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Failure of channelling to maintain low concentrations of metabolic intermediates

Athel CORNISH-BOWDEN

Centre de Biochimie et de Biologie Moléculaire, Centre National de la Recherche Scientifique, Marseille, France

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Computer modelling has been used to investigate the effect of direct transfer of metabolites between consecutive enzymes (channelling) on the free concentrations of the channelled metabolites. When a channelled intermediate cannot participate in any other reactions, any increase in channelling tends to increase its free concentration, albeit very slightly, unless the increase in net flux brought about by the channel is compensated for by a simultaneous decrease in the activity of the route through the free intermediate, in which case channelling has no effect at all on the free steady-state concentration of the channelled intermediate. If the free intermediate is capable of participating in side reactions, channelling can decrease these side reactions, but only slightly unless virtually all of the final product results from flux through the channel and the rate constants for the direct pathway are virtually zero. In general, channelling appears not to provide a useful mechanism for maintaining intermediate concentrations at low levels.

Numerous systems have been reported in which enzymes catalysing consecutive reactions in metabolic pathways are capable of forming loose complexes with one another in solution: for example, in a recent review Keleti [1] summarizes different kinds of experiment that suggest that aldolase forms complexes with glyceraldehyde-3-phosphate dehydrogenase and other glycolytic enzymes; likewise, complex formation has been reported [2] between ribulose-bisphosphate carboxylase and four other enzymes catalysing consecutive reactions of the Calvin cycle, and other examples may be found in recent reviews [3, 4].

When such complexes exist under physiological conditions, one has the possibility of channelling, i.e. the possibility that the product of the reaction catalysed by one enzyme may be directly passed as substrate of the next without being released into the bulk phase. Of course, demonstration that complex formation occurs *in vitro* does not prove that channelling occurs *in vivo* nor, if it does, that it has a definite physiological function. Even for experiments *in vitro*, there is by no means universal consent that channelling really occurs in the systems for which it has been reported [5 – 7]. Nonetheless, the fact that such complexes have often found between enzymes in the same pathway, rather than between random pairs of enzymes, suggests that they are more than just a haphazard consequence of protein stickiness without any particular function.

Correspondence to A. Cornish-Bowden, Centre de Biochimie et de Biologie Moléculaire, Centre National de la Recherche Scientifique, Boîte Postale 71, 31 chemin Joseph-Aiguier, F-13402 Marseille Cedex 9, France

Enzymes. Glyceraldehyde-3-phosphate dehydrogenase or D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase (phosphorylating) (EC 1.2.1.13); glutamate formiminotransferase or 5-formiminotetrahydrofolate:L-glutamate *N*-formiminotransferase (EC 2.1.2.5); ribulose-bisphosphate carboxylase or 3-phospho-D-glycerate carboxylyase (dimerizing) (EC 4.1.1.39); aldolase, fructose-bisphosphate aldolase or D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase (EC 4.1.2.13); formiminotetrahydrofolate cyclodeaminase or 5-formiminotetrahydrofolate ammonia-lyase (cyclizing) (EC 4.3.1.4).

For the purposes of the present paper, I shall not address the question of whether channelling occurs *in vivo*, but will instead ask what value may it be to the organism if it does occur. Does it, for example, decrease the free concentrations of channelled metabolites to the point where they do not strain the solvent capacity of the cell? Does it ensure that intermediates required in one pathway cannot be sequestered by enzymes of another? These questions are not as easy to answer by pure reason as they might appear at first sight, but they are simple to examine by means of computer modelling, and after such modelling it is not difficult to rationalize the results, however unexpected they may be if one only considers the questions superficially.

METHODS

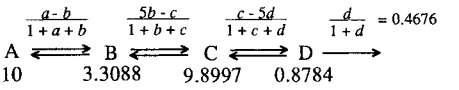
All of the results given in this paper were obtained by means of a program MetaModel [7a] that calculates the steady states of systems of up to 15 enzyme-catalysed or uncatalysed reactions (as well as calculating control and elasticity coefficients). This program is implemented on computers compatible with the IBM XT and AT computers, and is available on a 5¼-inch (13.3-cm) disk for a small cost to cover copying, production of a printed user's guide, and postage. The algorithm is essentially as described by Hofmeyr and van der Merwe [8] for their program METAMOD, but the user interface has been redesigned to be as easy and as efficient to use as possible.

One can hardly illustrate the interactive and error-trapping character of the program in a brief account, but one may see the simple way in which models are defined by considering the input needed to obtain the results shown in Fig. 1 (below). For this purpose, the model of Scheme 4 (below) was defined in terms of the nine reactions and rate expressions shown in Table 1 together with two 'moiety conservation equations' that ensure that the total concentrations of all forms of E_2 and E_3 are kept constant.

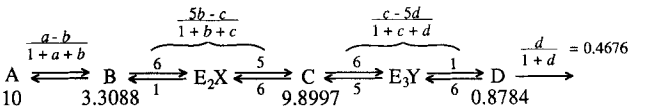
The following points should be noted: (a) it is sufficient to mention a metabolite in a reaction or rate expression to

Table 1. *Data as supplied to MetaModel for defining the model shown below in Scheme 4*

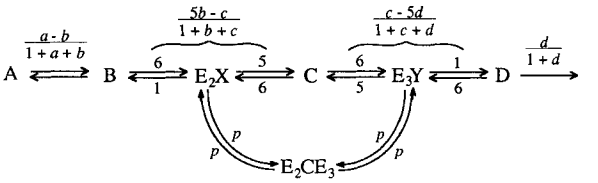
Step	Reaction	Rate expression	Conservation equation
1.	A = B	(A - B)/(1 + A + B)	
2.	B + E2f = E2X	6*B*E2f - E2X	
3.	E2X = E2f + C	5*Q*E2X - 6*Q*E2f*C	
4.	C + E3f = E3Y	6*Q*C*E3f - 5*Q*E3Y	
5.	E3Y = E3f + D	E3Y - 6*E3f*D	
6.	D = Z	0.5*D/(0.07818 + D)	
7.	E2X + E3f = EXY	P*E2X*E3f - P*EXY	E2f + E2X + EXY = 1
8.	EXY = E3Y + E2f	P*EXY - P*E3Y*E2f	E3f + E3Y + EXY = 1
9.	C = Z	C/(1 + 2*C)	



Scheme 1. *Pathway of four reactions*



Scheme 2. *The pathway of Scheme 1 expanded to show the intermediates in the second and third reactions*



Scheme 3. *The pathway of Scheme 2 with the addition of a channel from E_2X to E_3Y, by-passing realease of C into free solution*

define its existence to MetaModel; (b) names have a maximum of three characters, beginning with a capital letter, so p is entered as P and E_2CE_3 as EXY, for example; (c) ordinary steady-state rate expressions (e.g. steps 1, 6, 9) are sufficient, though one can write chemical equations for individual steps if one prefers (e.g. steps 2 – 5, 7, 8); (d) as enzyme conservation is implicit in the normal derivation of a steady-state enzyme rate equation (because it written in terms of total rather than free enzyme concentration) separate conservation equations are required only in special cases; (e) rate expressions are usually expressed with constant coefficients (e.g. 0.07818) but variables can be accommodated by means of dummy metabolites (P and Q representing p and q).

When a model is first defined all metabolite concentrations are defined as fixed, i.e. to be defined by the user, not by MetaModel. Before making a calculation one must define concentrations for A, Z, P and Q; redefine B, E2X, C, E3Y, D and EXY as variable, and E2f and E3f as conserved. For each conservation equation one concentration must be defined as conserved. However, the results are not affected by the particular choice one makes: one could equally well define E2X and E3Y as conserved instead of E2f and E3f.

As MetaModel is concerned only with finding steady states of systems, it is sufficient to work with the steady-state rate equations of the individual enzymes. This makes the mathematical problem much simpler than it would be if differential equations needed to be solved, but does not make it trivial because the simultaneous equations that need to be solved are not linear. As with all problems of this kind, one does not obtain an analytic solution for the steady state, but stops when a solution is found that satisfies a preset criterion. MetaModel normally assumes that a steady state has been found when all net rates of production of intermediates are less than 10^{-9}, in whatever units are implicit in the rate expressions, but this criterion can be made tighter or looser if desired. MetaModel does not provide information about the existence of multiple steady states. More information about the mathematical approach may be found in [8].

MODEL

As a starting point for studying the possible effects of channelling, let us consider the set of four enzyme-catalysed reactions shown in Scheme 1. The kinetic expression for each reaction is shown above the reaction, with a, b, c and d representing the concentrations of the metabolites A, B, C and D, respectively, and kinetic parameters and enzyme concentrations implicit in the coefficients (mostly unity). The last reaction, representing the totality of the metabolic processes that consume the end product D, is assumed to be irreversible and not subject to product inhibition; the other reactions are all written as reversible, with rate equations that allow for a back reaction and product inhibition. For a fixed concentration $a = 10$ of the starting material, the steady-state concentrations of the three intermediates are as shown under their symbols, i.e. $b = 3.3088$, $c = 9.8997$ and $d = 0.8784$, and the steady-state flux J through every step is 0.4676. All units are arbitrary, but will be consistent throughout the paper.

Without at this stage being very explicit about what we mean by 'too high', let us suppose that the value of $c = 9.8977$ is too high for the health of the organism, but that the concentration $a = 10$ and the flux $J = 0.4676$ correspond well with its needs. How can c be decreased without altering the values of a and J? The most obvious way is to increase the activity of the enzyme that catalyses the conversion of C to D, but one may also enquire whether it could be done by channelling, transferring C directly from the second enzyme to the third without the need for it to be released as a free intermediate.

Before we can introduce the possibility of channelling into Scheme 1, it needs to be expanded somewhat to show the enzyme-bound intermediates explicitly in reactions 2 and 3 (Scheme 2). The catalysed conversion of B to C has been expanded into two uncatalysed reactions (with reactant E_2 implied rather than drawn explicitly), from $B + E_2$ to E_2X, with forward and reverse rate constants 6 and 1, respectively, and from E_2X to $E_2 + C$ with forward and reverse rate constants 5 and 6, respectively. The third reaction is treated similarly. This model (with the particular rate constants given) is identical in its kinetic properties to that of Scheme 1, being just a more explicit way of writing the same reactions: this is illustrated by the fact that at the same concentration $a = 10$, both flux and intermediate concentrations are unchanged. However, it now allows for a mechanism for channelling, with a reaction from E_2X to E_3Y that does not involve free C. This proceeds in two steps, as illustrated in the expanded model shown in Scheme 3: the first is the binding of E_3 to E_2X to give E_2CE_3, a complex of the intermediate C with both enzymes; the second is the asymmetric breakdown of this com-

Table 2. *Effect on the concentration of C of increasing channel activity*

Flux via C	Channel flux	Total flux	c
0.4514	0.0065 (1.42%)	0.4580	10.0055
0.4025	0.0578 (12.6%)	0.4603	10.0408
0.1943	0.2760 (58.7%)	0.4704	10.1779
0.0317	0.4464 (93.4%)	0.4781	10.2706

Table 3. *Effect on the concentration of C of increasing channel activity in a model close to saturation*

Flux via C	Channel flux	Total flux	c
0.4512	0.0065 (1.42%)	0.4577	10.0151
0.4012	0.0573 (12.5%)	0.4585	10.1144
0.1941	0.2677 (58.0%)	0.4617	10.5241
0.0321	0.4319 (93.4%)	0.4641	10.8426

plex to produce E_2 and E_3Y, the same binary complex as could otherwise be produced by reaction of C with free E_3.

Scheme 3 now provides a basic model for channelling. To satisfy thermodynamic constraints, the rate constants in the channel reactions must give the same equilibrium constant for the conversion of E_2X to E_3Y by the direct route with C as intermediate; the particular choice shown, with all four of the new rate constants set to the same variable p, does satisfy this constraint, but it is only one of an infinity of possible choices. By varying p from zero to a value that causes the bulk of the flux to pass through the channel, one can begin to address the question of how the introduction of channelling affects the concentrations of intermediates.

All cases considered in this paper assume the existence of a 'well-stirred reactor', i.e. they ignore the possibility of steep local gradients in concentration. In addition to simplifying the analysis to the point where it becomes manageable, this may also conceal genuine properties of real systems. Thus real systems may display more interesting properties than those found here; nonetheless, it seems clear that one must have a good understanding of what can happens in well-stirred systems before it becomes profitable to speculate about what may happen in inhomogeneous ones.

RESULTS

Effect of channelling on the steady state when the rest of the system is unchanged

The simplest test is to study the effect of increasing p in Scheme 3 while leaving all other parameters (i.e. the fixed concentration a, all of the enzyme concentrations, and the kinetic parameters) unchanged. The results (Table 2) show that as the activity of the channel reaction is increased from a level where about 1.4% of the total flux passes through it to one where about 93% passes through it the effect on both the flux from A to D and the concentration of C is very slight; the flux changes only from 0.458 to 0.478, an increase of about 4%; the concentration of C changes from 10.0055 to 10.2706, an increase of about 2.6%.

This type of result is not a peculiarity of Scheme 3 nor of the particular numbers chosen: qualitatively the same results have been found in all cases tested. It illustrates two points: (a) the changes in steady state brought about by introducing a channel into a model are very slight; (b) the effect on the free concentration of the by-passed intermediate is to increase it, not, as superficial study of the model might lead one to expect, to decrease it. It is obvious, therefore, that introducing a channel into a model without making any other changes to it does not provide a mechanism for decreasing the concentration of the by-passed intermediate: it has very little effect of any kind; and such effect as it does have is in the wrong direction.

Before examining whether this is the most realistic model of a channel, we should enquire how this result can be understood, as at first sight it may appear counter-intuitive. The most obvious interpretation is that it arises from the increase in flux from A to D brought about by introducing a second mechanism in parallel to the original one: this increase in flux must increase the concentration of D; as this is already (see Scheme 2) almost at the half-saturation value (Michaelis constant) for the process that consumes it, increasing it further must noticeably increase the degree of saturation of the last enzyme and bring about additional product inhibition (and back reaction) in the conversion of C to D, thereby increasing the concentration of C.

If this interpretation is correct, we should expect that the effect would be more pronounced in a model in which the final enzyme is closer to saturation in the initial state with no channelling. This may be tested by replacing the expression $d/(1+d)$ for the rate of the last step by $0.5\,d/(0.07818+d)$, reducing the limiting rate from 1 to 0.5, so that instead of being about 50% of the limiting rate, a rate of 0.47 is more than 90%, the new Michaelis constant of 0.07818 being calculated to give the same value of d at this rate. With this model the results are altered to those shown in Table 3. As expected from the interpretation given above, the change in c is now greater than it was in Table 2, though it remains small.

I am grateful to a referee for pointing out a different way in which these results can be understood. It is obvious from elementary kinetic considerations that in the steady state the concentration of C can be expressed as $5([E_2X]+[E_3Y])/6([E_2]+[E_3])$, regardless of the value of p, i.e. regardless of the extent of channelling. Thus channelling can only change the concentration of an intermediate to the extent that it can change the proportions of enzyme-substrate complex and free enzyme in the unchannelled branch. In Scheme 3, where p enters symmetrically in four rate constants, such an effect can hardly be envisaged; but even in more complex models with more independence between the four additional rate constants, it is unlikely that varying these rate constants could bring about very large changes in the intermediate concentration.

Effect of channelling in a model with constant net flux

An obvious objection to the model explored in Tables 2 and 3 is that it violates, albeit slightly, the initial assumption that the concentration $a = 10$ and the constant flux of 0.47 from A to D satisfy the needs of the system: although the value of a has been maintained, the flux has been allowed to increase as an alternative parallel route became available. More properly, one should suppose that during evolution of the channelling route the activities of the direct-route reaction ought to decline to maintain a constant flux. This can be modelled by introducing a factor q into each of the four rate

Table 4. *Effect of channelling when the total flux is maintained constant*

Flux via C	Channel flux	Total flux	c
0.4543	0.0033 (0.72%)	0.4576	9.9999
0.4510	0.0066 (1.44%)	0.4576	10.0019
0.4445	0.0131 (2.86%)	0.4576	10.0058
0.4243	0.0333 (7.29%)	0.4576	9.9953
0.3912	0.0664 (14.5%)	0.4576	9.9972
0.3254	0.1322 (28.9%)	0.4576	10.0010
0.2278	0.2299 (50.2%)	0.4576	10.0064
1.1280	0.3296 (72.0%)	0.4576	10.0032
0.0921	0.3655 (79.9%)	0.4576	9.9966
0.0460	0.4116 (89.9%)	0.4576	9.9974
0.0228	0.4348 (95.0%)	0.4576	10.0038
0.0091	0.4485 (98.0%)	0.4576	10.0029
0.0046	0.4531 (99.0%)	0.4576	10.0065
0.0023	0.4554 (99.5%)	0.4576	10.0060

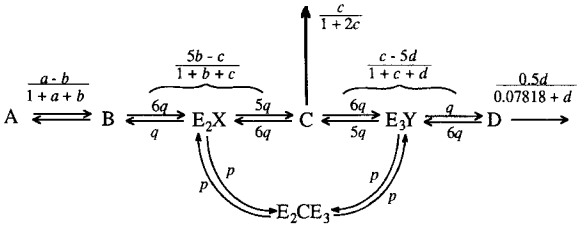
constants in the reactions from E_2X to C and from C to E_3Y. When this is done, and q is decreased concomitantly with the increase in p so as to maintain a constant flux from A to D, the results are as shown in Table 4. It is apparent that in this case there is no systematic effect on c, the small variations being a consequence of slight inaccuracies in the modelling, even though the proportion of flux through the channel increases from less than 1% to more than 99%. The conclusion, therefore, is very simple: channelling has no effect on the free concentration of a channelled intermediate in a pathway.

Does channelling decrease the possibility of leakage or toxic effects of intermediates?

In all of this, we have left unanswered the question of why the high concentration of an intermediate might be harmful. Examination of Scheme 1 suggests no obvious reason why a value of $c = 10$ might be considered less advantageous than $c = 5$; indeed, in the model as drawn no reason exists if we consider C in isolation. However, as noted in the Discussion, similar accumulations throughout an entire system imply tying up substantial amounts of resources unproductively, and straining the solvent capacity of the cell water. Even in isolation, high concentrations of C would be harmful it it were toxic, or even if were just too readily available as substrate of another pathway. Such possibilities may be summarized by including an additional reaction in the model to represent the consumption of C by unwanted reactions (Scheme 4).

Results with this model (Fig. 1) show that the concentration of c now decreases when the channelled flux increases from 0% to 100% of the productive flux to D. However, the improvement with respect to the earlier models is not impressive, corresponding to a decrease in c of only 10%, and the effect on the harmful reaction is much less, amounting to about 1% at most.

The numerical values here are, of course, determined by the particular assumptions made, but the tendency of the effect to be less on the harmful reactions as such than on the concentration that permits them is likely to be general. This is because most reactions in biological systems are saturable, so that most irreversible processes have elasticities less than unity with respect to the concentration of the starting material. In consequence, we can conclude that when one makes it explicit in the model why one considers a high intermediate



Scheme 4. *Model in which C can leak out of the pathway*

concentration to be harmful, one can demonstrate that the existence of a channel can be beneficial. However, this beneficial effect is likely to be so extremely small over the range considered in Fig. 1 that it is difficult to see how it can be very useful, or how it could lead to a significant selective pressure to allow channelling to evolve from a state in which there is no channelling.

However, it is obvious in the case of c, and perceptible in the other cases, that the curves become steeper as the channel flux approaches 100%, and this suggests that one ought to investigate what happens when this flux is increased beyond 100%, which is possible, of course, because the excess can reach the leak reaction by flux from E_3Y to C. The complete curve for c is shown in Fig. 2; the corresponding curve for the leak flux is qualitatively similar but turns more abruptly near the maximum channel flux of 132.4%. It is now apparent that for any channel flux in the range 100–132.4% of the productive flux there are two pairs of p, q values that give the same channel flux, but different (and over most of this range very different) values of the leak flux and concentration of C. It is instructive to compare the two pairs of values that give a channel flux of 100%: it is hardly surprising that the value of q is high (0.338) for a high leak flux, and zero for a zero leak flux; less obviously, however, the values of p are also quite different, 10.67 in the former case and 3.21 in the latter. Notice that changing from a useless channel to a useful one here implies decreasing the activity of the channel reactions by more than a factor of 3.

The flux diagrams in Fig. 2 illustrate how the distribution of fluxes between the different branches of the pathway varies along the curve. It is striking that as one varies the parameters between 0% and 100% channelling there is no significant effect on the leak flux: virtually the entire effect of such variation is to determine whether E_3Y is produced from C or from E_2CE_3, confirming the impression given by Fig. 1 that selective pressure could scarcely drive a system along this curve during the course of evolution. However, if a random saltation happened to place a system on the vertical part of the curve around 130% channelling, there might then be strong selective pressure in favour of eliminating the leak. Over the whole of this part of the curve, decreasing the amount of leak does not involve increasing the amount of channelling; on the contrary, the flux through E_2CE_3 decreases as the system approaches the state with no leak.

DISCUSSION

Srere [3] has commented that '80% of the shown metabolic intermediates {in a figure from Alberts et al. [9] illustrating graphically the connections between 520 metabolites} have just one use in the cell. It is apparent that it would be a wasteful process if these intermediates each had to fill the water volume

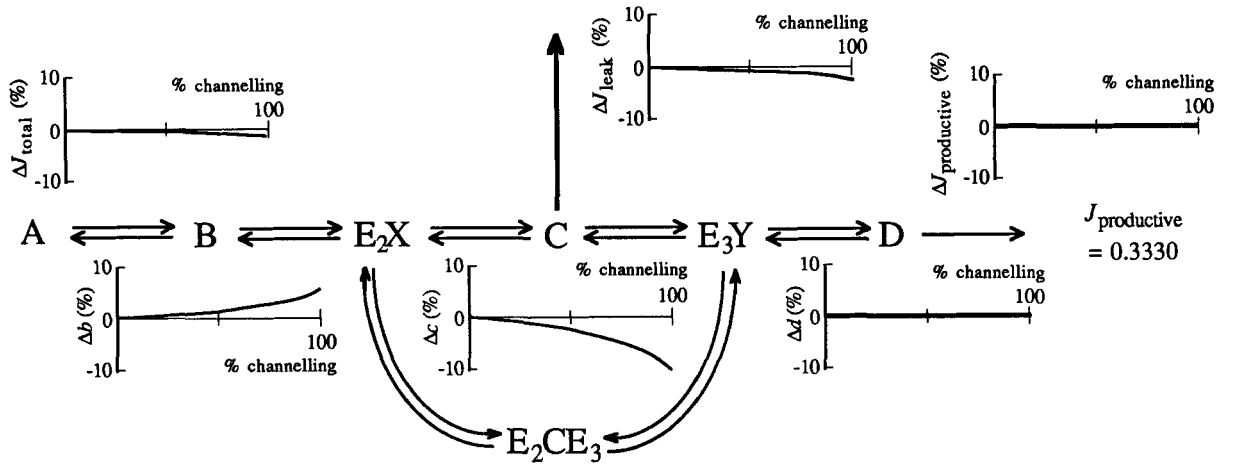


Fig. 1. *Effect of channelling on leakage of intermediates.* For the model shown in Scheme 4, a was held constant at a value of 10, and p and q were varied simultaneously in such a way as to maintain a constant flux of 0.3330 in the reaction from D, while varying the channelled proportion of this flux from 0% to 100%. The six inset graphs show the percentage deviations of three fluxes (shown above the pathway) and three concentrations (below) from their values in the absence of channelling over this range of channelling. In all cases the abscissa shows the channelled flux as a percentage of the productive flux

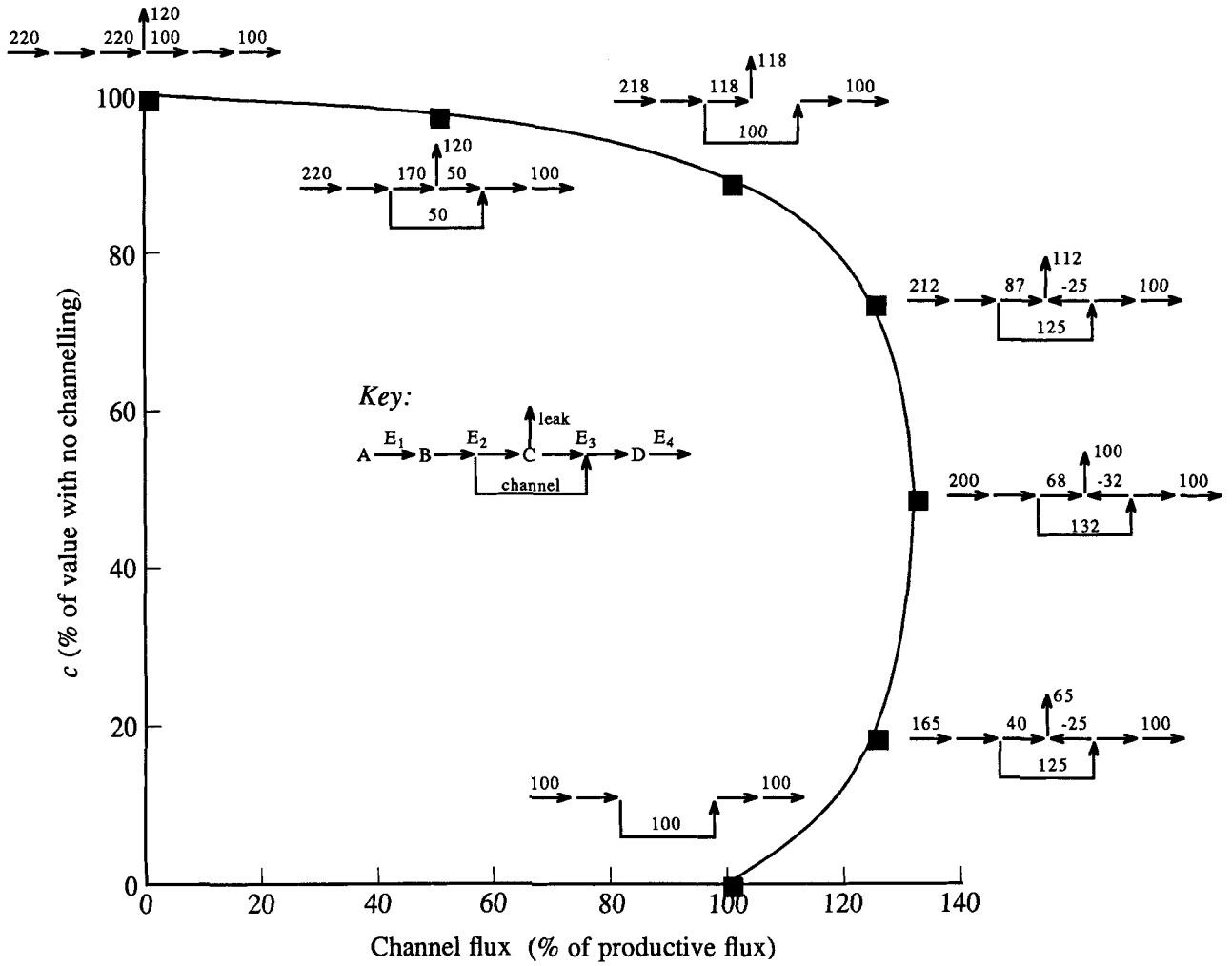


Fig. 2. *Effect of channelling over an extended range.* The figure shows the concentration of C for the model shown in Scheme 4, studied under the same conditions as in Fig. 1, except that the channel flux was allowed to exceed the productive flux (which is possible because the excess goes into the leak by right-to-left flux from E_3Y to C). At each of several points on the curve marked by (■) the partition of flux between the various branches is shown diagrammatically, the numerical values representing percentage of the productive flux of 0.333. The key in the centre of the figure shows the relationship between the arrows in these diagrams and the reactions shown in Fig. 1

of the cell to attain its operating concentration.' Referring to Atkinson's discussion of the limited solvent capacity of the cell [10], he argued that channelling would allow the difficulty to be overcome. Srivastava and Bernhard [4] argue similarly, and related ideas may be found elsewhere: Ovádi, for example, in listing the advantages of channelling, mentions 'segregation of competing pathways by microcompartmentation of intermediates' [11], and Easterby writes that 'less material... is wasted in generating unwanted intermediate pools' [12]. There is an implied assumption that channelling of intermediates allows enzymes to perceive usefully high concentrations of their substrates without requiring their concentrations in the bulk solution to be correspondingly high.

In contrast, the results obtained here indicate that the free steady-state concentrations of stable intermediates that are not used by other pathways should not be affected at all by channelling (and will even be increased if activation of the channel is not accompanied by flux-compensating inhibition of the diffusion route); the decreases in concentrations of unstable intermediates or ones that can be used by competing enzymes will be too small to have any practical consequence. How can these results be reconciled with the nearly universal assumption that channelling ought to decrease free pools? In reality there is almost no evidence that channelling decreases free concentrations, and the assumption that it ought to do so contradicts common experience with flowing liquids: do we expect, for example, that the water level in a smoothly flowing river can be lowered by pumping water rapidly around one side of an island? There may indeed be a transient drop in level, but once a new steady state is reached the new level will be the same as the original one unless there is an outflow from the by-passed region.

Nonetheless, 'channeling has been shown for the noncovalently bound intermediate in the bifunctional folate enzyme of formiminotransferase-cyclodeaminase. This channeling not only increases the efficiency of the enzyme, but also maintains a low concentration of a labile intermediate that has no other function' [3]. Here, apparently, we have experimental observation of a decrease in intermediate concentration brought about by channelling, but unfortunately the quotation goes beyond what was actually observed [13], a lack of any accumulation of one intermediate and a lag in the accumulation of others during 2 min. Observations over such a short period cannot reveal anything about what is maintained in the steady state, and thus do not conflict with the results from modelling reported in the present paper.

I have been concerned here only with the steady states of channelled systems, and note in passing that quite different behaviour may be apparent when considering the effect of channelling on transient times [12, 14]. It is not difficult to believe that an intermediate may take much longer to reach a new steady state if it is efficiently channelled than if it is not; this may have a significant long-term effect if one only considers intermediates in pathways that are active for periods that are brief in relation to the time required to reach a steady state. In the extreme case, a reaction in which the proportion of flux that proceeds through the bulk solution is vanishingly small may take longer to reach steady state than the life time of the cell, and, in this case, for practical purposes one has a perfectly channelled system with no leakage and with a 'true' steady state that is just a mathematical abstraction as it is never approached. However, the sources quoted above were not primarily concerned with near-perfect channels, Srere [3], indeed, being explicit that his principal interest was with leaky ones.

If loose association between consecutive enzymes in a pathway, and hence channelling of intermediates, does not provide a way to lower free metabolite concentrations, what role may it play? To examine this question, consider the complex formed between ribulose-bisphosphate carboxylase and four other enzymes of the Calvin cycle [2]. Ribulose-bisphosphate carboxylase is not simply an enzyme selected at random; it is by far the most abundant protein in the chloroplast, as abundant as water. Thus if it were advantageous to 'package' other enzymes in a complex to help to maintain their stability during periods of inactivity through lack of substrates (for example in the dark), ribulose-bisphosphate carboxylase would be the obvious choice of packing material. The idea of complex formation as a form of packaging for stability and storage may well appear less glamorous than as a mechanism for channelling and control of metabolite concentrations, but it should not be discarded on that account.

Conversely, how can excessive metabolite concentrations be avoided, if not by channelling? Atkinson [10, 15] was probably the first to draw attention to the limited solvent capacity of the cell: in detailed discussion of possible ways of overcoming the problem he did not mention channelling; instead, he suggested chemical activation of intermediates (to increase reactivity at low concentrations), coordinate derepression or induction, and enzyme modulation. These may well be sufficient.

I thank Dr María Luz Cárdenas for valuable comments on this paper.

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