

Information transfer in metabolic pathways

Effects of irreversible steps in computer models

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Various metabolic models have been studied by computer simulation in an effort to understand why allowing for the reversibility of the reaction catalysed by pyruvate kinase, normally considered as irreversible for all practical purposes, significantly altered the behaviour of the model of glycolysis in *Trypanosoma brucei* [Eisenthal, R. & Cornish-Bowden, A. (1998) *J. Biol. Chem.* **273**, 5500–5505]. Studies of several much simpler models indicate that the enzymes catalysing early steps in a pathway must receive information about the concentrations of the metabolites at the end of the pathway if a model is to be able to reach a steady state; treating all internal steps as reversible is just one way of ensuring this. Feedback inhibition provides a much better way, and as long as feedback loops are present in a model it makes almost no difference to the behaviour whether the intermediate steps with large equilibrium constants are treated as irreversible.

In the absence of feedback loops, ordinary product inhibition of all the enzymes in the chain can also transfer information; this is efficient for regulating fluxes but very inefficient for regulating intermediate concentrations. More complicated patterns of regulation, such as activation of a competing branch or forcing flux through a parallel route, can also serve to some degree as ways of passing information around an irreversible step. However, they normally do so less efficiently than inhibition, because the extent to which an enzyme or a pathway can be activated always has an upper limit (which may be below what is required), whereas most enzymes are inhibited completely at saturating concentrations of inhibitor.

Keywords: feedback inhibition; reversibility; metabolic modelling; steady state.

Starting with the pioneering work of Garfinkel & Hess [1], computer simulation of metabolism long remained highly specialized, being implemented with programs written by experts for experts to use. In the past decade, several programs have appeared that will run on widely available computer systems, including METAMODEL [2], SCAMP [3], GEPASI [4] and JARNAC [5], which have made metabolic simulation accessible to all. However, some precautions remain necessary for ensuring that a metabolic model is able to mimic accurately the behaviour of a real system. A complete model requires the values of a very large number of parameters, many of which may not be available from experimental measurements. The question then arises of how to handle missing information, e.g. the kinetic parameters for the reverse reaction of an enzyme that has only been studied experimentally in the direction regarded as physiological.

Usually, reversible rate equations are used for all processes, apart possibly from steps that remove metabolites from the system under study and steps considered to

represent 'the rest of metabolism'. To some degree, this just reflects chemical reality: all reactions have finite standard Gibbs energies and thus have at least some degree of reversibility. Physiological discussions of metabolism often take a broader view than this, however, and many reactions that have measurable reverse reactions are conventionally considered irreversible for physiological purposes. For example, in an account typical of those to be found in textbooks, Stryer [6] describes the reactions catalysed by hexokinase, phosphofructokinase and pyruvate kinase as 'virtually irreversible' and takes this as a reason to expect them to have roles in the regulation of glycolysis. As the equilibrium constant of the hexokinase reaction is less than 250 at pH 6.5 [7], characterizing the reaction as irreversible may appear rather extreme, but under normal physiological conditions the concentration of glucose is always much greater than that of glucose 6-phosphate and the concentration of ATP is usually greater than that of ADP. Under these conditions, the forward direction is thus favoured much more than the equilibrium constant alone might suggest. The equilibrium constant of the pyruvate kinase reaction is much larger, of the order of 10^5 , and this reaction is almost always regarded as irreversible, the back reaction being ignored completely.

It follows that for metabolic simulation it may seem quite safe to treat the pyruvate kinase reaction as irreversible, as has been done for in nearly all models incorporating this enzyme until now [1,8–13], with only rare and recent exceptions [14–16]. (In some of these models, less clearly irreversible enzymes like hexokinase and phosphofructokinase have also been treated as irreversible.) However, a

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Enzymes: hexokinase (EC 2.7.1.1); α -ketoglutarate dehydrogenase (EC 1.2.4.2); phosphofructokinase (EC 2.7.1.11); pyruvate kinase (EC 2.7.1.40).

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model of glycolysis in *Trypanosoma brucei* was found to give substantially different behaviour depending on whether the reversibility of pyruvate kinase was allowed for [17]. Apparently, even reactions with very large equilibrium constants cannot safely be modelled with strictly irreversible rate equations, implying that full reversible kinetic equations are needed for all the enzymes in a complex pathway even if no experimental data for the reverse reactions are available.

In view of the continued widespread use of irreversible reactions in metabolic modelling studies [10–13,18], we have thought it necessary to examine some simple models to understand why allowing for the reverse reaction of a reaction with a very large equilibrium constant may sometimes make a substantial difference to the way in which the model behaves. Without such understanding one would have to conclude that reversibility should always be allowed in all reactions, regardless of the magnitudes of their equilibrium constants. This would be a very inconvenient conclusion, for several reasons: it would require all conclusions drawn from previous models that included irreversible steps to be reconsidered; unnecessarily allowing for reversibility introduces extra complexity into a model that may already be undesirably complex; and lastly, for enzymes that are conventionally regarded as irreversible no experimental data may be available for the kinetics of the reverse reaction, forcing the kinetics to be guessed on the basis of knowledge of the equilibrium constant and the kinetics of the forward reaction.

We made a preliminary investigation of this problem [19] in the context of a simple straight-chain model with one almost irreversible step. We compared the effect of allowing for the small degree of reversibility with the effect of keeping it fully irreversible but introducing a feedback inhibitory effect of a metabolite that comes after the irreversible reaction on an earlier step in the pathway. Such feedback loops around near-irreversible steps often exist in real pathways, and we wished to establish whether allowing for reversibility was more or less effective than allowing for feedback inhibition for assuring the possibility of a steady state. Somewhat surprisingly, there proved to be very little difference in this example: allowing for reversibility and allowing for feedback both generated major changes in models initially set up with neither, and allowing for both gave results barely different from those obtained with just one or the other.

The most reasonable interpretation of this result is that a model can only achieve a steady state under conditions where the real system would do so if it includes a mechanism for transfer of information from the late steps to the early steps in a pathway, the exact nature of the mechanism being unimportant. If correct, this interpretation means that a small degree of reversibility in any reaction can safely be ignored (thereby allowing much simpler rate equations and avoiding the need to guess parameter values when no experimental data are available) provided that some other mechanism exists to allow early steps to sense the concentrations of metabolites near the end of the pathway. It is therefore important to know whether this interpretation of the earlier results is general: this is what we set out to determine in the present study by examining a broader range of models that include a variety of mechanisms for information transfer.

The most usual mechanism used in metabolism to supply early steps with information about the concentrations of downstream metabolites is, of course, feedback inhibition, and in very short pathways [20] ordinary product inhibition may also suffice. A recent investigation of product insensitivity in metabolism [21] showed how it affects the distribution of control in a pathway. This is an important consideration, but is somewhat different from our main concern here, the fact that if product sensitivity is omitted from a model it may be unable to achieve a steady state unless information is transferred around the insensitive enzyme in some other way.

EXPERIMENTAL METHODS

In the present investigation, we initially used version 3.21 of the metabolic simulation program GEPASI [4]. However, with this version we were unable to reproduce the results for the *T. brucei* model that had been obtained earlier [17] with what was then the current version, 3.01. The differences are numerically very large, and appear to be due to differences in the ways the two versions handle transport across membranes. The rates calculated by version 3.01 agree with those obtained with a hand calculator (whereas those calculated by version 3.21 do not) and they also agree with those given by the simulation program JARNAC [5], a

Table 1. Kinetic parameters for the model illustrated in Fig. 1. The symbols refer to those in Eqn (1) of the text, unless otherwise noted. Metabolite concentrations are shown by lower-case italic letters that correspond to the metabolite names, i.e. s_1 is the concentration of S_1 , etc. When numerical values are given for concentrations (i.e. for x_0 and x_6) these were treated as independent of the system, i.e. as constants; where no values are given they were treated as dependent variables and calculated by the simulation program. In all cases units are arbitrary (but consistent), but concentrations can be taken to be in mM if this is considered helpful for understanding.

Enzyme	a	p	K_{eq}	V	K_m	K_p
E_1^a	$x_0 = 10$	s_1	5	1.5	6	1.3
E_2^b			5	120		
E_{3a}^c	s_2		10	25	4	
E_{3b}	s_{3b}	s_{3a}	0.2	500	4	0.6
E_4^d	s_{3a}	s_4	1000	20	0.5	1.6
E_5	s_4	s_5	8	15	6	3
E_6^e	s_5	$x_6 = 0$	∞	0–2.4	2	∞

^a When E_1 was assumed to be feedback-inhibited by S_5 a term $(s_5/K_{fb})^2$ was added to the denominator of the rate expression. ^b The rate for E_2 was calculated from Eqn (2) of the text, with $K_{eq} = 5$, $V = 120$, $a_1 \equiv s_1$, $a_2 \equiv [ATP] = 8$ (constant), $p_1 = s_2$, $p_2 \equiv [ADP] = 2$ (constant), $K_{m1} = 7$, $K_{m2} = 5$, $K_{p1} = 3$, $K_{p2} = 0.8$. Note that as the concentrations of ATP and ADP are fixed, these metabolites were treated as external to the model. They could not be treated as components of a conservation relationship because the model as defined does not conserve the sum of their concentrations. ^c The rate for E_{3a} was calculated from a modified form of Eqn (1) in which p in the disequilibrium term represents $s_{3a}s_{3b}$ and the denominator is written as a product of three terms $(1 + s_2/K_m)(1 + s_{3a}/K_{p1})(1 + s_{3b}/K_{p2})$, with $K_m = 4$, $K_{p1} = 9$ and $K_{p2} = 7$. ^d In some simulations the equilibrium constant of E_4 was treated as infinite (i.e. the disequilibrium term in the numerator was treated as unity), and in some K_p , the Michaelis constant of the product, was also treated as infinite, i.e. the term in s_4 was omitted from the denominator. ^e Variation in demand for the end-product S_5 was simulated by varying the value of the limiting rate V for E_6 in the range 0–2.4.

Table 2. Kinetic parameters for the model illustrated in Fig. 3. The caption to Table 1 applies *mutatis mutandis* to this table.

Enzyme	<i>a</i>	<i>p</i>	<i>K</i> _{eq}	<i>V</i>	<i>K</i> _m	<i>K</i> _p
E ₁ ^a	<i>x</i> ₀ = 10	<i>s</i> ₁	3	5	4	2
E _{2a} ^{b,c}	<i>s</i> ₁	<i>s</i> _{2a}	104.6	20	1.7	8
E _{3a} ^d	<i>s</i> _{2a}	<i>s</i> _{3a}	30000	150	0.05	0.1
E _{4a} ^e	<i>s</i> _{3a}	<i>x</i> _{4a} = 0	∞	0–2	1.5	∞
E _{2b} ^f	<i>s</i> ₁	<i>s</i> _{2b}	62.5	75	0.3	2
E _{3b}	<i>s</i> _{2b}	<i>s</i> _{3b}	4.67	50	2.5	3.5
E _{4b}	<i>s</i> _{3b}	<i>x</i> _{4b} = 0	∞	25	3	∞

^aE₁ followed the reversible Hill equation [22] with a Hill coefficient of 2.5, so the values listed as *K*_m and *K*_p are actually the half-saturation concentrations of substrate and product, respectively. When feedback inhibition by *S*_{3a} was present the half-inhibition concentration of *S*_{3a} was 0.2. ^bWhen E_{2a} was inhibited by *S*_{3a}, i.e. in Fig. 3C, the inhibition was competitive with inhibition constant 0.5. ^cThe arbitrary appearance of the values for *K*_{eq} for E_{2a} and E_{3b} is an artifact of the transformation to the form of Eqn (1) of rate equations that were expressed in a different way at the time the simulations were done. ^dWhen E_{3a} was treated as irreversible, *K*_{eq} and *K*_p were infinite. ^eThe limiting rate of E_{4a} was varied in the range 0–2 to simulate variation in demand for the end-product *S*_{3a} of branch (a) of the pathway. ^fWhen E_{2b} was weakly activated by *S*_{3a}, i.e. in Fig. 3D, the specific activation constant was 12 and the other kinetic parameters were as follows: *V* = 450, *K*_m = 0.6, *K*_p = 1; when very strong activation was allowed, i.e. in Fig. 3E, *V* was increased to 45 000 and the activation constant to 1200. Note that none of these changes affect the value of the equilibrium constant *K*_{eq}.

successor program to SCAMP [3]. We therefore used JARNAC for the more recent simulations discussed here. For models that do not contain transport steps (i.e. most simple metabolic models) this change may well have no significance, but for models with transport steps it may be essential. JARNAC is available to download from <http://members.tripod.co.uk/sauro/biotech.htm>

The model used for *T. brucei* is described fully elsewhere [17], and was based on a very similar model developed by Bakker *et al.* [12].

The kinetic characteristics of the one-substrate-one-product enzymes incorporated into the various pathways considered are defined in Tables 1–3 by reference to the following generic equation:

$$v = \frac{V \cdot \frac{a}{K_m} \cdot \left(1 - \frac{p}{K_{eq}a}\right)}{1 + \frac{a}{K_m} + \frac{p}{K_p}} \quad (1)$$

which defines *v*, the steady-state rate of any enzyme at concentrations *a* and *p* of its substrate and product, respectively, in terms of *V*, the limiting rate of the forward reaction, *K*_{eq}, the equilibrium constant, and *K*_m and *K*_p, the Michaelis constants for the forward and reverse reactions, respectively. This way of writing a rate equation, discussed elsewhere [22], is not the most familiar, but it has the advantage of making a clear separation between the four component properties that contribute to the rate: the three factors in the numerator respectively express the limiting activity of the enzyme, its sensitivity to its substrate and the degree of disequilibrium; the denominator expresses its degree of saturation by its reactants.

For a two-substrate reaction Eqn (1) may be generalized as follows:

$$v = \frac{V \cdot \frac{a_1 a_2}{K_{m1} K_{m2}} \cdot \left(1 - \frac{p_1 p_2}{K_{eq} a_1 a_2}\right)}{\left(1 + \frac{a_1}{K_{m1}} + \frac{p_1}{K_{p1}}\right) \left(1 + \frac{a_2}{K_{m2}} + \frac{p_2}{K_{p2}}\right)} \quad (2)$$

where the meanings of the symbols follow those in Eqn (1). This equation contains only six adjustable parameters, whereas the equation for even the simplest form of the compulsory-order ternary-complex mechanism contains 13, of which eight are independent [23]. Thus Eqn (2) cannot express all the kinetic variability possible for such a reaction; however, it can express enough to allow realistic metabolic simulations in most circumstances.

RESULTS

Linear pathway with feedback

The pathway shown in Fig. 1 is more elaborate than the simple one examined previously [19], and incorporates more of the complications found in real metabolic pathways, but it has the same essential character as a linear biosynthetic pathway with a single end-product *S*₅, which is consumed by a demand pathway represented in vestigial form by the final step catalysed by E₆. To examine the importance of allowing for the reversibility of nearly irreversible steps the reaction catalysed by E₄ has an equilibrium constant of 1000 in favour of the forward direction, which was treated as infinite in some simulations (i.e. the back reaction was ignored). Feedback inhibition of E₁ by *S*₅ was likewise considered in some simulations and not in others. Ordinary product inhibition of E₄ by *S*₄ is shown explicitly in the figure because, although such

Table 3. Kinetic parameters for the model illustrated in Fig. 4. The caption to Table 1 applies *mutatis mutandis* to this table.

Enzyme	<i>a</i>	<i>p</i>	<i>K</i> _{eq}	<i>V</i>	<i>K</i> _m	<i>K</i> _p
E ₁	<i>x</i> ₀ = 10	<i>s</i> ₁	14.57	3.4	1	6
E _{2a}	<i>s</i> ₁	<i>s</i> _{2a}	∞	8	3.5	∞
E _{3a}	<i>s</i> _{2a}	<i>s</i> _{3a}	12.5	15	3	5
E _{4a}	<i>s</i> _{3a}	<i>s</i> ₄	980	21	0.5	7
E ₅	<i>s</i> ₄	<i>x</i> ₅ = 0	∞	0–2 (variable)	0.8	∞
E _{2b}	<i>s</i> ₁	<i>s</i> _{2b}	33.98	47	2	12
E _{3b}	<i>s</i> _{2b}	<i>s</i> _{3b}	1.63	35	2.8	1.5
E _{4b}	<i>s</i> _{3b}	<i>s</i> ₄	14.96	85	2	5

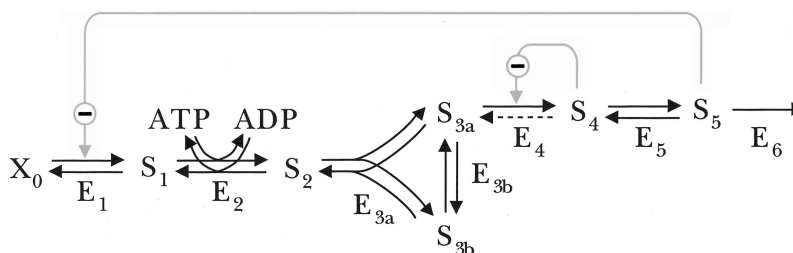


Fig. 1. Linear pathway with an irreversible step. The kinetic parameters for the component enzymes are listed in Table 1.

inhibition is an inevitable property of any reversible enzyme-catalysed reaction, it is sometimes ignored in steps considered to be irreversible. Details of the kinetic equations are given in Table 1. Variation in metabolic demand was simulated by varying the limiting rate of E_6 . Inclusion of ATP and ADP in the model at constant concentrations is in a sense superfluous, as their concentrations could, of course, be subsumed into the rate constants of a simpler model. Nonetheless, we include them to emphasize that the sort of conclusions we reach here do not depend on the use of oversimplified models in which every enzyme has just one substrate and just one product.

As seen in Fig. 2A, flux control in the complete model is concentrated in E_6 at low demand; as the demand increases it changes smoothly to majority control by E_1 , with a small contribution from E_2 . This means that at low demand the synthetic flux is essentially equal to the demand, falling significantly below it only when the demand starts to exceed what the supply can support (Fig. 2A, inset). This satisfactory degree of flux control is achieved without requiring enormous changes in metabolite concentrations (Fig. 2B), even though the feedback inhibition incorporated in the model is weak compared with what occurs in many real systems. The general implications of this sort of behaviour for the regulatory design of pathways in living organisms have been discussed previously [24,25], and we shall not consider them here.

As long as there is a feedback loop the small degree of reversibility in E_4 is irrelevant. Treating this reaction as strictly irreversible has no perceptible effect on the flux control coefficients (Fig. 2C), on the flux (inset to Fig. 2C), or on the metabolite concentrations (Fig. 2D). It is also irrelevant in the absence of a feedback loop if the irreversible enzyme is subject to ordinary product inhibition, as may be seen by comparing Fig. 2E,F with the virtually indistinguishable Fig. 2G,H. Thus reversibility as such is no more important in the absence of a feedback loop than it is in the presence of one.

The major change in the model of Fig. 1 comes on eliminating not only the feedback loop and the reversibility of step 4 but also the inhibitory action of S_4 on E_4 (Fig. 2I,J). The flux and flux control coefficients then do not change at all at high levels of demand (Fig. 2I), but at low demand there is no steady state at all; the concentrations of the late metabolites rise even more steeply than in Fig. 2F as the demand falls close to the critical level (Fig. 2J), and their uncontrolled increases at this level explain the loss of steady state. In simple terms it occurs because the supply steps produce S_5 faster than the limiting rate (or 'maximum velocity' in the terminology often used) of the demand pathway.

These results are consistent with previous observations [24] that apparently similar flux behaviour in different models may well conceal substantial differences in the behaviour of the metabolite concentrations. They suggest that if one is forced by lack of experimental information or by the need to avoid unnecessarily complicated rate equations to use models that are simpler than reality, it will normally be better to ignore reversibility in near-irreversible steps than to ignore feedback loops that go around such steps.

The fact that product inhibition must always be possible at high product concentrations does not exclude more complicated behaviour at low concentrations, such as product activation in nitrite reductase [26], but such behaviour is rarely reported and is probably rare in nature, so it is unlikely to be an important consideration in the design of metabolic models; in contrast, ordinary product inhibition is not only theoretically necessary but is also frequently detected experimentally, and must certainly be an important consideration in the design of metabolic models.

Branched pathway with feedback inhibition and cross-activation

Figure 3 illustrates a more complex model designed to show whether the conclusions drawn from the simple linear pathway apply more generally. It consists of a branched supply pathway that feeds two different demand processes. One end-product, S_{3a} , inhibits E_{2a} , the first committed enzyme in its branch, but it has no feedback effect on E_1 , the first enzyme in the complete pathway; as the reaction that produces it, catalysed by E_{3a} , is virtually irreversible it has no direct effect through the pathway either unless the small degree of reversibility is allowed for. However, it may have an indirect effect on E_1 if it activates E_{2b} , the first committed enzyme of the other branch, because activation of this enzyme tends to increase the concentration of the other end-product, S_{3b} , which does exert feedback inhibition on E_1 . If a flow of information from S_{3a} to E_1 is all that is required to ensure a satisfactory flux response to low demand in step 4a then one might expect that this circuitous route via activation of E_{2b} and inhibition of E_1 by S_{3b} could compensate for irreversibility of E_{3a} and lack of a feedback inhibition loop around it.

Results obtained with various versions of this model confirm this expectation to some degree, although they also indicate that reality is less simple than suggested by the simplest model. First of all, in the absence of any information transfer from S_{3a} to the beginning of the pathway – no reversibility in E_{3a} , no feedback inhibition of E_{2a} and no feedback activation of E_{2b} – there is no steady

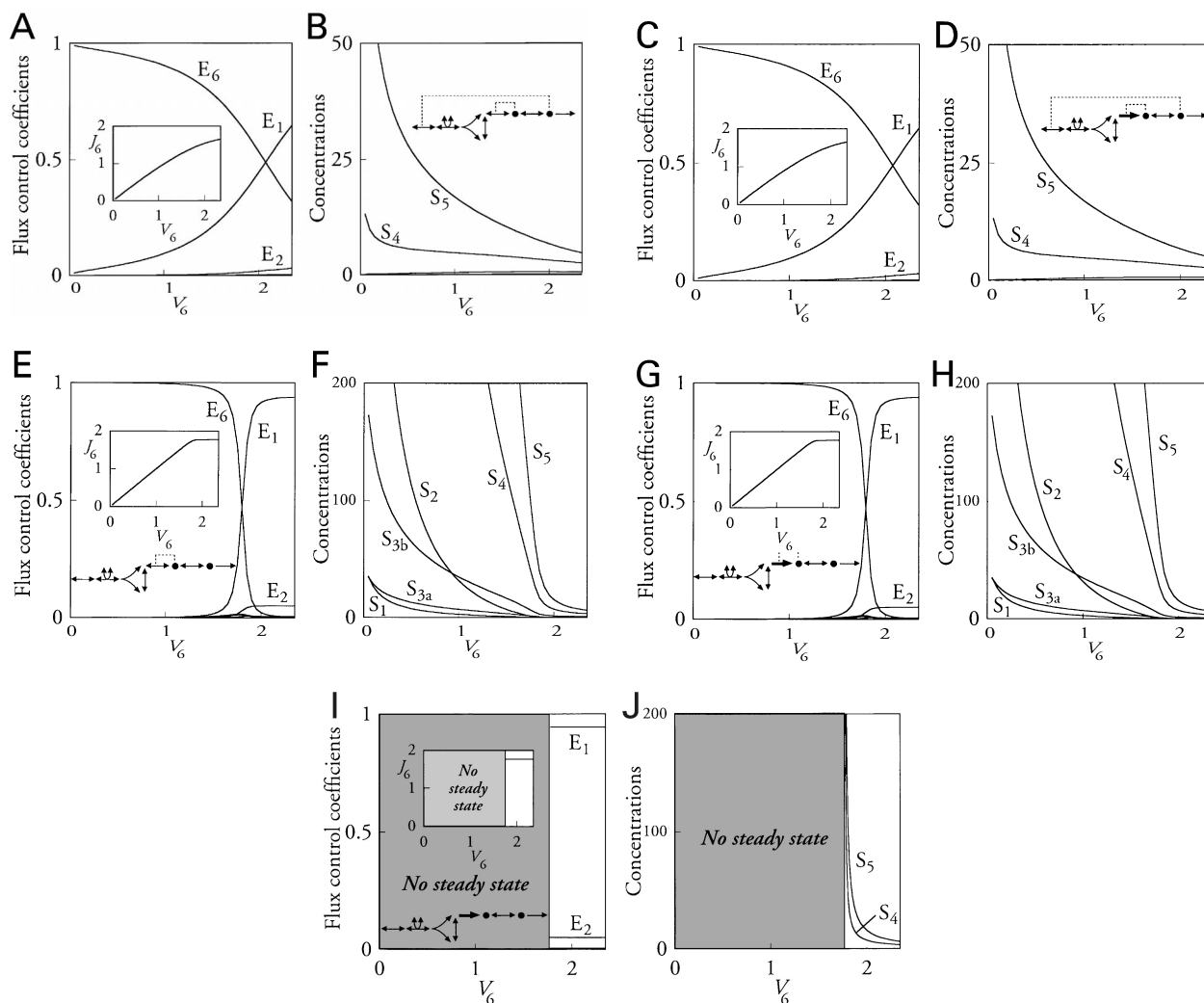


Fig. 2. Simulation of the model in Fig. 1. The left-hand (A,C,E,G,I) of each pair of panels shows flux control coefficients and (in the inset) the flux through E_6 as a function of the demand expressed by the value of V_6 and the right-hand panel (B,D,F,H,J) shows the metabolite concentrations in the same range of conditions. The five pairs of panels represent different versions of the model, and in each case a schematic version of Fig. 1 is added where there is room for it to give a quick indication of the properties considered, which were as follows: (A,B) full model as illustrated in Fig. 1, with feedback inhibition of E_1 by S_5 and E_4 treated as reversible; (C,D) E_4 treated as irreversible; (E,F) E_4 reversible but no feedback inhibition of E_1 ; (G,H) no feedback inhibition of E_1 , and E_4 irreversible, but still inhibited by its product S_4 ; (I,J) no feedback inhibition of E_1 , E_4 irreversible and no effect of S_4 on E_4 . Note that a more expanded scale for metabolite concentrations is used in panels B and D in order to make the much smaller variations in concentrations that occur in the presence of feedback inhibition more easily visible.

state at low demand and no variation in either flux or metabolite concentrations at high demand (Fig. 3A). Making E_{3a} reversible allows a steady state to exist at low demand, but the transition from one regime to the other is extremely abrupt (Fig. 3B). Introduction of feedback inhibition of E_{2a} by S_{3a} (without making the reaction catalysed by E_{3a} reversible) has a qualitatively similar effect (Fig. 3C) but the transition is now much smoother, closer to the way one would expect a real metabolic system to behave.

The question now is whether circuitous information transfer via activation of the other branch can compensate for a lack of feedback inhibition. As seen in Fig. 3D,E, this type of effect can give a satisfactory response, mimicking feedback inhibition of E_{2a} by S_{3a} to some degree, but to maintain a steady state at very low demand the activation

must be strong. If the maximum degree of activation is insufficient, the region in which there is no steady state is not completely eliminated (Fig. 3D), but it can be made negligible by allowing very substantial activation (Fig. 3E). This result contrasts with those in Figs 2B and 3C, in which weak feedback inhibition is seen to be sufficient to maintain a steady state at low demand.

This observation is easily rationalized, and it illustrates a practical difference between inhibition and activation that may have important implications for the regulatory design of metabolic systems in nature. Enzyme inhibition is usually linear or 'complete', which means that the rate approaches zero at saturating concentrations of inhibitor; the more complicated phenomenon of hyperbolic or 'partial' inhibition is reported much more rarely (although it is unclear if it is really rare in nature or just rarely noticed). In

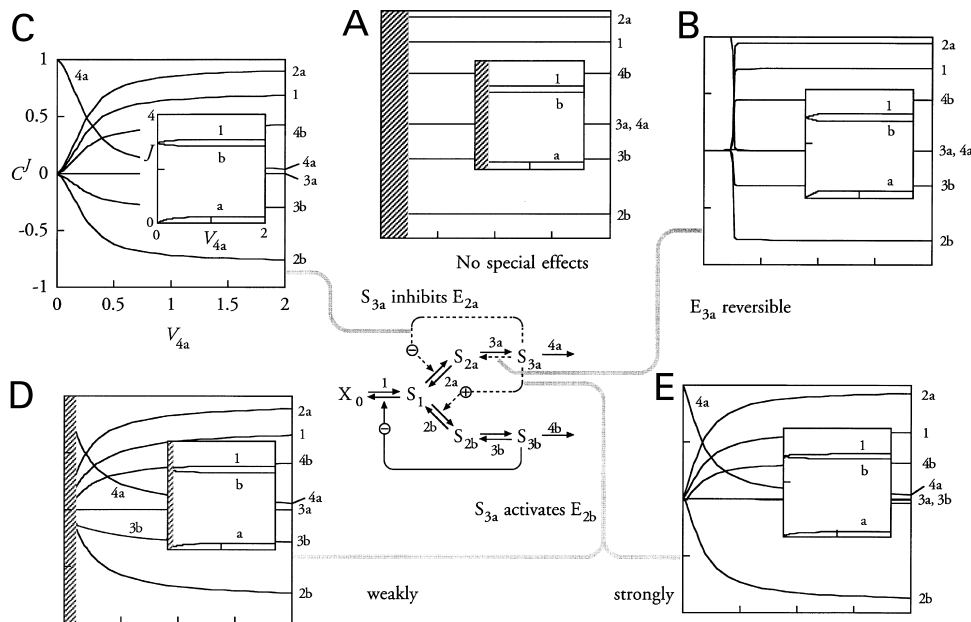


Fig. 3. Branched pathway with feedback inhibition and cross-activation. The model is shown in the central part of the figure, and kinetic parameters are listed in Table 2. Each of the panels (A) to (E) shows the response to changes in the demand in step 4a of the control coefficients for flux through branch a; in each case the inset shows the corresponding changes in the three fluxes over the same range of demand. In all cases the axes and scales are as shown explicitly in (C). The grey curves linking the panels to the scheme in the centre of the Figure show which special effects apply to each panel. In (A) there were no special effects, i.e. no reversibility in step 3a, no feedback inhibition of step 2a and no cross activation of step 2b. Each of the other panels shows the effect of adding just one line of communication from S_{3a} to E_1 . (B) shows the effect of allowing for the reverse reaction in step 3a (but no feedback or activation of the other branch); (C) shows the effect of feedback inhibition of E_{2a} by S_{3a} (while keeping step 3a irreversible); (D) shows the effect of moderate activation of E_{2b} by S_{3a} (with no feedback inhibition of E_{2a} or reversibility in step 3a); and (E) shows the effect of almost unlimited activation of E_{2b} by S_{3a} (again, without any other effects).

contrast, reported activation effects are often hyperbolic, in the sense that an enzyme subject to activation often shows some activity in the absence of activator, and in addition, the amount of activation possible is limited by the limiting rates

of the enzymes involved. In the case of Fig. 3, if the limiting rate of E_{3b} is not large enough to raise the concentration of S_{3b} sufficiently to produce a strong effect on E_1 then no amount of activation of E_{2b} will avoid the loss of the steady

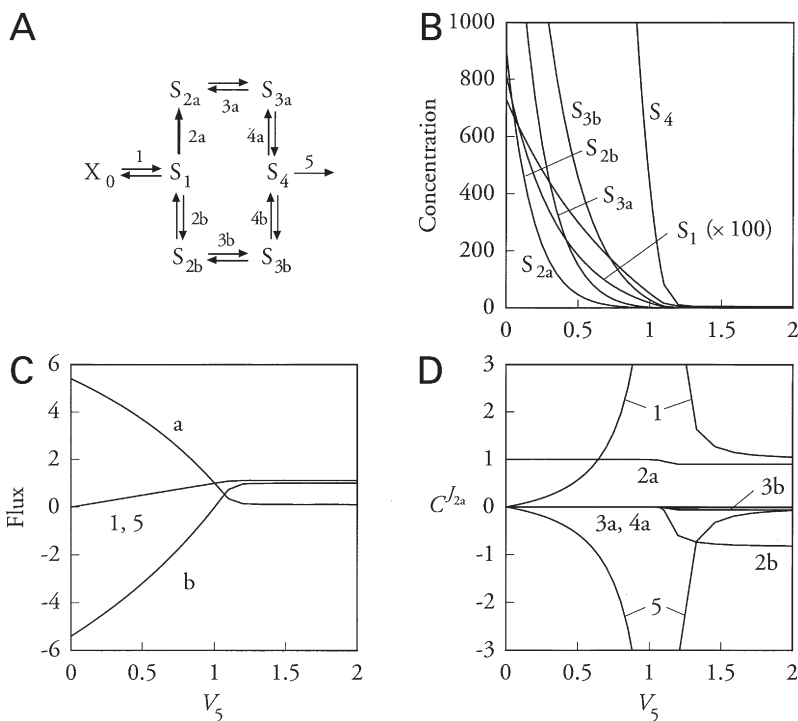


Fig. 4. Parallel routes between two metabolites. The reactions are shown in (A), and the kinetic parameters in Table 3. (B) shows the response of the intermediate concentrations to changes in the demand, represented by values in the limiting rate of E_5 in the range 0–2. (C) shows the variations in the three distinct fluxes over the same range of demand. Note that for about half of this range the flux in branch b is negative, i.e. the pathway operates as a cycle rather than as two parallel routes. (D) shows the control coefficients for the flux through branch a over the same range of demands.

state at low levels of demand for S_{3a} . This argument may appear very complicated, but the essential point is quite simple: effects that depend on linear inhibition can always decrease a flux to whatever is necessary to ensure a steady state, but effects that depend on activation may fail to do so if the fully activated pathway is not active enough to remove its intermediates as fast as they arrive.

Parallel routes

Figure 4 illustrates how information may be transferred around an irreversible reaction without requiring any regulatory effects at all in the usual sense of feedback or feedforward loops. In this model (Fig. 4A), the end-product S_4 is produced from the same precursor X_0 by two parallel routes, one of which contains an irreversible reaction catalysed by E_{2a} . (This implies, of course, that the equilibrium constant around the cycle is not unity, which in turn is only possible if additional metabolites are involved, such as ATP, ADP, water and inorganic phosphate. However, the conditions necessary for such 'futile cycles' to exist are well known [27] and do not require discussion here.) Although there is no feedback loop in the system, excessive production of S_4 by the irreversible route can still be signalled to E_1 by the reversible steps in the parallel route, and so in principle one may expect this system to be capable of reaching a steady state even at low demand for the product. This proves to be the case, although the stability of the flux response at low demand is achieved at the expense of enormous increases in the concentrations of all the intermediates, especially S_4 (Fig. 4B). Moreover, the apparently smooth response of the common flux through E_1 and E_5 conceals rather more complicated behaviour in the two parallel routes (Fig. 4C), which are seen to be truly parallel only at high demand, because at low demand the flux through branch a is much larger than the common flux and has to be balanced by negative flux in branch b. In other words, at low demand the flux in branch b is from right to left in the figure, and maintenance of the cycling must imply continuous hydrolysis of ATP or a similar process. Moreover, over the entire range, even at high demand, the flux in branch a changes in the opposite direction to the change in demand, i.e. it decreases as the demand increases, and vice versa. The smooth behaviour of the common flux likewise conceals behaviour in the control coefficients for flux through branch a that is not at all smooth (Fig. 4D): all of the flux control coefficients show abrupt transitions at the point where the flux in branch b changes sign, and two of them are infinite at this point.

This example of forcing flow into a parallel pathway is interesting, because although the model in Fig. 4 was conceived as a simple example of parallel pathways, it succeeds in switching off the net flow at low demand only by reversing the flow in the second route. This means that the central part of the pathway ceases to be a pair of parallel routes but becomes a cycle instead, implying a plausible evolutionary mechanism for the appearance of cycles in metabolism. It is easy to conceive how duplication of the genes coding for several successive enzymes in a linear pathway might produce several isoenzymes in succession. Subsequent mutations could then convert such a succession of isoenzymes into two distinct parallel routes between a given pair of metabolites (S_1 and S_4 in the symbolism of

Fig. 4). In this context it is interesting to note that although the tricarboxylate cycle is commonly represented in textbooks as a cycle, there are organisms such as *Methylococcus capsulatus* [28] where it does not operate as a cycle because α -ketoglutarate dehydrogenase is missing.

DISCUSSION

The main conclusion to be drawn from the examples examined in this paper is that a metabolic model able to reach a steady state, over the same range of conditions in which the real system that it models can do so, must allow for information about metabolite concentrations to flow from the end of the model to the beginning. Regardless of what other simplifications may be tolerated, this requirement must be met. The exact mechanism for the effect of late products on early steps may be unimportant if the ability to reach a steady state is the only concern, although with a broader perspective feedback inhibition is usually the most effective at generating satisfactory behaviour. If feedback inhibition is known to exist in a pathway, therefore, it will usually be unnecessary to allow for the small degree of reversibility of reactions with large equilibrium constants in favour of the forward direction.

Ordinary product inhibition requires special discussion, because it is often given insufficient attention in models, as we document below. An essential property of any metabolite is the capacity to inhibit the reaction that produces it, regardless of whether this reaction is effectively irreversible or not, as pointed out by Fell & Sauro [29], who also drew attention to its importance for distributing control in a pathway. For reactions with large equilibrium constants, the capacity to be inhibited is much more important than the capacity to be reversed, and failure to allow for effects of products can make a model misleading, as it will be unable to reach a steady state in some realistic conditions. For example, models in which some enzymes are assumed to be product-insensitive [21] can only reach steady state if the demand for the end-product is sufficiently high. Although this may not matter for simulating the metabolic behaviour of healthy cells, it may become important in the study of enzyme defects, when the high accumulation of intermediates may produce very large effects [14]. Even in healthy cells, natural selection will have ensured some robustness, i.e. that cells should be capable of achieving a steady state over a wider range of conditions than might be expected to occur in 'normal' conditions; a valid metabolic model ought to do the same.

As allowing for product inhibition is simpler than allowing for reversibility (and is anyway a part of allowing for reversibility), the construction of realistic models of complex processes is seen to be more straightforward than it appeared to be after the problem of reversibility of pyruvate kinase in models of *T. brucei* was first studied [17]. Provided that some inhibition of early steps by late products is allowed for, it appears unnecessary to make all the adjustments to the rate equations implied by making them fully reversible. For an enzyme like pyruvate kinase, with an equilibrium constant of about 10^5 , this is very helpful, because in such cases it is unlikely that any reliable kinetic constants for the reverse reaction are available from experimental measurements.

Once pointed out, the conclusions that we reach in this paper may appear to be obvious, and, indeed, with proper understanding of the conditions that allow a system to achieve a steady state they probably are obvious. It is important, therefore, to point out that in practice, since the first metabolic model was published in 1964 [1] until the present day, the basic knowledge that all biochemists have that every enzyme is subject to product inhibition has not been translated into general recognition that every enzyme in a metabolic model needs to be capable of being inhibited by any product whose concentration can rise to the level of the inhibition constant or higher. This has been ignored for various enzymes at one time or another, and almost always for pyruvate kinase [1,8–13], presumably because it has been forgotten that a very large equilibrium constant does not guarantee negligible product inhibition. In the first model of glycolysis [1], for example, hexokinase and phosphofructokinase were treated as reversible enzymes, but pyruvate kinase was not only treated as irreversible (probably a legitimate approximation) but also as totally incapable of responding to the concentration of pyruvate (an approximation which will certainly fail at low enough activities of the enzymes that follow pyruvate kinase in the model).

Many authors have referred to this pioneering model of glycolysis, but very little attention has been paid to the problems that are implied by the pyruvate-insensitivity of pyruvate kinase. Moreover, nearly all later models have had the same characteristic. For example, in the model of erythrocyte glycolysis described by Heinrich & Rapoport [8,9], the rates through hexokinase and phosphofructokinase were wholly determined by the concentration of ATP (and thus independent of glucose 6-phosphate, fructose 1,6-bisphosphate, etc.), and that through pyruvate kinase was wholly determined by the concentration of phosphoenolpyruvate (and thus independent of pyruvate). In discussing the model long afterwards in their book [11], Heinrich & Schuster made no comment on this characteristic. Other large-scale studies [10] of the metabolic dynamics of the erythrocyte also treated pyruvate kinase as pyruvate-insensitive, as did Bakker and colleagues in their most recent model of glycolysis in *T. brucei* [13,30]. Authors that have addressed the question at all [14,15] have tended to insist on the need to use a complete reversible rate equation, without considering the possibility that the much simpler solution of allowing for product inhibition might be equally effective.

A different example of modelling with irreversible reactions is provided by a substrate cycle that Newsholme & Start [31] argued would allow a higher degree of responsiveness to effectors than could be obtained with cooperative allosteric effects on a single enzyme in a pathway with no substrate cycle. As originally proposed their model had a product-insensitive first step, but in later analysis Fell & Sauro [29] introduced a nonzero elasticity for the effect of the first intermediate on the first enzyme. This was consistent with their general view expressed in the same paper that product inhibition was important for the distribution of control, and they also found that with a zero elasticity the sort of amplification effect described originally [31] could not occur. There was no suggestion that they made this change to ensure that the model would not break down at low activities of the later enzymes in the pathway.

A model of the Calvin cycle, both as described originally [32] and as later refined [33], used irreversible rate equations for several enzymes, but in all cases these allowed for product inhibition, to take account of 'reported inhibitory effects': this is of course a perfectly valid reason for including product inhibition, but according to our analysis it may not be the complete reason, because whether reported or not it must exist, and not taking it into account raises the danger that the model will fail to achieve a steady state in circumstances where the living plant would succeed. Thus in our view the authors took the correct steps for ensuring stable behaviour of their model, but the reason they gave was not the one we would have given.

In their analysis of the effects of product-insensitive enzymes on the distribution of control, Teusink & Westerhoff [21] noted that if an enzyme is the unique enzyme in a system that responds to a particular metabolite then it has no control at all over the flux. This is correct, as the only kind of metabolic intermediate that affects only the enzyme that uses it as substrate is one that does not inhibit the enzyme that produces it, and enzymes downstream from an irreversible step have no flux control if there is no inhibition of the irreversible enzyme by downstream metabolites [29]. In an extension of this idea, a feedback loop that allows a downstream metabolite to inhibit an enzyme upstream from the irreversible step reinstates the capacity for flux control by enzymes further downstream; all of the enzymes between the irreversible step and the feedback metabolite remain without any flux control. However, a model of this kind must fail to reach a steady state when the activity of the last enzyme is low, unless there is a feedback loop or another mechanism of information transfer: this applies to four out of the five models discussed (i.e. all of those illustrated in Fig. 1 of [21] apart from the one with a feedback loop, case D). Taken with the basic chemical necessity that product inhibition must always exist, and must always be capable of affecting even enzymes with such large equilibrium constants that the reverse reaction as such can be neglected, this suggests that the type of behaviour analysed [21] may be relevant only at high demand for end-products and may not take account of the capacity of living systems to adapt to a wide range of conditions.

More generally, as there are many metabolic processes that are irreversible to a first approximation, and for which product inhibition is often not considered, caution must be taken in modelling them. Any such model ought to include a mechanism of transfer of information to ensure the possibility of reaching a steady state under a wide range of conditions; in the absence of experimental information about the mechanism that actually exists, it will always be safe to postulate that product inhibition is present.

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