, the adequacy of the theories for making useful predictions, and the degree to which the quantitative measures of the theories do actually capture the relevant aspects of regulation and control. To some extent, this is a matter of semantics; a mathematical theory is more explicit about its underlying assumptions and the meaning of its statements, but regulation and control are two terms that have been used without strict adherence to any agreed definition in many different contexts (as noted in [1]). It is therefore inevitable that some will find that the use of these terms in the context of a mathematical theory places a narrower construction on them than they would like.

These theories all include a form of sensitivity analysis; that is, the magnitude of the effect of some small change in a parameter (such as an enzyme activity) on a metabolic system property (such as the flux or the concentration of a metabolite) is mathematically related to the properties of the components of the system. Sensitivity analysis is widely used for analogous problems in other fields, including economics, ecology [2], engineering [3] and chemical kinetics [4–6]. Its application in biochemistry was pioneered by Higgins [7], but three variants subsequently arose: Metabolic Control Analysis, Biochemical Systems Theory and Crabtree and Newsholme's 'flux-oriented' theory (the term used in [8]). It is not possible to give a succinct account of the differences between the approaches, which is a controversial area [9–18] even though the underlying mathematics is equivalent to a considerable extent. One area of difference is the choice of the type of parameter that is changed for the determination of sensitivities. In Metabolic Control Analysis, enzyme concentration (or activity) is usually chosen; the response to an external modifier of a metabolic pathway is derived from the resulting sensitivities. In Biochemical Systems Theory [19–25], the primary parameters for the sensitivities are the 'rate constants' for synthesis and degradation of metabolite pools. Savageau has given many reasons for this choice of parameter; Cornish-Bowden has articulated some of the problems with it [15]. Although using these 'rate constants' simplifies the analysis procedures within Biochemical Systems Theory, there is not a one-to-one relationship between them and the enzymes of the system, which can create a slight complication in determining the sensitivity to variation of an enzyme activity. Savageau's theory is part of an integrated system for stability analysis and simulation, in addition to sensitivity analysis. Crabtree and Newsholme's theory [8,10,26–30] is intermediate between the two others, and the primary sensitivities are to an external modifier (a hypothetical one if necessary), but its mathematical development is less rigorous. In this review, I shall concentrate on Metabolic Control Analysis. This is because, apart from considerations of space, approximately two-thirds of the literature citations of theories of metabolic regulation in the past 5 years have been to Metabolic Control Analysis. This may relate to perceived ease of use, which has been compared using the different approaches on the same set of experimental results [31].

In the following review, I will not give a complete derivation and description of the basic concepts of Metabolic Control Analysis; clear accounts can be found in previous articles and reviews [9,32–39]. Instead I will try to indicate areas of disagreement, the scope of the basic theory and where it has been modified or extended, and recent approaches to experimental applications.

ASSUMPTIONS

The current terminology of Metabolic Control Analysis arose in 1984 when it was agreed that the two independent approaches originating with Kacser & Burns [32] and Heinrich & Rapoport [40,41] could be described in the same terms [42]. Naturally, this means that the original publications use terminology that is not in current use; a glossary is given in [43].

In its basic form, Metabolic Control Analysis depends on certain assumptions about the nature of the metabolic system. Although some of these can be relaxed, as will be described later, the theory becomes more complicated as a result. In its simplest form, the theory assumes that:

1. The metabolic system under study is a single connected unit. i.e. all the reactions are connected, via common metabolites (including coenzymes), into an interdependent whole. This is not a necessary condition, though it is implicit in most treatments of the theory. Kahn & Westerhoff have shown that the theory can be extended to cover separate blocks of metabolism connected only by regulatory or catalytic interactions [44].

2. The metabolic system can be studied in a stable steady state. The steady state is a mathematical abstraction; it requires that the rate of formation of every metabolite in the system is equal

to its rate of degradation, so that all concentrations remain constant with time. For this to happen whilst there is a flow of material through the metabolic system (a dynamic steady state), there must be at least one substance (a *source*) that provides a reservoir of matter (or pool), and at least one other (a *sink*) into which the output of the pathway flows, as in the following scheme where the X_i are the pools and the S_i are the variable metabolites:

$$X_0 \rightarrow S_1 \rightarrow S_2 \rightarrow X_1$$

Scheme 1

The pools are the boundaries of the system and Metabolic Control Analysis applies to the processes between these boundaries: transport steps and spontaneous reactions as well as enzyme-catalysed reactions. Unfortunately, no real system is likely to be in a true steady state, since this can only be approached asymptotically; instead we have to settle for a quasi-steady state, which depends on the concepts of a time hierarchy in metabolism [25,45–49] and of the limited accuracy of observations of concentrations and fluxes. In outline, relative to the time period of the observations, reactions have to be classified into one of three groups:

(*a*) rapid reactions whose time scale for reaching a state indistinguishable from the steady state is very much shorter than the time scale of observation;

(*b*) reactions whose time scale for approaching a steady state is close to the time scale of observation, and

(*c*) reactions that cause significant changes in concentrations on time scales longer than that of the observation.

Within a metabolic system, processes in the first group could include ionic equilibria and binding of substrates and effectors by enzymes. Indeed, Metabolic Control Analysis usually assumes that all enzyme-bound intermediates are at a steady state and does not consider the molecular details of enzyme reactions. In the third group might be the rate of depletion of a pool metabolite, the rate of synthesis or degradation of the common part of a coenzyme group needed for the reactions (e.g. the adenosine of AMP, ADP, ATP), or even the synthesis or degradation of an enzyme. Entities affected only by these slow reactions can be treated as invariants, or as part of the parameter group, in the analysis. Thus it is only the middle group that must be allowed time to approach sufficiently close to a steady state. The system is then in a quasi-steady state because the metabolites associated with reactions in this group are still approaching steady state, even though the rate of change is unobservable, and because the variation of other substances (such as pools) would become significant if the time scale of observation were to be extended. The fluxes and metabolites governed by this middle group must be treated as variables in the analysis. Whilst it may seem that the vagueness of this division detracts from the utility of the analysis, there has to be some demarcation of its scope, based on what questions are to be answered. There is no point in extending the time scale to allow everything to approach a steady state, partly because more and more reactions will have to be included in the system (dealing with smaller and smaller fluxes within metabolism), partly because the domain of the problem will move out of biochemistry, first into physiology, then into ecology and so on, and finally, because it is unreasonable to expect external conditions to remain constant for ever.

The requirement for stability is analogous to mechanical stability: if a metabolic system is stable, a slight perturbation in the amount of a metabolite, or some other condition, will result in the system eventually returning either to its original state (dynamic stability, when a variable metabolite has been perturbed), or to one close by (structural stability, when a parameter such as enzyme concentration has been perturbed). If the system is unstable, even a small perturbation makes it diverge from its original state, though in some cases it may oscillate around the unstable point. The analysis of stability is important on theoretical grounds, for example in comparing behaviour of feedback inhibition systems [25]; it is less important experimentally because it is difficult to see how reproducible experimental results could be obtained from an unstable system, which conversely makes it likely that most biochemical systems are studied under conditions where a stable steady state exists. The only exceptions are oscillating systems, of which a few examples are known, e.g. [50–52].

There are modifications of Metabolic Control Analysis that deal with time-varying systems [53–57]. Stability analysis can be incorporated into Metabolic Control Analysis [58,59], but it is better integrated in Biochemical Systems Theory [21,25], and can be considered separately, e.g. [47,48]. None of these topics will be considered further here.

3. Metabolites must be distributed homogeneously over the enzymes that act on them. There is no problem about subcellular compartments, but within a compartment there must be a single concentration value that describes the level of each metabolite. In other words, Metabolic Control Analysis does not deal with reaction–diffusion systems that would require positional information to describe the state of the system.

4. Rates of enzyme action should be directly proportional to enzyme concentration, and one enzyme should affect only one reaction. Again, there are ways of modifying Metabolic Control Analysis to cope with non-proportionality between enzyme concentration and activity [60–63], action of one enzyme on more than one step [64] and enzyme–enzyme interaction [61,65], including the enzyme–enzyme complexes involved in metabolite channelling. Opinions vary about whether enzyme–enzyme interaction, and in particular metabolite channelling, is a sufficiently general phenomenon to render use of the unmodified, simpler form unjustifiable [66].

5. Enzymes do not appear in the analysis as variables but as parameters. Where an enzyme acts in the scheme as a catalyst, it cannot also be a metabolite. This creates complications in describing schemes with covalent modification or synthesis of an enzyme. Metabolic Control Analysis can be modified to deal with these cases [44,67]; the method proposed by Kahn & Westerhoff [44] involves dividing the total scheme into separate blocks, with the enzyme appearing in one block as a metabolite and having a regulatory or catalytic interaction with another block containing the metabolites of the pathway. Biochemical Systems Theory does not need any special extension and may therefore be simpler for formulating models of such schemes.

6. All metabolite concentrations are for the free form; the amounts of enzyme-bound metabolites are presumed not to affect the properties of the steady state in most cases [68]. Obviously, this may lead to problems in relating to those experiments that involve measurements of total substrates, though other techniques such as n.m.r. may give free concentrations directly.

A common misunderstanding is the belief that Metabolic Control Analysis is only applicable to linear chains of enzymes operating in the pseudo-first-order domain; this appears to have arisen through some readers failing to distinguish between the presentation of the general theory and the analysis of a simple example in the original article by Kacser & Burns [32].

FUNDAMENTALS OF CONTROL ANALYSIS

Metabolic Control Analysis attempts to relate the overall properties of a metabolic system to the properties of its component parts, in particular, the enzymes. The system proper-

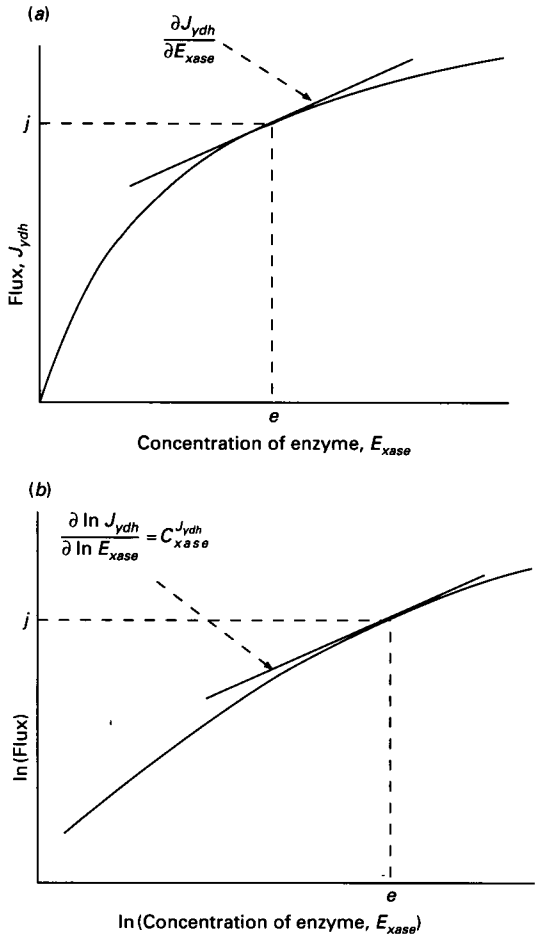

Fig. 1. The flux control coefficient

(*a*) The flux control coefficient at e, j is the slope of the tangent to the curve $\partial J_{ydh}/\partial E_{xase}$ times the scaling factor e/j. (*b*) On a logarithmic plot of the same curve, the flux control coefficient is the slope of the tangent to the curve.

ties that are at the centre of the theory are the control coefficients. These describe how a variable or property of the system, typically a metabolic flux or the concentration of a metabolite, will respond to variation of a parameter, typically enzyme concentration. Thus we have *flux control coefficients* [42] (formerly known as *sensitivity coefficients* [32] or *control strengths* [40]) for the effects of each of the enzymes (or transporters, etc.) on pathway flux, or each pathway flux where there is more than one flux that can be chosen (as for example in a branched pathway). For each variable metabolite in the system, there are also concentration control coefficients for the effects of each of the enzymes on that concentration. Mathematically, all the control coefficients represent the fractional change of a system property, $\delta V/V$, in response to a fractional change in a parameter, $\delta P/P$, in the limit as δP tends to zero:

$$C^V_P = \lim_{\delta P \to 0} \frac{\delta V/V}{\delta P/P} = \frac{\partial V}{\partial P} \cdot \frac{P}{V} = \frac{\partial \ln |V|}{\partial \ln P} \quad (1)$$

where $|V|$ indicates the absolute value of V, i.e. its magnitude without regard to sign. The advantage of this definition of the control coefficient, as a relative or logarithmic sensitivity, is that it is dimensionless. Unscaled or absolute sensitivities, $\partial V/\partial P$, have been used in chemical kinetics [4,5]. Reder has also argued in favour of unscaled control coefficients in Metabolic Control Analysis, though her definition of them was an unscaled form of Eqn. 3 [69]. In the case of the *flux control coefficient*, the variable is a flux, such as J_{ydh} measured through a particular reaction, catalysed by enzyme *ydh*, of the metabolic pathway with respect to the concentration, E_{xase}, of an enzyme *xase* giving (with concentration brackets omitted here as elsewhere for clarity):

$$C^{J_{ydh}}_{xase} = \frac{\partial J_{ydh}}{\partial E_{xase}} \cdot \frac{E_{xase}}{J_{ydh}} = \frac{\partial \ln |J_{ydh}|}{\partial \ln E_{xase}} \quad (2)$$

(Theoretical papers on Metabolic Control Analysis usually use numbers or counting variables rather than enzyme and substrate names in the subscripts and superscripts, but this is merely a convenience to allow compact description of the mathematical operations.) The flux control coefficient can be visualized as shown in Fig. 1. Note that the hypothetical response curve shown appears to approximate to a rectangular hyperbola; although this seems to be justified by experimental observations of such curves [70–73], there are theoretical grounds for believing that these curves cannot be perfect rectangular hyperbolas (D. A. Fell *et al.*, unpublished work) except in the case of a chain of Michaelis–Menten enzymes all operating in the pseudo-first order region [32,74].

Heinrich, Rapoport and Rapoport [48], followed by Reder [69], gave a more general definition of a flux control coefficient in terms of any parameter p that acts exclusively on an enzyme *xase* as:

$$C^{J_{ydh}}_{xase} = \frac{v_{xase}}{J_{ydh}} \cdot \frac{\partial J_{ydh}}{\partial p} \Big/ \frac{\partial v_{xase}}{\partial p} \quad (3)$$

If E_{xase} is chosen as p, the advantage of this is that it reduces to the standard definition, provided that $\partial \ln v_{xase}/\partial \ln E_{xase}$ is 1 (i.e. that enzyme activity is directly proportional to enzyme concentration, as in normal enzyme kinetics). Under circumstances where this is not the case, then there are implications for both the values and interpretation of the flux control coefficients [61–63,68,75,76]. Finally, note that although the partial differentiation notation has been used above, this is in the context that the control coefficient is one of a set that is obtained by taking each enzyme (or other parameter) in turn, at constant values of all the others. Mathematically, there are reasons for regarding it as a total derivative (as in [43]), particularly when it is desired to decompose the effects of the enzyme into its component parts.

There have been objections both to the name and concept of the control coefficients. Some of the principal grounds for this have been the claims that:

1. Enzyme concentration is not a parameter that is particularly relevant to metabolic control e.g. [8,77], compared with the action of effectors that bind to allosteric enzymes. The general definition of a control coefficient in eqn. (1) does allow for a control coefficient for the sensitivity of a flux to an effector; this has been termed a *response coefficient* by Kacser & Burns [32] or a *net sensitivity* by Crabtree & Newsholme [29]. One of the reasons for giving emphasis to the flux control coefficients with respect to enzyme concentration is that these can be shown to be components of the response coefficients, or net sensitivities, and the partitioning of the response into its components is useful information. The use of the response coefficient as a measure of regulatory importance has recently been considered in some detail by Hofmeyr & Cornish-Bowden [1]. The other reason is that the actions of genes, DNA manipulation, diet and hormones on metabolism can be mediated through the modulation of the concentration of the active form of an enzyme, and these are hardly insignificant as means of metabolic control.

2. The sensitivity of a flux to an enzyme concentration is not a measure of whether that enzyme is a *control* or *regulatory* enzyme [13,8,77], so the term is misleading. There is some justification for this criticism, which can be considered in relation to an enzyme that is subject to feedback inhibition by a metabolite further along a metabolic pathway. Since the effect of the inhibition is to tend to stabilize the concentration of the feedback effector by modulating its synthetic flux to match the rate at which it is being used, the enzyme is acting as a regulator according to the accepted usage in science and engineering [3,78–80] for an element acting to maintain a constant set point (a concentration) for long periods. (See [1] for the link between this definition and the concept of homeostasis.) The apparent paradox that a regulator enzyme has a low flux control coefficient was specifically discussed in the original paper by Kacser & Burns [32], so it is common ground that flux control coefficients do not identify regulatory enzymes, in the sense of all those that have an effector site. However, much of the qualitative writing about metabolic regulation has implied that such regulatory enzymes are potential candidates for the 'rate-limiting step(s)' of the pathway. Now a major point of Metabolic Control Analysis is to replace this misleading and limited concept, but in so far as the flux control coefficient can be regarded as a measure of degree of rate-limitingness, the implication of the paradox is that 'regulatory enzyme' and 'rate-limiting enzyme' are not necessarily related categories, but deal with separable properties [81]. Newsholme & Start [82] considered definitions of regulatory enzymes; they noted that one possibility was to regard a regulatory enzyme as a non-equilibrium enzyme whose activity depends on metabolites other than its substrates (which is in large part an identification of the molecular characteristics of a regulatory element, since the only part of the requirement that depends on the system properties is that of not being close to equilibrium); they also stated that: "The crucial question is, which is the enzyme which responds to the original metabolic signal and thereby initiates subsequent changes in the activities of the remaining enzymes." It will be argued later, as in [1], that the flux control coefficient does identify potential sites for control in this sense, which corresponds to the usage of the term control to signify adjusting power level (i.e. flux) [79,80] or obtaining a desired response [83], even though these definitions imply an identifiable purpose, which is more problematical in biology than in engineering.

3. The flux control coefficient has limited predictive value because it is only valid under the conditions of measurement, and as the state of the system changes, the values of the flux control coefficients change [8]. This cannot be disputed, though Metabolic Control Analysis has not been promoted as a tool for system modelling in the same way as Biochemical Systems Theory. Nevertheless, for a change in active enzyme concentration from $E_{xase,1}$ to $E_{xase,2}$, small enough for $C^{J_{ydh}}_{xase}$ to be effectively constant, the integrated form of eqn. (2) is:

$$\Delta \ln J_{ydh} = C^{J_{ydh}}_{xase} \Delta \ln E_{xase} \tag{4}$$

where $\Delta \ln E_{xase} = \ln E_{xase,2} - \ln E_{xase,1}$. As pointed out by Higgins [7], in connection with the equivalent set of coefficients that he termed *reflexion coefficients*, this corresponds to the power law form:

$$J_{ydh} = a E^{C^{J_{ydh}}_{xase}}_{xase} \tag{5}$$

Eqn. (4) describes the tangent in Fig. 1(*b*), and it is clear that use of the flux control coefficient to predict the metabolic flux at other enzyme concentrations becomes less and less accurate the further away the new enzyme content is from the one at which the coefficient was measured. However desirable it would be to have an equivalent theory that would simplify making predictions of the effects of large changes in concentration of an external substrate, or effector, or amount of active enzyme, this is an extremely difficult problem because of the non-linear nature of enzyme kinetics. In spite of claims to the contrary [84], it is unlikely that any other form of sensitivity analysis will perform better in all circumstances ([15]; D. A. Fell, unpublished work). If the control coefficients change significantly between two points, they could be remeasured at a number of steady states corresponding to varying strengths of the perturbation being studied. In any case, however large the overall change, the stability properties of a steady state mean that is necessary to explain how the system came to leave its initial steady state, and it is difficult to see how a large physiological response could be initiated by an effector without there being a finite response coefficient with respect to the effector at the initial point, which in turn requires that the step on which the effector acts has a non-zero flux control coefficient (for reasons explained later in connection with eqn. 8).

As well as flux control coefficients, there are the *concentration control coefficients*, where the variable affected by the chosen parameter, such as the enzyme *xase*, is a metabolite concentration, say S:

$$C^{S}_{xase} = \frac{\partial S}{\partial E_{xase}} \cdot \frac{E_{xase}}{S} = \frac{\partial \ln S}{\partial \ln E_{xase}} \tag{6}$$

In addition to the control coefficient, another measure that plays an important role is the *elasticity*. Unlike the control coefficient, this is not a property of the metabolic system, but of an individual enzyme, though the form of the definition is analogous to that of eqn. (1). Thus the elasticity coefficient for the effect of metabolite S on the velocity v of enzyme *xase* is the fractional change in rate of the isolated enzyme for a fractional change in substrate S, with all other effectors of the enzyme held constant at the values they have in the pathway:

$$\epsilon^{xase}_{S} = \frac{\partial v_{xase}}{\partial S} \cdot \frac{S}{v_{xase}} = \frac{\partial \ln |v_{xase}|}{\partial \ln S} \tag{7}$$

Elasticities can be regarded as apparent kinetic orders of the rate of the reaction with respect to the metabolites, for integration and conversion to the power law form (by analogy with eqns. 4 and 5) shows them to be equal to the power law exponents used in Biochemical Systems Theory [19,20,25] (provided that the reaction has not been aggregated with others in analysis under the latter theory). They have positive values for metabolites that stimulate the rate of a reaction (substrates, activators) and negative values for those, like products and inhibitors, that slow the reaction (Fig. 2). Their relationship to enzyme rate laws is considered in more detail later in connection with their measurement. Their function in Metabolic Control Analysis is as a quantitative replacement for the vague concepts of responsiveness of an enzyme to a metabolite that are used in qualitative explanations of metabolic regulation (such as: 'The substrate S controls the rate of reaction because its concentration in the cell is well below the K_m of the enzyme for S...' or 'The rate of the enzyme will be relatively unresponsive to variations in the concentration of S because it is well above the K_m...'). One reason why values of elasticities are more useful than such statements is that they are determined with the concentrations of other substrates, products and effectors at the values they have in the cell in the metabolic state being analysed. The rectangular hyperbola of the single-substrate enzyme in the absence of products has little application to enzymes *in vivo* where the majority have more than one substrate, are in the presence of an appreciable concentration of product, and may be catalysing a significant flux in both directions. The value of an elasticity, however, reflects the results of all these influences.

It was mentioned previously that it was possible to define a

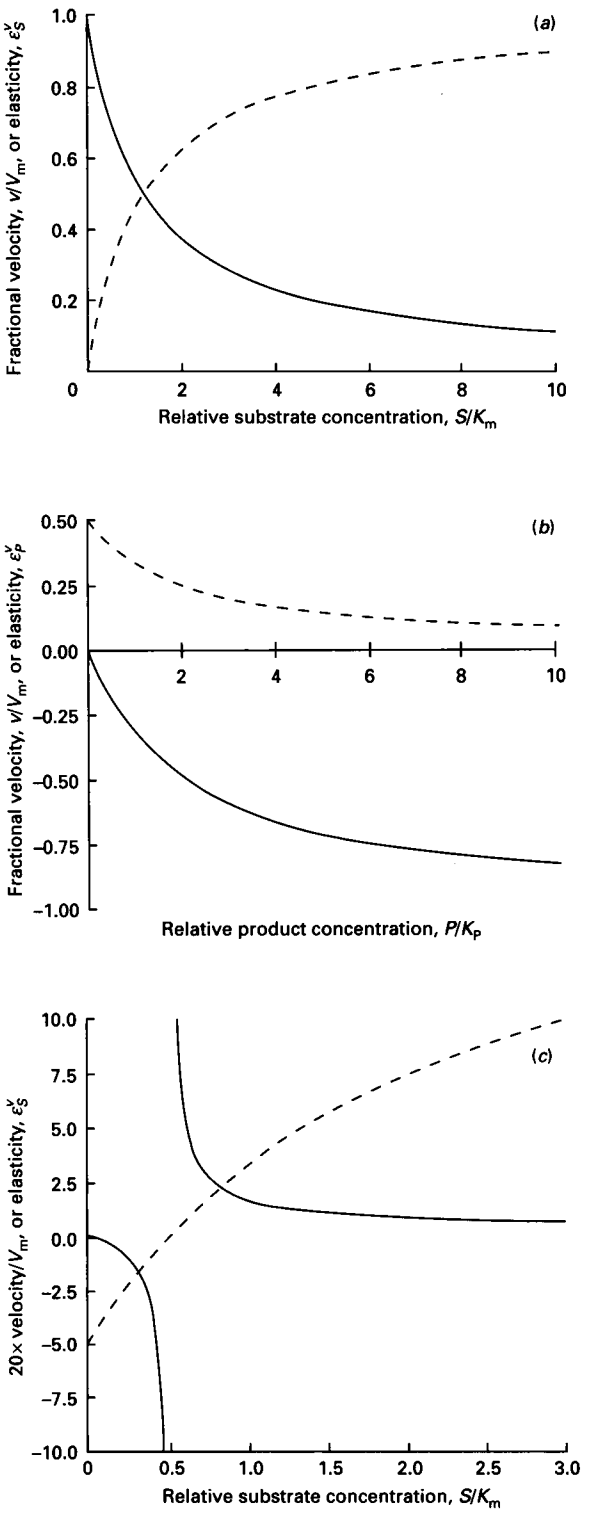

Fig. 2. Metabolite dependence of elasticities of a reversible Michaelis–Menten enzyme

The enzyme is assumed to obey the rate function of eqn. (22) in the text. (*a*) Elasticity with respect to substrate as a function of substrate concentration, reaction far from equilibrium. ——, ϵ_S^v; — — —, fractional velocity, v/V_m. Parameter values: $K_{eq.} = \infty$; $P/K_P = 0.2$. (*b*) Elasticity with respect to product as a function of product concentration, reaction far from equilibrium. ——, ϵ_S^v; — — —, fractional velocity, v/V_m. Parameter values: $K_{eq.} = \infty$; $S/K_m = 1$. (*c*) Elasticity with respect to substrate as a function of substrate concentration, reaction near equilibrium. ——, ϵ_S^v; — — —, fractional velocity, v/V_m. Parameter values: $K_m = 1$; $P = 5$; $K_P = 5$; $K_{eq.} = 10$. The elasticity is undefined at equilibrium ($S = 0.5$).

control coefficient, or *response coefficient*, for the effect of a metabolite external to the system being considered. Kacser & Burns [32] pointed out that the response coefficient for the effect of such an external, constant metabolite X on the flux J_{ydh}, where X acts through being an effector of the pathway enzyme *xase*, is composed of the flux control coefficient with respect to *xase* and the elasticity of *xase* with respect to X:

$$R_X^{J_{ydh}} = C_{xase}^{J_{ydh}} \epsilon_X^{xase} \quad (8)$$

If X acts on more than one enzyme, the total response will be the sum of the responses from each enzyme [1]. Crabtree & Newsholme [8,29] favour the use of the term *net sensitivity* that generally corresponds to the response coefficient rather than giving prominence to its components. Nevertheless, eqn. (8) for the response coefficient makes it clear that a pathway will not respond to control by an external effector that acts on an enzyme unless that enzyme has a non-zero flux control coefficient. Since feedback inhibition on an enzyme reduces its flux control coefficient, it renders that enzyme less effective as a site for external control. This is the justification for the claim above that a flux control coefficient does give information about an enzyme's potential for control, and that this attribute is differentiable from its status as a regulatory enzyme.

If it is the effectiveness of feedback regulation that is to be characterized, then measures other than the flux control coefficients must be used. Savageau has used Biochemical Systems Theory to analyse this problem [22,24,25], and approaches have been developed within Metabolic Control Analysis [1,85,86].

THEOREMS OF CONTROL ANALYSIS

The values of the control coefficients and elasticities of a metabolic system are subject to a number of constraints and inter-relationships that are termed the theorems of Metabolic Control Analysis. Their derivations will not be given here. For most of them, there is a derivation via an examination of the effects of a hypothetical, vanishingly small perturbation of a specimen pathway; this has the advantage of illustrating the connection between the mathematics and the physical system (e.g. the derivations given in [32]), but the disadvantage that there is no proof that the derivation would be valid for all possible configurations of metabolic pathway. There are more mathematically rigorous proofs where it is shown that the theorems are necessary properties of the sensitivity coefficients of a generalized metabolic system (e.g. [69,75,76,87–89]), but these are less accessible to many biochemists.

The *summation theorem* for flux control coefficients [32] states that the sum of all the control coefficients on any one chosen flux J for all the n enzymes in a metabolic system is 1; i.e.:

$$\sum_{i=1}^{n} C_i^J = 1 \quad (9)$$

This theorem is linked to the concept that the enzymes of the pathway can share the control of flux. In a linear pathway consisting of enzymes with normal kinetics (i.e. substrates activate and products inhibit), all the flux control coefficients must be zero or positive, so that the maximum value any enzyme could have is 1, when all the other enzymes would necessarily have flux control coefficients of zero. In such a case, the one enzyme could be said to be 'rate-limiting', for a flux control coefficient of 1 corresponds to a proportional relationship between the activity of an enzyme and the pathway flux. The summation theorem shows that this is not a necessary feature of the pathway, for it would be possible for some or all of the enzymes to have values greater than zero but less than 1. In the general case, however, the concept of the possibility of shared control of the flux

remains, but it is not possible to put bounds on the values of individual flux control coefficients because, even with normal kinetics, negative flux control coefficients will arise in pathways with branches and cycles, leaving open the possibility that some coefficients have values greater than 1 [34].

For every enzyme that responds to the concentration of metabolite S, the *connectivity theorem* states that the flux control coefficients are related to the elasticities of the enzymes with respect to S in the following way [32]:

$$\sum_{i=1}^{n} C_i^J \epsilon_S^i = 0 \tag{10}$$

This is widely regarded as the most meaningful of the theorems, for it provides the route to understanding how the kinetics of the enzymes (represented by the elasticities) affect the values of the flux control coefficients. For example, consider a short section of glycolysis:

$$\cdots \xrightarrow{\text{Enolase}} \text{Phosphoenolpyruvate} \xrightarrow{\text{Pyruvate kinase}} \cdots$$

Scheme 2

The specific connectivity theorem involving phosphoenolpyruvate is obtained by writing a term for every enzyme that has a non-zero elasticity for phosphoenolpyruvate; in this case, we assume this involves only enolase (denoted *eno*) and pyruvate kinase (*pk*), giving:

$$C_{eno}^J \epsilon_{PEP}^{eno} + C_{pk}^J \epsilon_{PEP}^{pk} = 0 \tag{11}$$

or

$$\frac{C_{eno}^J}{C_{pk}^J} = -\frac{\epsilon_{PEP}^{pk}}{\epsilon_{PEP}^{eno}} \tag{12}$$

Without knowing anything about the rest of the pathway, we cannot tell the magnitude of either flux control coefficient, but we can see that the relative values of two successive flux control coefficients depend on the elasticity representing the product inhibition and the substrate activation by the intermediate metabolite. The tendency of large elasticities (e.g. for enzymes catalysing reactions near equilibrium) to be associated with small flux control coefficients is apparent. There are as many pairs of terms in a connectivity equation as there are enzymes in the pathway affected by the metabolite under consideration, so the relationships are not always so straightforward. Also, the involvement of metabolites in a moiety-conserved cycle requires that the connectivity theorem be modified for each of the metabolites containing the conserved group [69,87,90,91]; in essence, the right-hand sides of eqn. (10) no longer equal zero, though the equations can be combined in pairs to give a zero result, with the side effect that the number of independent connectivity equations is reduced by one.

The summation theorem and the set of connectivity theorems for all the metabolites of a linear pathway provide exactly the number of simultaneous equations needed for solution for the flux control coefficients of all the enzymes in terms of the elasticities, but this is not the case for branched pathways or pathways containing certain types of cycle (such as substrate cycles). For these pathways, Fell & Sauro [90] proposed additional equations relating the flux control coefficients and the relative fluxes through different parts of the system. These equations, known as the branch-point and substrate cycle theorems, make the flux control coefficients expressible in terms of the elasticities and the fractional fluxes in the system. Reder [69,87] showed that such branch point equations belong to the same set of structural constraints as the summation theorem itself; they are termed structural because they are independent of the detailed kinetics of the pathway and arise merely from the form of the network; her analysis also showed that there will be sufficient of these equations to combine with the connectivity equations to allow expression of the control coefficients in terms of elasticities and relative fluxes for any metabolic system capable of reaching steady state. (The concept of structural constraints on the possible steady states of a reaction network has been developed in chemical kinetics by Clarke, e.g. [92].) Further work on the branching equations has involved methods of deriving them that avoid explicit calculation of a basis for the null space of the stoichiometry matrix [93–97] as Reder had proposed (though implicitly, these methods must give rise to results that are consistent with Reder's equations).

A corresponding set of theorems exist for the concentration control coefficients. Thus there is a summation theorem [40]:

$$\sum_{i=1}^{n} C_i^{S_j} = 0 \tag{13}$$

where S_j represents any one of the variable metabolites of the pathway. The connectivity theorem [98] becomes slightly more complex in that it has one form when the metabolite whose concentration is the subject of the control coefficients (say A) is different from the one on the elasticities (say B):

$$\sum_{i=1}^{n} C_i^A \epsilon_B^i = 0 \tag{14}$$

but the following form when they are the same:

$$\sum_{i=1}^{n} C_i^A \epsilon_A^i = -1 \tag{15}$$

As with the flux control coefficients, the form of the equation changes when metabolites in conserved cycles are involved [93]. Generally, less attention has been given to concentration control coefficients. However, in the original derivation by Heinrich & Rapoport [40], as *elements of the control matrix*, they were used to derive expressions for the flux control coefficients by means of the relationships:

$$C_i^{J_i} = 1 + \sum_{j=1}^{m} \epsilon_{S_j}^i C_i^{S_j}$$

$$C_i^{J_k} = \sum_{j=1}^{m} \epsilon_{S_j}^k C_i^{S_j} \tag{16}$$

These equations, which have a more compact but impenetrable matrix formulation, actually show how the systemic response of the flux to a modulation of an enzyme can be broken into its components. Thus, the first one shows that the response of the flux through step i to modulation of enzyme i is composed of a proportional change from the change in the amount of enzyme (the '1'), on which are superimposed the changes in activity of the enzyme because of the changes in each of the metabolites S_j, with each of these effects calculated from the concentration control coefficient of enzyme i on substrate S_j (to show how much the steady state concentration changes) and the elasticity coefficient for the effect a change in substrate S_j has on the activity of enzyme i. In the second equation, because the effect of a change in enzyme i on the flux at k is sought, there is no term for a direct effect of the change in the amount of enzyme. Another application of the concentration control coefficients and their associated theorems has been in theoretical analysis of the potential effects of metabolite channelling in enzyme–enzyme complexes on free metabolite concentrations [99].

Opinions have differed over whether the theorems are any use. Fell and co-workers [90,93] used the theorems to derive expressions for the control coefficients. Kacser and colleagues have used the theorems for their explanatory value [32,33], and many workers agree that they can be used to provide explanations of how control coefficients come to have the values that they do. On the other hand, other derivations of the properties of the

control coefficients do not rely on the theorems [8,60,69,76, 87,88,95]. Savageau and colleagues [14] have claimed that they are unnecessary because they are implicit in the mathematical description of a metabolic system at steady state. This reflects the philosophical debate, discussed by Ayer [100], about the proposition that all mathematical theorems are tautologies because they are implicit in the axioms; his conclusion that they are useful because of the limitations of our reason seems appropriate in the context of the theorems of Metabolic Control Analysis.

EXPERIMENTAL METHODOLOGY FOR CONTROL ANALYSIS

Manipulation of enzyme activity

Since the control coefficients express the effect of changing the amount of enzymes on system properties such as metabolic fluxes or metabolite concentrations, the only direct method of determination of their values is to make a change to the amount of an enzyme and observe the consequences whilst all other conditions are kept constant. A number of methods of doing this have been used, but they all share the problem that the control coefficients are defined as the change for an infinitesimally small perturbation, but the finite precision of experimental measurement requires them to be approximated from changes large enough to produce responses that can be measured. One possibility is to make a number of finite changes and extrapolate the results to an infinitely small change; ideally, balanced increases and decreases of enzyme activity should be used to avoid bias.

Alteration of expressed enzyme activity by genetic means. The classical genetic methods of breeding homozygotes and heterozygotes for different alleles of the gene for an enzyme can be used either to alter the amount of active enzyme expressed (through change in the effective gene dosage when an inactive mutant form is used) or the catalytic activity of the expressed enzyme (when polymorphic alleles of different specific activity are used). The technique often does not allow very fine adjustment of the enzyme activity, so the results can often only be interpreted as an indication of whether the control coefficient is large or small. This was the case in the studies by Flint *et al.* [70], where heterokaryons of the fungus *Neurospora* were formed to vary gene dosage of various enzymes of arginine synthesis, but it was only considered safe to conclude that the flux control coefficients of the enzymes studied were below 0.1.

Middleton & Kacser determined the effect of varying activities of alcohol dehydrogenase (EC 1.1.1.1) on the catabolism of ethanol in the fruit fly, *Drosophila melanogaster*, exploiting several allozymes [101]. It was concluded that the enzyme's flux control coefficient was close to zero, as also was its effect on the fly's ability to tolerate ethanol. These results suggest it is unlikely that the polymorphism at the alcohol dehydrogenase locus is maintained through selective pressure on alcohol metabolism.

Dykhuizen *et al.* [73] used the effects of constitutive production of different alleles of the highly polymorphic β-galactosidase (EC 3.2.1.23) of *Escherichia coli* on the catabolism of lactose, assessed by relative fitness of competing strains in a chemostat, as part of their study of the control of lactose metabolism (discussed further below).

Stitt and coworkers have been using a variety of approaches to estimate the flux control coefficients for the photosynthetic production of sucrose and starch. One of these has been the use of mutants of the flowering plant *Clarkia xantiana* with reduced activity of cytosolic or plastid isoenzymes of glucose-6-phosphate isomerase (EC 5.3.1.9) [102]. There are duplicate genes for the cytosolic enzyme so that mutants with 18, 36 and 64% of wild-type activity could be isolated. Mutants with 50 and 75% of the activity of the wild-type plastid content were also studied. Although the changes in enzyme activity were large, the flux control coefficients were calculated from a finite difference equation the authors derived on the assumption of a hyperbolic relationship between flux and enzyme content:

$$C^{J_1}_{E_1} = \frac{\frac{E_2}{E_1}\left(1-\frac{J_2}{J_1}\right)}{\frac{J_2}{J_1}\left(1-\frac{E_2}{E_1}\right)} \qquad (17)$$

where the subscript 1 signifies the flux (J) and enzyme (E) levels at the first point, and 2 those at the second. The cytoplasmic enzyme had low flux control coefficients for the fluxes of carbon fixation, sucrose synthesis and starch synthesis. The plastid enzyme had larger flux control coefficients that varied between low and high light intensities, but these could not be estimated accurately. Similar studies were made with mutants of *Arabidopsis thaliana* (L.) Heyhn. (thale cress) with 7% and 50% of wild-type activity of glucose-1-phosphate adenylyltransferase (EC 2.7.7.27) and 0% and 50% of plastid phosphoglucomutase (EC 5.4.2.2) [103]. Again, eqn. (17) had to be used to estimate the flux control coefficients for starch synthesis, which were 0.28 and 0.64 for the first enzyme in low and high light levels, and 0.01 and 0.21 for the second under the same conditions. Under high light conditions, the two enzymes account for 0.84 of the summation theory total; however, as the photosynthesis pathway is branched, with a significant flux to sucrose that will have a negative control coefficient on starch synthesis, other enzymes could also have significant flux control coefficients.

Techniques of genetic manipulation allow extra copies of a gene to be inserted on a plasmid to increase the amount of enzyme expressed. Again, it is often difficult to make small incremental changes, particularly with the variable copy number plasmids of yeast, and there is the danger of pleiotropic effects from the expression of large amounts of one enzyme [104]. Heinisch overexpressed 6-phosphofructo-1-kinase in yeast [105] but observed no effect on glycolytic flux to ethanol; this finding was later extended first to phosphoglycerate kinase [106] and then to most of the other enzymes of glycolysis [107]. Davies & Brindle [108] showed in a similar experiment that although extra phosphofructokinase had no effect on anaerobic glycolysis, it stimulated it in aerobic conditions, though the flux control coefficient must necessarily be small.

In the tryptophan biosynthesis pathway of yeast, five of the enzymes were down-modulated by varying the gene dosage in tetraploid yeast. Four of the flux control coefficients were less than 0.05, and the fifth (for anthranilate phosphoribosyltransferase, EC 2.4.2.18) was about 0.17 [109]. Up-modulation of the enzymes using multicopy vectors produced little or no increase in flux, except when all five genes were simultaneously expressed on one plasmid.

One technique for obtaining finer control over the amount of expressed protein is that used by Walsh & Koshland [110,111] for varying the expression of citrate synthase (EC 4.1.3.7) in *E. coli*; the gene, carried on a plasmid, was placed under the control of a *tac* promoter (a hybrid of the *trp* and *lac* promoters), and the plasmid also carried the *lac* repressor so that the expression of the enzyme could be varied with the gratuitous inducer isopropylthiogalactoside. These authors were not aiming to measure flux control coefficients, and interpretation is made more difficult by the interaction between the amount of citrate synthase and the amount of the active form of isocitrate dehydrogenase [111], but flux control coefficients can be estimated from the results in the first paper [110] (H. M. Sauro, personal

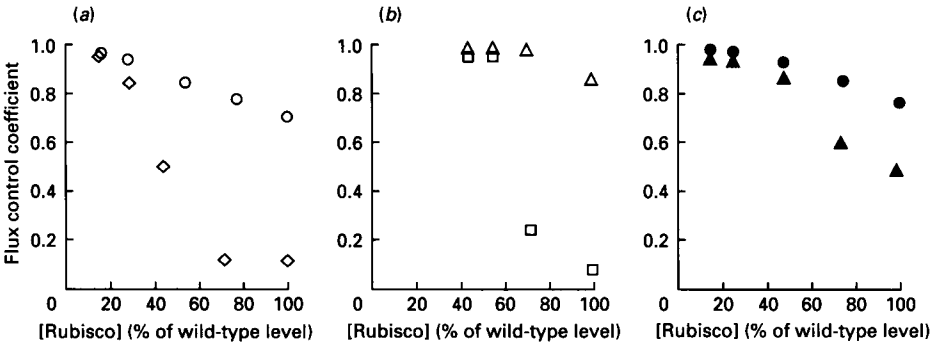

Fig. 3. Flux control coefficients of rubisco on photosynthetic carbon assimilation in transgenic tobacco plants

The diagrams have been normalized from results in [114]. The amount of rubisco was reduced below wild-type levels by using transformed plants expressing antisense RNA. (*a*) Variation of the flux control coefficient at (○) high (1050 μmol quanta·m^{-2}·s^{-1}) and (◇) moderate (350 μmol quanta·m^{-2}·s^{-1}) light intensities. Other conditions: 35 Pa CO_2, 65% relative humidity, 22 °C. (*b*) Variation of the flux control coefficient at (□) above ambient (45 Pa) and (△) below ambient (25 Pa) CO_2. Other conditions: 1050 μmol quanta·m^{-2}·s^{-1}, 65% relative humidity, 22 °C. (*c*) Variation of the flux control coefficient at (●) 85% and (▲) 35% relative humidity. Other conditions: 1050 μmol quanta·m^{-2}·s^{-1}, 35 Pa CO_2, 22 °C.

communication). At wild-type levels of expression, cells grown on acetate have a flux control coefficient for citrate synthase on overall cycle flux of 0.63, but of less than 0.2 for the flux to CO_2. The results show some unusual features in that the fluxes do not depend on the enzyme content in a hyperbolic manner, and the control coefficients appear to increase with enzyme content. Nevertheless, they offer only qualified justification for the authors' description of citrate synthase as a 'rate-controlling' enzyme under these conditions. Ruyter *et al.* [112] used a similar plasmid construct to vary the expression of one of the glucose-specific components (enzyme II^{Glc}, EC 2.7.1.69) of the phosphoenolpyruvate-dependent phosphotransferase system in *E. coli*. They could vary the level of II^{Glc} between 20 and 600% of its wild-type level by the concentration of isopropyl-thiogalactoside with little effect on the expression of other proteins of the system. Slight variations in II^{Glc} activity near wild-type levels had little effect on the rates of glucose oxidation and growth, so the control coefficients were very low. The control coefficients on methyl-α-glucoside transport and phosphorylation were about 0.6 at wild-type levels.

Portillo & Serrano used site-directed mutagenesis to create a set of yeast plasmids that carried mutant alleles of the yeast plasma membrane H^+-transporting ATPase (EC 3.6.1.35) that they expressed in an engineered yeast strain in which the chromosomal copy of the enzyme had been placed under control of a galactose-dependent promoter. Six of the mutant enzymes resulted in yeast with between 10 and 70% of the wild-type level of ATPase and proton-pumping activity. The effect on growth rate depended on the pH of the growth medium. At pH 4, it seems that the ATPase had a control coefficient of 1 on growth, but the value was lower (though not determinable exactly) at pH 6. Since the transported protons do not participate in the mass flows sustaining growth, these particular control coefficients are not constrained by the summation relationship, and are more analagous to response coefficients. (An alternative form of analysis would be to divide the system into a proton-transporting block and a block for metabolism supporting growth [44], interacting through the effects of pH on the enzymes.) Part, though not all, of the effect might represent the summation of the responses of many different enzyme activities to intracellular pH.

Modern molecular genetics techniques also allow reduction of the amount of expressed enzyme by inserting a gene that expresses anti-sense RNA. Stitt and co-workers [113,114] have used tobacco plants transformed with 'antisense' *rbcS*, the gene for the nuclear-encoded small subunit of ribulosebisphosphate carboxylase (rubisco, EC 4.1.1.39) to determine the flux control coefficients of this enzyme on photosynthesis in the leaves in varying conditions of light, CO_2 and humidity. Varying degrees of expression of the enzyme were obtained in the transformed plants, with minor changes in the contents of other photosynthetic enzymes. Although rubisco is often referred to as the 'rate-limiting' enzyme of photosynthesis, the experiments showed that the maximum flux control coefficient was about 0.8 for the wild-type range of enzyme contents; the highest values were observed with strong illumination, high humidity and low CO_2, whereas the control coefficient fell to about 0.1 at high levels of CO_2 or low light intensity (Fig. 3).

As well as the contribution that genetics can make to metabolic control analysis, there is a significant contribution the other way, for Metabolic Control Analysis shows why so many deleterious mutations in the genes for enzymes are recessive [115,116].

Alteration of expressed activity by inducers or dietary and environmental means. If it is possible to alter the degree of expression of a specific gene by some means, then the enzyme's control coefficients can be determined. The obvious problem is the degree of specificity of the effect. Dykhuizen *et al.* [73] used variable induction of the *lac* operon of *E. coli* to produce co-ordinate changes in the activities of the lactose permease and the β-galactosidase. The results were combined with those cited above to conclude that for the induced level of expression in wild type *E. coli* K12, the control coefficients on fitness (as a proxy for catabolic flux) were for the permease $C_{perm} = 0.55$ and for the β-galactosidase $C_{\beta\text{-}gal} = 0.018$.

Salter *et al.* [72] used dietary and hormonal influences to change the degree of induction of an enzyme of tryptophan catabolism, tryptophan 2,3-dioxygenase (EC 1.13.11.11), in hepatocytes isolated from treated rats. The control coefficient for catabolic flux varied from 0.75 in the controls to 0.25 in the maximally induced state.

Titration with purified enzyme. Enzyme can be added directly to a system if permeability problems can be overcome, as for example with homogenates or permeabilized cells. Applications so far of this 'enzyme titration' method have mainly involved the former. One of the first was the doubling of hexokinase, 6-phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase activities in red cell homogenates

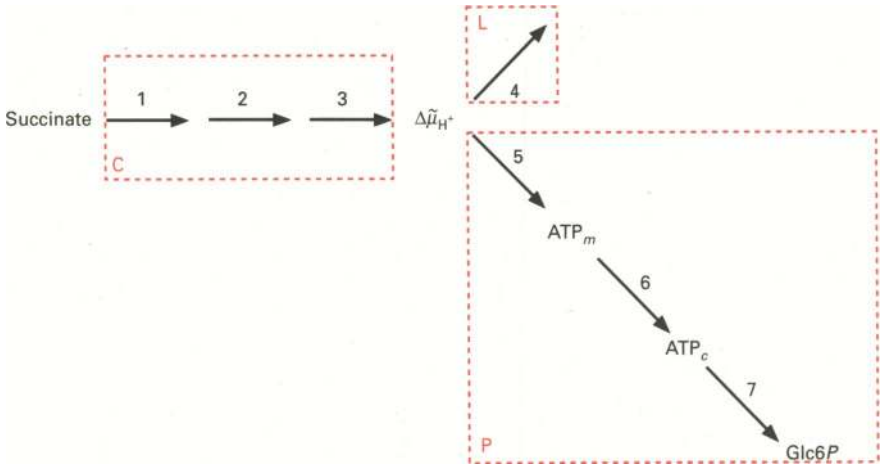

Fig. 4. Schematic representation of mitochondrial oxidative phosphorylation system

The following steps were studied by Groen *et al.* [123]: 1, dicarboxylate carrier; 2, bc_1 complex (succinate dehydrogenase not measured); 3, cytochrome *c* oxidase; 4, proton leak; 5, ATP synthase (not measured); 6, adenine nucleotide translocase; 7, hexokinase. These steps were grouped by Hafner *et al.* [144] into the three blocks outlined in red: C, respiratory chain; L, proton leak; P, phosphorylation system. $\Delta\tilde{\mu}_{H^+}$ is the protonmotive force.

[117]; though the amounts added were too large to give an accurate estimate of the control coefficients directly, application of eqn. (17) suggests the flux control coefficient of the first two would be about 0.3 each. This result is broadly consistent with the predictions of a kinetic model that the first two enzymes would have the major effect and pyruvate kinase very little. In a modification of this approach, erythrocytes were haemolysed and resealed with about a 15-fold excess of hexokinase, resulting in a near doubling of glycolytic flux [118], which corresponds to a flux control coefficient of 0.5 if eqn. (17) is applicable.

The distribution of control coefficients between the first three enzymes of glycolysis was studied in rat liver homogenates with excess (fructose-bisphosphate) aldolase and glycerol-3-phosphate dehydrogenase added to shorten the pathway [71]. Hyperbolic responses were obtained on titration with hexokinase (*HK*, for supplementing glucokinase) and 6-phosphofructokinase (*PFK*), but virtually no response to extra glucose-6-phosphate isomerase (*GPI*). The flux control coefficients obtained were $C_{HK} = 0.77$, $C_{GPI} = 0.0$ and $C_{PFK} = 0.24$. Subsequently, similar experiments have been performed to calculate the control coefficients for the transition times in the pathway [119] and to determine how the flux control coefficients varied with glucose concentration [120].

Titration of enzymes by specific inhibitors. The activity of a specific enzyme in a pathway can be decreased by the addition of an appropriate inhibitor. Rognstad suggested that if pathway flux did not initially respond to an inhibitor of an enzyme, but showed a stronger response at higher inhibitor concentrations, then the enzyme was originally 'non-rate-limiting' but became rate-limiting as its activity was reduced [121]. A rate-limiting enzyme, on the other hand, was expected to give rise to an immediate hyperbolic inhibition of pathway flux. This interpretation was criticized by Groen *et al.* [122] because the effect of an inhibitor, *I*, of some enzyme *xase*, on flux is described by a response coefficient R_I^J, composed of the enzyme's flux control coefficient and the elasticity of *I* on *xase*, i.e.:

$$R_I^J = C_{xase}^J \epsilon_I^{xase} \qquad (18)$$

Both factors must be taken into account in interpreting the results of inhibition experiments, and the shape of the inhibition profile is not a reliable guide. It is the initial slope of the graph of flux against inhibitor concentration that must be measured and combined with an estimate of the inhibitor elasticity; the equations for inactivators (i.e. irreversible inhibitors) and competitive and noncompetitive inhibitors have been given by Groen *et al.* [122,123]. They [122] used Rognstad's data [121] for the inhibition of gluconeogenesis by the action of mercaptopicolinate on phosphoenolpyruvate carboxykinase (EC 4.1.1.32), and applied their equation to conclude that the flux control coefficient of this enzyme was only 0.08, whereas Rognstad had classed the enzyme as rate-limiting because of the form of the inhibition curve.

Inhibitor titrations have been applied extensively to the determination of the flux control coefficients on respiration in isolated mitochondria and cells. Groen *et al.* [123] carried out the original study on rat liver mitochondria and demonstrated the futility of the previous arguments in the literature about whether the 'rate-limiting' step was cytochrome *c* oxidase or the adenine nucleotide translocator in State 3 respiration and states intermediate between 3 and 4. The system analysed is shown in Fig. 4. The adenine nucleotide translocator was titrated with carboxyatractyloside, the dicarboxylate carrier (which transported the respiratory substrate succinate into the mitochondria) with phenylsuccinate, cytochrome *c* oxidase with azide, and the bc_1 complex with hydroxyquinoline-*N*-oxide; the flux control coefficients of these steps on respiration were 0.29, 0.33, 0.17 and 0.03 respectively in State 3, but all varied with the respiration rate as shown later in Fig. 6. Groen *et al.* also gave figures of 0.04 for the control coefficient of the proton leak and 0.0 for hexokinase (representing ATP consumption) which leads to an overall sum of 0.86 for the measured coefficients, with those for succinate dehydrogenase and ATP synthase undetermined. Their methodology for determining the control coefficient of the proton leak by titrating with an activator (FCCP) was later criticized [124] on the basis that it contained a hidden implication that the flux control coefficient of the proton leak was 1.0 in State 4. Brand *et al.* found by a different approach [124] that the control coefficient of the leak on State 4 respiration was between 0.66 and 0.75. Several other groups have applied the inhibitor titration methodology to mitochondria, including those from yeast [125] and a plant (turnip) [126]. With submitochondrial particles, inhibitor titrations of the electron transport chain and the ATP synthase gave flux control coefficients on the phosphorylation flux of 1 [127]; although the control coefficient

of the competing proton leak on phosphorylation was not measured, arguments were presented that it could not have the value of -1 that would be required if the summation theorem were to be valid. If the summation theorem could be shown to have been violated, this would be evidence against a completely delocalized form of the chemiosmotic hypothesis [128,129].

The flux control coefficient of diacylglycerol acyltransferase (EC 2.3.1.20) on the flux from glycerol and fatty acids to triacylglycerol in rat hepatocytes has been determined by an inhibitor titration with 2-bromo-octanoate [130], giving a value of 0.76. An associated experiment with permeabilized hepatocytes, so that the actual inhibitor 2-bromo-octanoyl-CoA could be added directly, purported to show the same coefficient to lie between 0.8 and 0.99. However, in this experiment, the control coefficient was ostensibly determined from the flux response coefficient of the inhibitor divided by the elasticity of the enzyme with respect to the inhibitor (eqn. 18); unfortunately, the elasticity measurement taken was in fact a second, independent measure of flux response coefficient of the inhibitor, and so the ratio was near 1 because of the close agreement between the two measures. The authors' conclusion that the enzyme was rate-limiting was therefore not strictly validated.

Inhibitor titrations have also been used as one technique amongst others in a variety of metabolic studies that have involved control analysis:

1. To examine citrulline synthesis by isolated rat liver mitochondria from ornithine, ammonia and CO_2 [131], ornithine transcarbamoylase (EC 2.1.3.3) was titrated with norvaline and carbonic anhydrase (EC 4.2.1.1) with acetazolamide. The flux control coefficients of both enzymes were found to be less than 0.02. The flux control coefficient of carbamoyl-phosphate synthetase (EC 6.3.4.16) was the only large one found, and was estimated as 0.96, not by inhibitor titration, but by its ratio to that of ornithine transcarbamoylase using the connectivity theorem (cf. eqn. 12) and the elasticities of the two enzymes to carbamoyl phosphate, which were determined by using inhibitor titrations for the single modulation method described later.

2. The flux control coefficient of phosphoenolpyruvate carboxykinase on gluconeogenesis in hepatocytes in the presence of glucose has been determined by titration with mercaptopicolinate [132] and was found to be 0.24, in close agreement with the value obtained by these workers using a different methodology developed by Groen *et al.* [133,134] (see later). The value is higher than both that reported above, and that obtained in the studies by Groen *et al.*, though this was attributed to differences in the activity of this enzyme in the hepatocytes.

3. In the study of aromatic amino acid catabolism by hepatocytes [72], the flux control coefficient of tryptophan uptake on tryptophan catabolism was determined by inhibition with phenylalanine to be 0.22 in basal conditions and 0.70 after induction by dexamethasone. The flux control coefficient of tyrosine aminotransferase (EC 2.6.1.5) on tyrosine catabolism was estimated as 0.71 and 0.29 in basal and induced conditions by titration with amino-oxyacetate.

4. To investigate their potential as targets of chemotherapy, the flux control coefficients of steps in the pathway of *de novo* purine biosynthesis in a human leukaemia cell line [135] are being measured. The flux control coefficient of the third step, phosphoribosylglycinamide transformylase (EC 2.1.2.2) was titrated with the inhibitory substrate analogue 5-deazaacyclotetrahydrofolate. The flux control coefficient is near zero, since an appreciable inhibition of flux was not detectable until the enzyme was 90% inhibited, though the flux control coefficient then started to rise.

5. A study of the role of the glucose phosphotransferase system in the regulation of glycolysis in *Clostridium pasteurianum* [136] involved the determination of its flux control coefficient (0.14) by an inhibitor titration with xylitol.

6. An inhibitor titration of glyceraldehyde-3-phosphate dehydrogenase with iodoacetate had no effect on the glycolytic flux in yeast even though the enzyme activity was reduced to about 25% of its original level, showing the flux control coefficient of this enzyme is very small [106].

7. Another pathway with a history of unresolved arguments about whether or not a rate-limiting step exists is ethanol catabolism in mammals. A recent inhibitor titration study [137] showed that in rat hepatocytes, the maximum value of the flux control coefficient of alcohol dehydrogenase is 0.7, confirming once again that no single factor determines the rate of a pathway. This study also gave equations for evaluating inhibitor titrations with a mixed inhibitor of an irreversible Michaelis–Menten enzyme and an inhibitor of a reversible, ordered two-substrate two-product enzyme.

A problem with the inhibitor titration method is that the initial slope of the flux-against-inhibitor curve must be extrapolated to zero inhibitor for insertion into the equations for the flux control coefficient. Analysis of simulated experimental results with varying amounts of random error suggested that the procedure would be very sensitive to the errors [138] (as has been reported in practice [135]). Fitting a line to the quasi-linear initial part of the curve is unreliable; fitting a polynomial curve of low degree to a greater range of the data makes some improvement. In addition, further errors arise when the end-point of the titration is used in the calculations [139], and an analysis of inhibitor titrations of mitochondrial respiration showed it was preferable to fit an inhibition equation to the full range of the results, as the initial slopes appeared to overestimate the control coefficients. The potential problem with this approach would be uncertainty about whether the model equation were appropriate. Inhibitors of pathways can be used in other ways in control analysis (such as determination of elasticities; see below) that are possibly less sensitive to the experimental errors [138].

Control coefficients from elasticities

As explained in the section on the theorems of control analysis, control coefficients are expressible in terms of elasticities, and, for certain types of pathway, relative fluxes and concentrations. Therefore, it is in principle possible to measure the latter and calculate all the control coefficients of a pathway. This was first achieved by Groen *et al.* for hepatic gluconeogenesis [133,134]. Two major assumptions underlying this approach are firstly that the metabolic system has been adequately described in terms of component reactions and identification of all significant interactions between enzymes and metabolites, and secondly that the theorems of control analysis apply to the system (i.e. it satisfies the conditions listed in the section on assumptions of control theory). It is therefore preferable to support results obtained in this way by direct measurement of one of the control coefficients.

It is also possible to use mixed approaches, where measurement of one or two elasticities can be combined with another measurement to yield a control coefficient. One example is the use of elasticities and the connectivity theorem to calculate a second flux control coefficient when one is already known, as described above for citrulline biosynthesis [131]. Another example is the determination of the flux control coefficient of the first step in a pathway by determining the response of the pathway flux to the concentration of the pathway substrate, and the elasticity of the first step to this same substrate. The flux control coefficient is then obtained from eqn. (8). This was used by Groen *et al.* [134] in their study of gluconeogenesis from pyruvate in hepatocytes. It was also used to determine the flux control coefficients of the

aromatic amino acid transporters on catabolism of the amino acids in hepatocytes [72].

Measurement of fluxes and concentrations is standard methodology in metabolic biochemistry. Problems of intracellular compartmentation and the relationship between total and free metabolite concentrations are neither more nor less of an issue for Metabolic Control Analysis than for any other approach to interpretation of experiments in metabolic biochemistry. The novel requirement of Metabolic Control Analysis is for the values of elasticities, and there have been a number of different approaches to their estimation.

Experimental measurement *in vivo*: double modulation. This methodology for measuring elasticities was suggested by Kacser & Burns [33]. Suppose we were considering the elasticities of glucose-6-phosphate isomerase (GPI) in glycolysis:

$$\cdots \rightarrow \text{glucose 6-phosphate} \xrightarrow{GPI} \text{fructose 6-phosphate} \rightarrow \cdots$$

Scheme 3

The rate of glucose-6-phosphate isomerase depends solely on the two glycolytic metabolites glucose 6-phosphate (*G6P*) and fructose 6-phosphate (*F6P*), i.e. $v_{GPI} = f(G6P,F6P)$, and at steady state, $v_{GPI} = J$, where J is the glycolytic flux. In a control experiment, J_c and the concentrations $G6P_c$ and $F6P_c$ are measured, whilst in a parallel experiment, the rate of glycolysis is perturbed in some way by a small amount, for example by altering the input glucose level, and the new levels of flux, J_{e1} and concentrations, $G6P_{e1}$ and $F6P_{e1}$ measured at steady state. A Taylor series expansion of the expression for v_{GPI} about the control conditions, including only the first derivatives, gives:

$$\Delta J_1 \approx \frac{\partial v_{GPI}}{\partial G6P}\Delta G6P_1 + \frac{\partial v_{GPI}}{\partial F6P}\Delta F6P_1 \qquad (19)$$

where $\Delta J_1 = J_{e1} - J_c$ etc. Scaling this equation, by dividing throughout by $J_c = v_{GPI}$, leads to:

$$\frac{\Delta J_1}{J_c} \approx \epsilon^{GPI}_{G6P}\frac{\Delta G6P_1}{G6P_c} + \epsilon^{GPI}_{F6P}\frac{\Delta F6P_1}{F6P_c} \qquad (20)$$

If a second independent change can be made in the glycolytic flux, say by inhibition of an enzyme downstream from fructose 6-phosphate, leading to another equation:

$$\frac{\Delta J_2}{J_c} \approx \epsilon^{GPI}_{G6P}\frac{\Delta G6P_2}{G6P_c} + \epsilon^{GPI}_{F6P}\frac{\Delta F6P_2}{F6P_c} \qquad (21)$$

then the pair of equations can in principle be solved for the two unknown elasticities. It is important for the accuracy of the method that the two imposed changes result in mathematically independent equations $\left(\text{i.e. } \frac{\Delta G6P_1}{\Delta F6P_1} \neq \frac{\Delta G6P_2}{\Delta F6P_2}\right)$ otherwise the result will be dominated by the experimental error. Unfortunately, simulation studies suggest it might be difficult to obtain independent changes particularly for steps in the middle of a pathway [138], though the chances are maximized if one perturbation is made upstream and one downstream. The equation is only valid for small changes, but these are most likely to be dominated by the experimental error; however, it might be possible to make a series of changes of the same type but different sizes, and to estimate the initial slope of the graph at the control point [138]. If the activity of the enzyme is affected by three of the variable metabolites (for example, if the enzyme is subject to feedback inhibition), then three independent perturbations are needed, and this may be even more difficult to arrange.

Groen *et al.* [133,134] used this method to determine the elasticities of the transport of oxaloacetate by the malate/apartate shuttle between mitochondria and cytoplasm, with changes in the pathway substrate pyruvate and inhibition of phosphoenolpyruvate carboxykinase as the two perturbations. Along with other methods of elasticity determination, they were able to calculate the flux control coefficients for gluconeogenesis from pyruvate in rat hepatocytes. Their conclusions were that, in the presence of glucagon, the largest flux control coefficient was that of pyruvate carboxylase (0.83–0.89); no other enzyme (including phosphoenolpyruvate carboxykinase) had a control coefficient above 0.1. In the absence of glucagon, the largest control coefficient was still that of pyruvate carboxylase (0.56 in the presence of 5 mM-lactate), but there were a number of other significant values: pyruvate kinase, −0.19; the enolase to phosphoglycerate kinase group, 0.32, and the triosephosphate isomerase to fructose bisphosphatase group, 0.27. Similar methods were used, and comparable results obtained, in a study of the effect of glucose on gluconeogenesis [132], except that, as mentioned earlier, a larger value was obtained for the flux control coefficient of phosphoenolpyruvate carboxykinase; this value was independently validated by an inhibitor titration of the enzyme.

Kruckeberg *et al.* [102] attempted to use the double modulation method to determine the elasticities of glucose-6-phosphate isomerase in photosynthesis, but found that the measurement errors dominated the results. Stitt and coworkers have used the technique in a series of studies to determine the distribution of control between cytosolic fructose bisphosphatase (EC 3.1.3.11) and sucrose-phosphate synthase (EC 2.4.1.14) in the cytosolic pathway for utilization of photosynthetic triose phosphates (Fig. 5). The analysis is complicated by the need to take into account the role of fructose 2,6-bisphosphate through an 'effective' elasticity for the hexose phosphates, on the basis that the levels of the effector molecule are linked to those of the hexose phosphates [140]. In *Spinacia oleracea* L. (spinach) the elasticities of the two enzymes to substrates, products and effectors were determined by double modulations using several different light levels and different sucrose contents as the two modulations [140,141]. At low irradiances, application of the connectivity relationship (*cf.* eqn. 12) shows the flux control coefficient of the fructose bisphosphatase block to be 0.5–0.7, and that of the sucrose-phosphate synthase block 0.3–0.5 (Strictly these are relative values because only part of the complete pathway has been analysed.) The two modulations in *Clarkia xantiana* were the light level and the level of cytosolic glucose-6-phosphate isomerase [140,142] using the mutants mentioned previously. The estimates were reasonably consistent with elasticities based on kinetic measurements *in vitro* [140], and show that the control coefficients of the two enzymes will both be about 0.5, though the exact distribution of control between the two will depend on conditions such as light intensity.

Experimental measurement *in vivo*: single modulation. When one term in eqn. (20) is known or insignificant, only a single perturbation type is needed. This has the advantage that several modulations of the same type but different sizes can be made; the elasticity is then the gradient of a graph of logarithm of rate against logarithm of the metabolite concentration taken at the point corresponding to the control value.

Groen [133,134] used this method to estimate the elasticity of pyruvate kinase with respect to phosphoenolpyruvate and the elasticity of pyruvate carboxylase with respect to cytoplasmic oxaloacetate as part of the experiments cited in the previous section. In the studies of citrulline synthesis described earlier, the elasticity of ornithine transcarbamoylase to carbamoyl phosphate was determined by modulating the pathway flux at carbamoyl

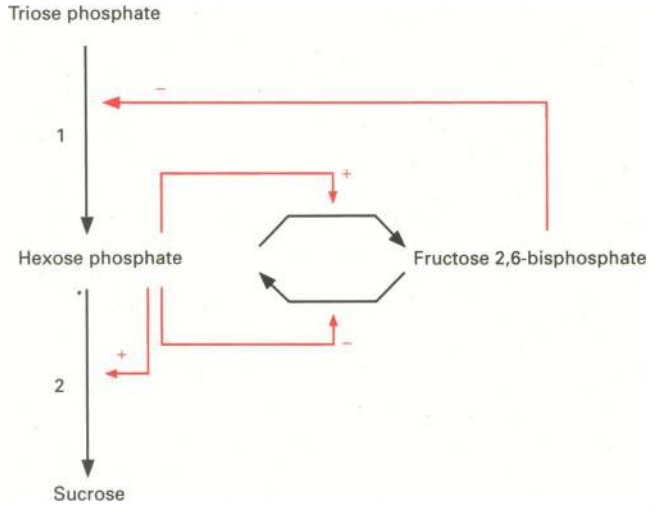

Fig. 5. Photosynthetic sucrose synthesis in the cytosol

A schematic representation of the cytoplasmic pathway investigated by Stitt and coworkers. Black lines indicate enzymic reactions: 1, fructose bisphosphatase plus aldolase and triosephosphate isomerase; 2, sucrose phosphate synthase plus UDPG synthase and sucrose phosphatase.

phosphate synthase via alteration of the ATP level through titration with the inhibitor malonate [131]. Similarly, the elasticity of carbamoyl phosphate synthase to carbamoyl phosphate was determined by modulation of the pathway flux by titration of the other enzyme with its inhibitor norvaline.

Brand, Brown and coworkers have used this method as a central feature of their 'top-down' approach to Metabolic Control Analysis [143,144], which they have specifically applied to the control of mitochondrial respiration and oxidative phosphorylation [124,144,145,146]. Their argument is that a complete control analysis of all the enzymes in a pathway is too demanding a study; therefore, since it is known that is possible to have elasticities and control coefficients for a block of enzymes in metabolism [34,90,93], it is preferable to start the analysis by dividing the system into a small number of blocks. When the system has been analysed in this way, the next stage is to look inside the blocks in increasing detail. Thus a linear pathway might be divided into two about a single metabolite that is the product of one block and the substrate of another, and has no other interactions with components of the block, i.e.:

$$X_0 \overset{\text{Block 1}}{\rightarrow} S_1 \overset{\text{Block 2}}{\rightarrow} X_1$$

Scheme 4

The relationship between the block elasticities and the elasticities of the component enzymes in the block has been described theoretically [90,93] and the regulatory properties of this model considered [1]. Brown, Brand *et al.* treated respiration, phosphorylation and the proton leak rate as the three blocks in the metabolism of isolated mitochondria [144] and rat hepatocytes [146] (Fig. 4). The common metabolite is regarded as the protonmotive force. The elasticity of any one block with respect to the protonmotive force was determined by inhibiting or stimulating one of the other blocks; thus if the elasticity of the phosphorylating system was required, it was obtained from the slope of the phosphorylation-coupled component of the respiration rate as the respiratory chain block was titrated with the inhibitor malonate. The justification of the use of elasticities with respect to the protonmotive force rather than a metabolite concentration follows from the work of Westerhoff and colleagues [147,148]; since the chemical potential of a species, or an associated electrochemical gradient, depends linearly on the logarithm of concentration, the denominator term $\ln S$ in eqn. (7) can be replaced by a chemical or electrochemical potential. The summation and connectivity theorems still remain valid with this substitution.

An application of this approach was the determination of the elasticity of mitochondrial cytochrome *c* oxidase with respect to protonmotive force and degree of reduction of the cytochrome *c* pool [145]. This was followed by determination of the elasticities of the proton leak and the respiratory chain to protonmotive force in non-phosphorylating mitochondria [124], to show, by application of the summation and connectivity theorems that the control of respiratory flux was shared by the leak (0.66–0.75) and the respiratory chain (0.34–0.25). The study of phosphorylating mitochondria mentioned above, which divided the process into three blocks [144] (Fig. 4), gave flux control coefficients for each of the three blocks on each of the three fluxes: respiration (C), phosphorylation (P) and the proton leak (L) at a range of respiration rates between states 4 and 3. The range of the coefficients for the respiration flux between the ends of the range was approximately: $C_L^{J_c} = 0.9–0.0$; $C_P^{J_c} = 0.0–0.5$, and $C_C^{J_c} = 0.1–0.5$ (Fig. 6). The profile between these points resembles the results Groen *et al.* obtained by inhibitor titrations [123]. The control of mitochondrial respiration in resting hepatocytes has also been analysed by this technique [146]. In one set of experiments, the $NAD^+/NADH$ couple was taken as the central metabolite, dividing the system into an NADH-producing block (glycolysis and Krebs' cycle) and an NADH-consuming block (the respiratory chain etc). $C^{J_c}_{supply}$ was estimated as 0.15–0.3, with $C^{J_c}_{demand}$ as 0.85–0.7. Experiments using the mitochondrial membrane potential as the central 'metabolite' showed that the flux control coefficient of ATP synthesis and consumption was about 0.5, so that it constituted the largest part of the 'demand' control coefficient. Overall, however, the control of respiration is divided between NADH generation, the respiratory chain and ATP synthesis and consumption, with no single factor being anything near 'rate-limiting'.

One criticism that might be made of the results of Brand's group is that they assume the applicability of the summation and connectivity theorems. This requires that the assumptions stated earlier in this paper apply to mitochondrial oxidative phosphorylation, and, in addition, that the protonmotive force is indeed delocalized (as noted by the authors themselves). However, another assumption is that the stoichiometry of the system should not be dependent on the variables, as is evident in the derivation of the theorems by Reder [69], where it is assumed that the terms in the stoichiometry matrix are not functions of either the variables or the parameters of the control coefficients. Westerhoff & Kell [129] considered the effect of the violation of this requirement by a dependence of the proton stoichiometry of phosphorylation on the protonmotive force. They concluded that it introduced a new term involving the scaled slope of the proton stoichiometry against protonmotive force into the control coefficients. The summation theorem should still apply and the connectivity theorem will be valid for the elasticities of the proton fluxes with respect to the protonmotive force but not those for respiration and phosphorylation (H. V. Westerhoff, personal communication). Brand *et al.* state that their analysis would not be affected by variable stoichiometry from pump slippage [124], which would only affect the interpretation of the elasticity coefficient and not the applicability of the connectivity theorem, provided that all the fluxes are measured in terms of the oxygen flux and not the proton fluxes. Probably the lack of mechanistic detail in the three blocks of the top-down approach allows the problem, if it exists, to be hidden in the blocks, but

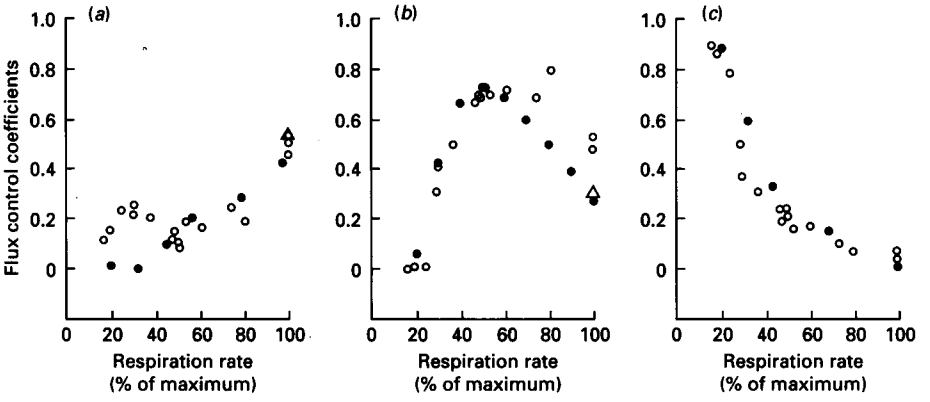

Fig. 6. Flux control coefficients for mitochondrial respiration rate as a function of respiration rate

The results of Hafner *et al.* [144] are compared with the corresponding results of Groen *et al.* [123]. (*a*) The flux control coefficient of the respiratory chain (block C, Fig. 4): ○, Hafner *et al.*; ●, results of Groen *et al.* for the dicarboxylate carrier only (step 1 in block C, Fig. 4); △, sum of the coefficients obtained by Groen *et al.* for steps 1 to 3 of block C. (*b*) The flux control coefficient of the phosphorylation block (block P, Fig. 4): ○, Hafner *et al.*; ●, sum of the coefficients for the adenine nucleotide translocator and hexokinase (steps 6 and 7, block P) obtained by interpolation of smooth curves drawn through the experimental points reported by Groen *et al.*; △, sum of actual values reported by Groen *et al.* for these two steps. (*c*) The flux control coefficient of the leak (block L). ○, Hafner *et al.*; ●, Groen *et al.*

whether it could be circumvented in the same way in an analysis at a greater level of detail remains to be seen. Although dependence of stoichiometry on protonmotive force has been reported, a potential error was identified in certain of the studies (e.g. [149]), and a recent reinvestigation concluded that there was no significant dependence of P/O stoichiometry [150]. There was also the indication quoted earlier that the summation total of the control coefficients on phosphorylation flux in submitochondrial particles was greater than 1 [127]. Nevertheless, the similarity between the results obtained for mitochondrial respiration by inhibitor titrations and a disputed method of measuring the control coefficient of the leak [123] and those by the 'top-down' approach using elasticity determinations and the theorems of control analysis [144] surely suggests that the results are relatively insensitive to any of these potential complications (Fig. 6).

Calculation of elasticities. For most of this century, enzyme kineticists have attempted to determine appropriate mathematical functions to describe the dependencies of the rates of reactions of enzymes on metabolites and then to evaluate the parameters of the equations experimentally. Where a suitable function for an enzyme has been determined, then its elasticities can be derived by analytical partial differentiation with respect to each of the metabolites in accordance with eqn. (7). Although differentiation of these functions is often tedious by hand, the wide availability of symbolic algebra programs for computers has removed the need for a high order of mathematical skill. For the purposes of control analysis, it is not necessary that the function used for the kinetics is soundly based mechanistically; it suffices that it describes the enzyme's responses to its metabolites over the physiological concentration range. The final stage in the calculation of the required elasticity value is to insert numerical values for the parameters in the equation, such as K_m values, and the values for the concentrations of all the necessary metabolites. This raises all the usual problems of interpretation. Are the values of the kinetic parameters determined *in vitro* applicable in intracellular conditions? Fortunately, $V_{max.}$ values turn out not to be required because they cancel out when the elasticities are scaled to their dimensionless form. Are the measurements of cellular metabolite concentrations representative of the actual concentrations experienced by the enzyme in the cell? Have all the significant effectors of the enzyme's activity been accounted for? None of these problems is unique to Metabolic Control Analysis; they apply equally to other forms of biochemical explanation, and the reductionist ethos of much biochemistry (to seek an understanding of the functioning of biochemical systems through the study of the molecular properties of its components) is undermined if there is no confidence in the applicability of such results. Nor are the results of Metabolic Control Analysis necessarily particularly sensitive to inaccuracies in such calculated elasticities. For elasticities smaller in magnitude than 1, the value of the elasticity is likely to be a less sensitive function of the metabolite concentration than is the rate of the enzyme. Furthermore, the values of the control coefficients are not equally sensitive to the values of all the elasticities; the dependence of the control coefficients on the elasticities has been analysed [138,151] and a methodology for identifying those elasticities that have the greatest influence on the results devised. The analysis confirmed the expectation that elasticities of large magnitude would have little influence on most flux control coefficients, and showed that small feedback and product inhibition elasticities would be particularly important. Thus approximate values of many of the elasticities will often suffice, so calculation with its attendant problems may well be acceptable. Unfortunately, the literature base for enzyme kinetics is stronger on information for the less important substrate elasticities than for the more important weak product and effector elasticities. Apart from the experimental difficulties, this seems to reflect an unwillingness to face up to the very limited relevance of initial rate studies of enzymes in the absence of products to conditions *in vivo*. Only with the advent of quantitative analysis of metabolic systems such as Metabolic Control Analysis and Biochemical Systems Theory [22,25] has the significance of adequately characterizing apparently weak inhibition effects been apparent.

For enzymes that are near to equilibrium, Groen showed [43,133] that the elasticities with respect to substrate and product are dominated by the degree of displacement from equilibrium rather than the kinetic details of the reaction. For example, consider the reversible Michaelis–Menten equation for the conversion of S to P:

$$v_{net} = \frac{V_{m,f}/K_{m,S}(S - P/K_{eq.})}{1 + S/K_{m,S} + P/K_{m,P}} \qquad (22)$$

where v_{net} is the net rate (positive for formation of P), $V_{m,f}$ is the maximum velocity in the forward direction, $K_{m,S}$ and $K_{m,P}$ are

the two K_m values and $K_{eq.}$ is the equilibrium constant for the reaction. Differentiation and scaling of this equation gives:

$$\epsilon_S^v = \frac{1}{1-\Gamma/K_{eq.}} - \frac{S/K_{m,S}}{1+S/K_{m,S}+P/K_{m,P}}$$

$$= \frac{1}{1-\Gamma/K_{eq.}} - \frac{v_f}{V_{m,f}} \quad (23)$$

where Γ is the mass action ratio P/S and v_f the total forward rate. The first term on the right-hand side depends on the degree of displacement from equilibrium, going from 1 in the absence of product to infinity at equilibrium, and the second term represents the fractional saturation of the enzyme with S, going from 0 in the absence of S to a maximum value of 1, giving the overall result illustrated in Fig. 2. (But note that $S \gg K_{m,S}$ is not a sufficient condition for the saturation term to reach 1 if $P \gg K_{m,P}$; 'saturated' can be a vague concept with a near-equilibrium reaction.) In many practical cases, the elasticity of a near-equilibrium reaction will be given with sufficient accuracy by the term $1/(1-\Gamma/K_{eq.})$. The product elasticity is given similarly by:

$$\epsilon_P^v = \frac{-\Gamma/K_{eq.}}{1-\Gamma/K_{eq.}} - \frac{P/K_{m,P}}{1+S/K_{m,S}+P/K_{m,P}}$$

$$= \frac{-\Gamma/K_{eq.}}{1-\Gamma/K_{eq.}} - \frac{v_r}{V_{m,r}} \quad (24)$$

Where the saturation terms are negligible, it follows that the two elasticities are related as:

$$\epsilon_S^v + \epsilon_P^v = 1 \quad (25)$$

Groen also showed [133] that similar equations apply to multisubstrate rate equations having numerators of similar form to that of eqn. (22).

Several of the studies mentioned previously have relied on calculation methods for some of the elasticities. The first was the study of the control of gluconeogenesis in hepatocytes by Groen *et al.* [133,134] whose overall result was cited earlier. Salter *et al.* used calculated elasticities in their study of the control of aromatic amino acid metabolism by hepatocytes [72]. In particular, they calculated the elasticities of the amino acid transporters to their amino acids from the above equations, and combined them with the response of the catabolic fluxes to the external levels of the amino acids to calculate the control coefficients of the transport steps. These varied from about 0.25 for transport of tyrosine and tryptophan at basal catabolic levels to 0.93 for phenylalanine transport when catabolism was induced.

Fell & Snell [152,153] analysed the three enzyme pathway for the synthesis of serine from the glycolytic intermediate 3-phosphoglycerate using published information on the metabolite concentrations in rabbit liver and the kinetics of the final enzyme phosphoserine phosphatase (EC 3.1.3.3). In normal rabbit liver where there is a relatively high biosynthetic flux, this pathway is unusual in that it is the final enzyme of the pathway, phosphoserine phosphatase, that has the largest flux control coefficient (0.97), whereas the first two enzymes are close to equilibrium and have negligible flux control coefficients. Phosphoserine phosphatase is inhibited by the pathway product serine, for which a response coefficient of -0.63 on the pathway flux can be calculated. In contrast, the response coefficient of the flux to the pathway source, 3-phosphoglycerate, is about a tenth of this value, so the flux is largely determined by the serine concentration through inhibition of the final step. In conditions where the biosynthetic flux is smaller, the control coefficient of the phosphatase is decreased at the expense of the first two enzymes of the pathway.

A mixture of experimental measurements of glycolytic intermediates by n.m.r., modelling of the glycolytic pathway, and calculation of the elasticities of the model equations at the observed levels of intermediates was used to determine the flux control coefficients for glycolysis in suspended and immobilized *Saccharomyces cerevisiae* [154]. The results varied for the different incubation conditions used, though the largest control coefficients were shown by glucose entry into the cell, phosphofructokinase, and ATP consumption processes. However, the conclusions depend strongly on two elasticities that are not well-characterized: the feedback inhibition of glucose transport by glucose 6-phosphate and the group elasticity of ATP-utilizing processes with respect to ATP.

Elasticities have also been calculated in their own right as a useful measure of the strength of various feedback inhibitors on branched-chain 2-oxoacid dehydrogenase complex (2-oxoisovalerate dehydrogenase, EC 1.2.4.4) [155]. Appropriate kinetic measurements were made to determine the parameters of the rate equation, which was differentiated to give the equation for the elasticities, and solved numerically for metabolite concentrations representative of those found *in vivo*.

CONTROL COEFFICIENTS FROM MODELS

There is increasing interest in the construction of mathematical models that simulate some aspect of metabolism. Few of these models can be solved algebraically, but the wide availability of increasingly powerful computers and libraries of appropriate numerical routines make numerical solution feasible in many cases. The computer simulation of metabolism merits a review in its own right, so only those studies of relevance to Metabolic Control Analysis can be mentioned here. Once a model of a pathway has been constructed, it is possible to perturb one of the parameters that represents an enzyme activity, and, by observing the calculated effect, determine a control coefficient faster and with greater accuracy than by experiment. The question of whether the calculated coefficients reflect those of the original system must be addressed. The essence of a model is that it is a simplification of reality; it is likely that a model will reproduce some aspects of the behaviour of the real system with acceptable accuracy, yet fail to represent other aspects correctly, so some validation of the results is required. On the other hand, it could be argued that even a model that makes poor predictions of the control coefficients can have heuristic value. Indeed, regardless of the accuracy of any of the models to be discussed below, it is notable that they generally show several enzymes having moderate flux control coefficients rather than the existence of a single rate-limiting enzyme.

A metabolic model will usually consist of a set of ordinary differential equations, usually nonlinear, that describe the net rates of production and utilization of metabolites. There are two types of numerical solution: a time course can be simulated from some set of starting concentrations, so that, if the model has a steady state solution, the concentrations may eventually converge on it; alternatively, the set of equations describing the production and utilization of each variable metabolite can be solved, using a general routine for nonlinear equations, for the set of concentrations that make the net rates zero. The latter process is less robust computationally, and may require a good initial estimate of the solution, but when it works, it is a direct route to the solution. There is a slight risk of finding a steady state that would not be attainable because it is unstable, but this can be tested. Simulations of time courses can be simpler, though they can be inaccurate or excessively slow if an inappropriate choice has been made of numerical method for integrating the equations. The main danger is that, since the steady state is approached

asymptotically, a decision has to be made as to when the solution is no longer very far from the steady state; if there is a wide spread of rates of reaction in the model, as is common, then it is possible to overlook the long term effects of apparently insignificant slow changes taking place after the early stages where the rapid reactions dominate. Therefore, for determination of control coefficients in a model, there may be advantages to using steady state solutions, though this has probably been less common because computer packages for simulation have been more widely available. However, some recent biochemical simulation programs do determine steady state solutions [156,157], and like some others [158], calculate control coefficients automatically.

Many of the contributions from Heinrich and colleagues to the theory of Metabolic Control Analysis have been linked to the development of models of the metabolism of the human erythrocyte from an initial form modelling just the glycolytic pathway [159] through a series of versions to one that includes the 2,3-bisphosphoglycerate bypass and, via the membrane ATPase, ion fluxes and volume changes [117,160,161]. Control coefficients on the fluxes and metabolite concentrations have been calculated. Other models of erythrocyte metabolism have included theoretical examination of the effects of inherited enzyme deficiencies [85,162] and the regulation of the hexose monophosphate shunt [163,164]. The control coefficients of the period of glycolytic oscillations in yeast have been compared between the experimental system and a model [165].

Ottaway & McMinn [166,167] used control analysis to interpret the behaviour of their model of the citric acid cycle in mitochondria under different conditions. An apparent breach of the summation theorem was traced to the effects of sequestration of conserved metabolites by the high concentrations of enzymes (cf. [68]).

Kohn and colleagues have used the concepts of metabolic control analysis, extended to include time-dependence in transients [53], in the sensitivity analysis of some of the metabolic models developed by Garfinkel's group [168,169].

A number of models of photosynthesis have been proposed to aid in understanding how its rate responds to environmental conditions. Some of these have been subject to sensitivity analysis, and Metabolic Control Analysis has been taken as the methodology in some of these [170–173]. The dependence of the control coefficients for the photosynthetic flux on the conditions predicted by the models is complex, but, like the experimental studies reported above, they show that a high flux control coefficient for ribulose bisphosphate carboxylase is obtained in some conditions, but in others, it declines and other steps have comparable or higher coefficients.

Finally, a point of contrast between Metabolic Control Analysis and Biochemical Systems Theory is that the latter was developed with system modelling as part of its aims [21], so simulation and sensitivity analysis can be closely linked in it, taking advantage of the computational gains that can arise from having all the equations expressed in the same power-law form [84,174,175].

THEORETICAL APPLICATIONS

Theoretical development of Metabolic Control Analysis has accelerated during the past 10 years. Much of this has been mentioned earlier in the context of investigating the effects of relaxing some of the assumptions and constraints behind the original versions of control analysis, such as proportionality between enzyme concentration and activity, and will not be returned to here. Other broad lines of development can be recognized in other groups of papers.

My group attempted to make the application of control analysis to different pathway configurations easier by developing rules for writing matrix equations that could be solved for the control coefficients [90,93,176], as have others [94,97]. A variety of approaches were adopted by authors who wished to establish the mathematical validity of such matrix equations and the theorems of control analysis without reference to particular pathway configurations, e.g. [60,69,75,87,76,88,89,95,98,177]. The search for more accessible ways to determine the relationships between control coefficients and elasticities has led to graphical or diagrammatic methods [178–181] and the use of analogies from electrical circuits [182]. As happened in enzyme kinetics, the use of computers to generate and solve the equations is being developed [183,184,185].

Another line of theoretical development has been the metabolic control analysis of particular pathway structures. Often this builds on work that has used other methods of sensitivity analysis, and part of the aim is to bring these different approaches into a consistent framework under Metabolic Control Analysis. The application to substrate cycles has been reviewed previously [186]. Covalent modification cascades have been studied although they are a difficult subject for Metabolic Control Analysis because of the multiple levels created by an enzyme in one pathway being a metabolite in another [44], and because their behaviour needs to be followed over large changes in enzyme activity. The properties required of the modifying enzymes in cascades in order to generate high sensitivity to effectors have been explored [67,187,188]. The links between Metabolic Control Analysis and theoretical studies of the evolution of metabolic pathways have been covered in a recent review [189].

Finally, Delgado & Liao have developed the theory of a potential new experimental method of determining flux control coefficients [190]. A simpler and more reliable variant was then devised [191]. It assumes the possibility of measuring the concentrations of metabolites as a pathway approaches a steady state after a perturbation. There will be technical difficulties in implementing the method because of the requirement for accurate measurements at a sufficiently closely spaced set of time points. Spectroscopic measurements would be ideal, and it is unfortunate that n.m.r. at present probably cannot offer speed and sensitivity simultaneously. The analysis relies on mathematical links established between the decay of the perturbation and the control coefficients in a pathway sufficiently close to the steady state that a linear approximation to the kinetics is valid.

CONCLUSION

This review, by classifying published studies by the experimental techniques employed, may give a rather fragmentary view of the results for particular metabolic systems. (In contrast, a recent review has focused on the applications of control analysis in microbial metabolism [192].) Nevertheless, some general points can be discerned in the work cited. Firstly, whatever criticisms might be made about any one of the experimental studies, it is significant that none have provided support for the concept of the existence of unique 'rate-limiting enzymes' in pathways. Occasionally a particular enzyme may exhibit a flux control coefficient close to 1 under a certain range of conditions, but generally there is a distribution of control that varies with circumstances. The simulations of model systems reinforce this conclusion. It is therefore not surprising that most attempts to change the flux of a metabolic pathway by producing more of a particular enzyme from additional copies of a gene introduced into the cells have had little effect (e.g. [105,107]). Thus if an aim in biotechnology is to increase the flux in a target pathway, knowledge of the system properties of the metabolic

pathway, such as that derived from Metabolic Control Analysis, can indicate the likelihood of success if only a single enzyme is altered either in activity or kinetic properties [94]. In the absence of such an analysis, the default assumption must surely be that the chance of success is poor, or that at best the results will be modest. This is not the case in targeting enzymes with drugs designed to inhibit them and thus to suppress a metabolic pathway for therapeutic purposes; with sufficient inhibitor, any enzyme will eventually come to have a flux control coefficient of 1 (i.e. be rate-limiting) on the flux in its own branch of a pathway, and in principle the pathway can be completely stopped. Therefore the identification of enzymes with high flux control coefficients is not essential, though it might help to ensure a good response of the flux to low levels of the drug.

Even if the flux control coefficients cannot be readily determined for a pathway, it might be more useful to determine the elasticities of the enzymes with respect to substrates, products and effectors rather than the traditional kinetic parameters for assessment of their regulatory and control properties *in vivo*. Elasticity is a more useful indicator of strength of metabolite–enzyme interaction in the operative conditions of a pathway than qualitative descriptions predicated on a generally inappropriate model (the single-substrate irreversible Michaelis–Menten equation) of enzyme action in metabolic pathways *in vivo*. Control analysis has shown the importance of assessing even apparently weak product inhibition characteristics of irreversible enzymes.

There is no doubt that experiments to determine control coefficients can be difficult to plan and execute, though the experimental techniques required are the same as those generally used in metabolic biochemistry for other purposes. Even so, the number of experimental applications is increasing rapidly. The theoretical development of Metabolic Control Analysis has advanced into topics that have not yet been explored experimentally, encouraged perhaps by critics who have claimed that Metabolic Control Analysis would not be adaptable to deal with this or that situation. Continued elaboration of the theory would probably now be counter-productive before the need for it is apparent experimentally. Where theory could make a significant contribution would be to solve the main limitation of the existing theory of Metabolic Control Analysis so that the response and control coefficients, which are only reliable predictors of the effects of small perturbations, can be related to the response to large changes encountered in certain genetic or physiological experiments. A recent exploration of this topic has shown that, under certain circumstances, the large deviations measured can be related to certain functions of the control coefficients (J. R. Small & H. Kacser, personal communication). Otherwise, the major need is to evaluate existing experimental techniques and to suggest new ones, so that the body of experimental examples continues to increase.

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