METABOLIC CHANNELING IN ORGANIZED ENZYME SYSTEMS: EXPERIMENTS AND MODELS

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ABSTRACT

The intracellular *milieu* is not a simple, homogeneous, aqueous state: protein concentration is high in eukaryotes, and even higher in prokaryotes and in organelles such as mitochondria, and membrane surfaces are clearly abundant. Evidence gathered with various techniques indicates that the cellular water does not have the same properties as water in dilute aqueous solutions. These findings support the view that classical enzymological studies may not provide sufficiently relevant information for generating a correct understanding of cellular physiology. Cellular organization exists at the molecular level: enzymes aggregate in clusters and in many cases this affects their catalytic activity. Consecutive enzymes in a number of metabolic pathways can channel their common intermediates without release to the "bulk" solution. This process can occur either via stable (static) multienzyme complexes or via short-lived (dynamic) enzyme–metabolite–enzyme complexes. Static complexes are found in anabolic pathways such as amino acid, nucleotide, and protein biosynthesis, where most of the intermediates have no other function or destination in the cell; dynamic complexes occur in amphibolic pathways where there are various flow-bifurcations. Channeling between dynamic complexes of enzymes is in some ways harder to demonstrate since the enzyme–enzyme complexes are not stable and are thus not isolatable. Theoretical developments, and simulations of existing metabolic channeling models, are not abundant. We review such studies and propose how modeling should evolve, the better to match the evolution of physiological experiments from *in vitro* to *in situ* to *in vivo*.

I. *IN VIVO* IS NOT THE SAME AS *IN VITRO*

Essentially since the beginning of modern biochemistry itself (Schlenk, 1985), enzymologists have studied enzymes *in vitro*. This (reductionist) approach to understanding cellular behavior is based on the belief that the phenomena observed in cells can be attributed solely to the properties of the cell components. The quest then has been that of isolating the thousands of different enzymes in the living world and studying their physicochemical properties *in vitro*, with the implicit assumption that after this knowledge had been attained one could somehow "reconstitute" the properties of the cell, if not in practice at least in principle. Throughout the last three decades (and some would say the whole century), a large amount of evidence has accumulated that suggests this approach is essentially flawed. Two main arguments are as follows: (1) following the pioneering studies of Kacser and Burns (1973) and of Heinrich and Rapoport (1974) it has been shown that the steady-state behavior of fluxes and metabolite concentrations within a cell are *systemic* properties not properly accountable in terms of the behavior of single enzymes, but instead by the concerted action of *all* of them (indeed even by noncatalyzed processes); and (2) the conditions used for *in vitro* assays are so far from those observed in cells (which are generally unknown) as to make extrapolations from *in vitro* to *in vivo* at best

hazardous and at worst completely misleading. While the first point is very important (it is discussed in detail in the Cornish-Bowden chapter of this volume), we shall concentrate on the second. Since this book is about enzymology *in vivo*, it is worth discussing some of the findings that lead to the conclusion that a reductionistic analysis of cell biology is doomed to fail, an analysis which may be seen as an implication of the Humpty Dumpty effect (Kell and Welch, 1991). It is worth rehearsing the general argument.

The notion of "analytical reductionism" is intimately associated with the principles of irreversibility and boundary conditions. As Prigogine and Stengers (1984) point out, "Irreversibility is either true on all levels or on none: it cannot emerge as if out of nothing, on going from one level to another," but as nicely delineated by Coveney and Highfield (1990), irreversibility remains a philosophical enigma. Newtonian physics is time-reversible; if we watch a film of billiard balls colliding, we cannot tell whether the film is running forwards or backwards. By contrast, if we observe a film of a bull in a china shop, we may be fairly confident that the film is running in one (the "forward") direction; bulls do not normally reassemble broken crockery and emerge smiling from retail stores. Thus, as one sees with Humpty Dumpty, there are many ways of breaking things, but only one way of putting them together correctly. The key point is that the successively higher levels of the hierarchically organized, complex living cell are dependent, reductionistically, not so much on the *elements* at the lower levels, but on the nature and existence of boundary constraints. If one removes the constraints at a given level, the systemic (or holistic) properties of all higher levels potentially collapse. Thus, while individual protein molecules can be persuaded to refold to their "native" states (Anfinsen, 1973), though not reversibly in the sense of *microscopic* reversibility, no one has succeeded in making a cell do so, let alone an organism such as Humpty Dumpty, and there are straightforward combinatorial arguments why they are unlikely to succeed (Kell, 1988a,b; Kell and Welch, 1991).

In recent decades, we have come to appreciate some of the boundary constraints extant *in vivo*. To begin with, the intracellular medium is not a simple, homogeneous, aqueous state. Its protein content is extremely high (100–300 mg/ml in eukaryotes, and maybe double that in prokaryotes), and membrane surfaces are clearly abundant. Electron microscopy has revealed a complex and diverse particulate infrastructure in living cells, especially in the larger eukaryotic cells. This structure encompasses not only an extensive membranous reticulation but also a "ground substance" which is laced with a dense array of proteinaceous cytoskeletal elements. The protein density in association with these membranous and fibrous structures is akin to that in crystals (Sitte, 1980). In particular, the work of Porter and collaborators (see for example Porter and Anderson, 1982; Porter and Tucker, 1981) has revealed an intricate network structure in the cytoplasm of eukaryotic cells. This network has been named the microtrabecular lattice (MTL) and it is observable in high-voltage electron photomicrographs. The existence of the MTL does not, by itself, exclude the hypothesis that the enzymes found in the soluble

fraction would also be in solution in the cytoplasm. The extra evidence needed can be found in the experiments of Kempner and Miller (1968a,b) with *Euglena gracilis*. Kempner and Miller found that due to their hard cellular wall, *E. gracilis* cells can be centrifuged at 100,000 × *g* for 1 hour without disruption, after which the various cellular components become stratified inside the cell. An important aspect is that the cells remained viable under these conditions. Kempner and Miller analyzed quickly frozen stratified *E. gracilis* cells by cytochemical methods for the presence of 19 different enzymes and found that none of these enzymatic activities were present in the ostensibly "soluble" aqueous phase, but rather in denser layers. However, if the cells were homogenized *before* the centrifugation, all of those enzymes were then found in the 100,000 × *g* supernatant. These experiments undoubtedly show that most of the "soluble" enzymes are in fact not in solution at all within *E. gracilis* cells. Similar experiments made with *Neurospora* (Zalokar, 1960) and ultracentrifugation and biochemical studies on *Artemia* cysts (Clegg, 1982) produced similar results. We have no reason to think that other eukaryotic cells would be much different from these. Another strong piece of evidence for the bound state of cytoplasmic proteins in the cell comes from studies with cells whose plasma membranes were made permeable (Kell and Walter, 1986; Clegg and Jackson, 1988; 1990). In some cases the pores in the plasma membrane were big enough to allow molecules of ~400 kDa to pass through them; nevertheless the loss of protein from these cells was small, indicating that most proteins are associated with some structure (or at least in complexes bigger than 400 kDa) (Clegg and Jackson, 1988; 1990).

Additional evidence for the cytoskeletal infrastructure comes from electron spin resonance (ESR) (Mastro and Hurley, 1987), fluorescence recovery after photobleaching (FRAP) (Luby-Phelps et al., 1988) and microfluorimetric (Fushimi and Verkman, 1991) studies *in situ*, which each show the interstititial voids (200–400 Å in diameter) to contain a medium akin to a dilute aqueous *milieu* of low macromolecular density.

It is widely understood that in order to reproduce *in vitro* the properties of enzymes that in their native cellular *milieu* are rigidly membrane-associated one must provide them with some sort of proteolipid environment, frequently by isolating them in fragments of the original membrane or otherwise by incorporating them into proteoliposomes. Unfortunately, the same belief is not so commonly held for the so-called soluble enzymes that are present in the 100,000 × *g* supernatant fraction. In not seeking to emulate more closely the native microenvironment *in vitro*, we take the risk of building models of cells which have little resemblance to reality. One immediate consequence of this extensive organization of enzymes in the cytoplasmic compartment (and others) is that the classic, bulk-phase, scalar concept of concentration is no longer very helpful. Instead we may have to start thinking in terms of "local concentrations" (Welch, 1977).

Available evidence from ESR (Mastro and Keith, 1981), nuclear magnetic resonance (NMR) (Seitz et al., 1981), quasi-elastic nuclear scattering (Trantham et

al., 1984), and microwave dielectric measurements (Clegg et al., 1982; 1984; Bruni et al., 1989) indicate that the cellular water does not have the same properties as water in dilute aqueous solutions. In the late 1930s, Kopac and Chambers (reviewed in Chambers, 1940), using microinjection of oil droplets in echinoderm egg cells, observed an absence of the Devaux effect (the surface of the droplet crinkles due to adsorption of proteins), which should be observable if the intracellular proteins were in aqueous *solution*. These findings support the view that classical enzymological studies may not be terribly relevant to what goes on inside a cell.

II. ORGANIZATION LEADS TO CHANNELING

In parallel to these findings about the structure of the cytoplasmic matrix, there is a considerable amount of evidence for enzyme–enzyme interactions, both in this compartment and inside other cellular organelles such as the mitochondrion, and within prokaryotic cells. These interactions could of course simply be a consequence of proximity effects if enzymes are part of the MTL or are adsorbed to it in the cytoplasmic compartment, or because the enzyme concentration is high (as in mitochondria and prokaryotes). However, a very interesting and important set of enzyme–enzyme interactions, for the existence of which there is large body of evidence (e.g., Friedrich, 1984; Srivastava and Bernhard, 1986; Srere, 1987; Keleti, et al., 1989), is that in which two (or more) sequential enzymes in a pathway transfer their common intermediate (the product of the first and the substrate of the second) *directly*, i.e. without releasing it to the bulk solvent. The evidence for this phenomenon, known as *metabolic channeling*, has been gathered by several methods, from X-ray crystallography to kinetic experiments *in vitro* (Keleti et al., 1989).

III. STATIC VERSUS DYNAMIC CHANNELS

One obvious situation in which metabolic channeling can easily occur is that of the "static" multienzyme clusters, complexes of several polypeptides with different enzymatic activities. There are many well-known examples of such clusters, including the pyruvate dehydrogenase and fatty acid synthase complexes and the ribosome. In such cases it is common knowledge that intermediates of the reactions catalyzed by these clusters are not released to the solvent. Of particular interest is the case of tryptophan synthase, whose structure (from *Salmonella typhimurium*) has been determined by X-ray crystallography to 2.5 Å resolution (Hyde et al., 1988; Hyde and Miles, 1990). This cluster is an $\alpha_2\beta_2$ tetramer; the α chains convert indole 3-glycerol-phosphate to indole and D-glyceraldehyde 3-phosphate, and the β chains convert indole and L-serine to L-tryptophan. The images reconstructed by Hyde and co-workers show a tunnel going from the active center of one α polypeptide chain to the active center of one β chain (there are two of these tunnels per cluster). It is through this tunnel that indole undergoes "facilitated diffusion"

from one active center, and is thus not released to the solvent. One obvious advantage of this mechanism is that if indole were to be released to solution it would be able, due to its hydrophobic character, to diffuse freely across the plasma membrane and thus escape (Manney, 1970) (as indeed it does in strains harboring the tryptophanase gene (Goodacre and Kell, 1993)). This former fact is particularly nicely illustrated in a recent paper by Murdock et al. (1993), who were interested in the biotechnological production of indigo from carbohydrates, via indole as a cellular intermediate within the cell. Only after site-directed mutagenesis of the tryptophan synthase, *to cause indole to leak from its channel*, were satisfactory yields achieved.

The channeling of metabolic intermediates, here known as *static* channeling or channeling via static complexes is not, however, restricted to stable multienzyme complexes; it can also take place via short-lived enzyme complexes referred to as *dynamic* channels (Friedrich, 1974; 1984; 1985). Channeling between dynamic complexes of enzymes is in some ways harder to demonstrate since the enzyme–enzyme complexes are not stable and are thus not isolatable. Of course this classification describes two extreme situations, and one may consider that in reality enzymatic complexes have properties that are a continuum between the two extremes (Ovádi, 1991). Ovádi (1991) proposed a general mechanism which can account for this continuum, and although not absolutely complete (see e.g., Ryazanov, 1988), this general mechanism is the best and most convenient description to date.

There is a remarkable correlation between the organizational state and the functionality of supramolecular enzyme complexes (Welch and Marmillot, 1991): static complexes are found in anabolic pathways such as amino acid, nucleotide and protein biosynthesis. In these pathways most of the intermediates have no other function or destination in the cell (Srere, 1987; Alberts et al., 1989). In some cases, as for indole in the tryptophan synthase reactions in wild-type strains, there is essentially no leakage of the metabolite to the solvent (perfect channeling). Dynamic complexes occur in amphibolic pathways like glycolysis and the Krebs tricarboxylic acid (TCA) cycle where there are various flow-bifurcations. In this case there are metabolites at branch-points, which are known (e.g. from NMR measurements) to exist in soluble pools. However this does not rule out the possibility that a proportion of these substances is bound to enzymes (either individually or in complexes); in some cases, e.g. for oxaloacetate in mitochondria (Atkinson, 1969), the number of binding sites exceeds the number of molecules extractable using perchloric acid. Indeed, there is evidence that the role of enzymes is not simply catalytic but also structural (Wistow et al., 1987). Based on the high concentration of enzymes and the relatively low concentration of some of their intermediates in glycolysis, it has been argued that an important role of the cytoplasmic enzymes may be that of buffering the concentrations of their ligands (Betts and Srivastava, 1991; Srivastava, 1991). In this case, enzyme–intermediate

complexes may act as better substrates for the next enzymes in the pathway than are the soluble intermediates themselves (Srivastava and Bernhard, 1985).

IV. SOME CONTROVERSIES ABOUT DYNAMIC CHANNELING

In a series of elegant *in vitro* kinetic experiments, Srivastava and Bernhard observed channeling of NADH among some dehydrogenases (reviewed in Srivastava and Bernhard, 1986; see also Srivastava et al., 1989). The basis of their experiments is the buffering of NADH by the first dehydrogenase, such that if channeling occurs then the rate of the reaction of the second dehydrogenase calculated from the concentration of free NADH present will be different from the experimental rate. A requirement for these experiments is that the first dehydrogenase must be in large molar excess with respect to NADH which is in turn in molar excess over the second dehydrogenase; also the dissociation constant of the NADH from the first dehydrogenase must be known. Srivastava et al. (1985) and Srivastava and Bernhard (1986) also showed a remarkable correlation between the stereospecificity of NADH binding to dehydrogenases and the channeling of NADH between pairs of dehydrogenases. It turns out that in order for channeling to occur the two dehydrogenases must be of opposite chiral specificity to NADH (there are two possible stereospecificities, A and B). Chock and coworkers reported inconsistencies of this analysis, based in part on their own repetition of these experiments (Chock and Gutfreund, 1988; Wu et al., 1991), but it is now clear that this was due to the fact that they failed to reproduce key features of the original experiments, such as the ionic strength (Srivastava et al., 1989; Srivastava, 1991). The remarkable correlation between enhanced catalysis and opposite chiral specificity of the dehydrogenases (and no enhancement for equal specificities) is the major factor supporting the channeling model and this was never addressed by Chock's group.

There are a number of other cases of experimental results that point to channeling in dynamic complexes, but which have not universally been accepted. Ovádi and Keleti (1978) proposed channeling of glyceraldehyde-3-phosphate from aldolase to glyceraldehyde-3-phosphate dehydrogenase but Kvassman et al. (1988), although reproducing Ovádi and Keleti's results, argued that these were also consistent with a free diffusion mechanism. Vértessy and Ovádi (1987; see also Srivastava and Bernhard, 1986) showed data that suggested the channeling of dihydroxyacetone phosphate from aldolase to glycerol-3-phosphate dehydrogenase, although Chock and Gutfreund (1988) offered an alternative interpretation. Weber and Bernhard (1982) suggested direct transfer of 1,3-diphosphoglycerate between glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase; however Kvassman and Pettersson (1989a,b) argued that if autocatalytic effects of NAD^+ were taken into account then it would not be necessary to invoke channeling. Unfortunately, in most of these reports the data were not obtained under the same

conditions by the different groups; there has also been a certain tendency among those who argue against channeling to do so despite the fact that only data which *also* support free diffusion are shown.

There are, however, cases of metabolic channeling which occur through transient enzyme complexes and whose existence is unchallenged, despite the fact that, as in the previous cases, the actual multienzyme complex has never been detected. One example is that of electron transfers between enzyme-bound flavin coenzymes (Steenkamp, 1987).

The Krebs tricarboxylic-acid cycle (TCA), has been extensively studied in terms of its supramolecular organization (Sumegi et al., 1992 and references therein; see also Srere et al., this volume). Srere has developed the concept of the *metabolon*, a complex of the enzymes of the TCA cycle which is anchored to the inner mitochondrial membrane (Srere, 1985). The *metabolon* can be obtained intact from sonicated mitochondria (Srere, 1985; 1987) and there is evidence that the intermediates of the TCA cycle are channeled in the *metabolon*. Data that support this view were obtained *in situ* with gently disrupted (sonicated) mitochondria (Robinson and Srere, 1985; Robinson et al., 1987; Sumegi et al., 1992) and *in vivo* in yeast cells using NMR techniques with [3-^{13}C]-propionate as the substrate (Sumegi et al., 1990; 1992).

Using chromatographic techniques, Gontero et al. (1988) isolated a complex of five enzymes of the Calvin cycle from spinach leaves while Persson and Johansson isolated a 6-enzyme complex from the same source, using partition in an aqueous two-phase system (Persson and Johansson, 1989). Interestingly enough, no interaction between purified enzymes of these complexes could be demonstrated *in vitro*. This strengthens the argument that one should study viable cells, and that if for technical reasons this is not possible, that one should try to emulate the relevant physiological environment as much as possible. The words of Henrik Kacser (1986) could not be more appropriate: "But one thing is certain: to understand the whole, you must look at the whole."

One of the reasons that it is arguable is dominant in determining the difficulty in demonstrating dynamic complexes *in vitro* is the possibility that, in the intact cell, these complexes might be stabilized by other cellular structures (Clegg and Jackson, 1988). The MTL is an attractive candidate for such interactions (Clegg, 1984) but membranes can also play an important role (Wilson, 1978). The interaction of muscle cell glycolytic enzymes with actin (Clarke and Masters, 1976; Knull and Walsh, 1992) is a remarkable example, but other specialized cell structures are certainly also involved. It may also be that enzyme–enzyme complexes can affect the strength of allosteric effects (or even create new ones) (Welch, 1977). It is also worth pointing out that the inability of a cloned enzyme to affect the flux through a pathway may be due either to the fact that they have a low flux-control coefficient or because they are unable to participate in channeling due to their expression at unsuitable concentrations or in an unsuitable location; to hope to distinguish these,

one should clone the relevant genes down as well as up (Brindle, 1988; Kell and Westerhoff, 1990).

For pathways such as glycolysis there is a distinct structure–function duality. This is evidenced for example in the reversible, "ambiquitous" partitioning of glycolytic enzymes between cytomatrix-bound and aqueous cytoplasm-free forms (Masters, 1981; Knull and Walsh, 1992; Uyeda, 1992). The direct-transfer "channeling" scheme is probably immaterial for the *unbound* enzyme fraction in the bulk cytoplasmic solution. Depending on the cell type, the "whole cell" concentration of the individual glycolytic enzymes is in the range 1–100 μM. Considering the high, *bulk* "viscosity" of the cytoplasm relative to the translational motion of the macromolecles (Mastro and Hurley, 1987; Luby-Phelps et al., 1988), it is unlikely that the binary complex, consisting of enzyme$_1$ bound to its nascent product molecule, would last long enough to form the requisite ternary complex with enzyme$_2$ (Keizer and Smolen, 1992). However, for the *cytomatrix-associated* enzyme population, the microenvironmental situation is quite different. The "local" enzyme concentration is probably much higher than the averaged, "whole-cell" value. In addition, the total macromolecular density at the cytomatrix–"cytosol" interfaces (≤ 100 Å into the bulk phase) is extremely high (Sitte, 1980), probably similar to that within the mitochondrial matrix (viz. 250–500 mg/ml; Srere, 1985). Recent *in situ* measurements of the mitochondrial matrix bulk viscosity show the diffusion coefficient for small-metabolite-sized particles to be as much as 30-fold smaller than that for normal water (Abney et al., 1993). With such high "local" enzyme concentrations, along with the high microenvironmental viscosity (Siegbahn et al., 1985) and high activity coefficients for protein-based reactions in such a "crowded" medium (Minton, 1990), the efficacy of formation of channeling ternary complexes has been calculated to be greatly enhanced (Keizer and Smolen, 1992). The size of the free metabolite pools for the dynamically interacting (pairwise) enzyme systems will also depend on molar ratios of the metabolically consecutive enzymes, as well as on the respective enzyme kinetic mechanisms; moreover, the physiological grounds for channeling may not always entail a kinetic flux advantage for the multienzyme system (Srivastava, 1991; Welch and Easterby, 1994).

Meanwhile, one should be cognizant of the weight of the *in vivo* evidence for channeling in these pathways, obtained from the many whole-cell studies (Paul, 1989; Clegg and Jackson, 1990; Srere, 1992; Sumegi et al., 1992), as well as the correlation with *in vitro* indications of a "physiological" regulation of enzyme–cytomatrix associations (Masters, 1981; Knull and Walsh, 1992; Uyeda, 1992) and of the control of enzyme–enzyme interactions at pathway branchpoints (Tompa et al., 1987; Neuzil et al., 1990). The ambiquitous character of the enzyme organization—and more specifically the variability in the degree (and spatial locale) of channeling—in the branching amphibolic pathways may reflect the need to maintain adequate catabolite pools for proper responsiveness of such multifunctional processes to varying cellular demands (Easterby, 1991). It is within the localized

cytomatrix microenvironments that channeling of amphibolic flow may therefore be expected.

V. MODELING STRATEGIES FOR STUDYING ENZYMOLOGY *IN VIVO*

All interpretations of experimental results are based on models, and metabolic channeling is no exception. Generally, enzyme models exhibit nonlinear behavior, and it is very difficult (and dangerous) to make predictions from those models by simple reasoning alone. For that purpose one has to formulate the model in mathematical terms and use the equations to calculate the values that the variables in question will take as a function of different starting conditions (parameters). These calculations are best carried out using computer programs to minimize errors and to process the calculations at an acceptable speed. A number of such programs are available (see e.g. Letellier et al., 1990; Kell et al., 1993; and Cornish-Bowden, this volume); in some of our own work on the simulation of metabolic channeling (Mendes et al., 1992), we have used the program GEPASI (Mendes, 1993).

Smolen and Keizer (1990) simulated a model of dynamic channeling with the direct transfer of NADH between dehydrogenases in mind. This group concluded from their simulations that the conditions for the ternary complex dehydrogenase$_1$–NADH–dehydrogenase$_2$ to form are in accordance with the estimates of K_m for the oxidation of the complex dehydrogenase$_1$–NADH by dehydrogenase$_2$. More recently these authors have extended their analysis to complexes of three dehydrogenases which would cycle the redox couple NAD^+/NADH. They concluded that there would be novel methods for regulation of the redox state of the couple which are consequent upon the channeling mechanism (Keizer and Smolen, 1992).

One consequence of metabolic channeling that has frequently been mentioned is that the operation of the catalytic path through the channel would be expected to reduce the size of the soluble pool of the intermediate compared to the case where the reaction proceeded exclusively via diffusion in the solvent (Srere and Mosbach, 1974; Kell, 1979; Heinrich et al., 1991; Ovádi, 1991). Cornish-Bowden (1991) showed that this is not always the case by simulating a model of metabolite channeling through a dynamic bi-enzyme complex. He then argued that this reduction of soluble pool size could *never* happen with a dynamic channel, but it was later shown that this generalization does not hold: channeling can decrease the pool size substantially (Mendes et al., 1992). It was also shown that if channeling operated through a static bi-enzyme complex then the size of the soluble pool could be decreased to an arbitrarily low level by increasing the proportion of flux through the channel (Mendes et al., 1992).

Brooks and Storey (1991) investigated the possibility of the existence of a complex of glycolytic enzymes in muscle cells. They used *in vitro* data for the association constants of the several complexes (enzyme–enzyme, enzyme–F-actin

and enzyme–calmodulin) and relative activities of these complexes to conclude that complexes of enzymes on F-actin "may not exist;" complexes of *some* sequential glycolytic enzyme pairs could exist to a significant degree and increase the glycolytic flux, and the binding of phosphofructokinase with F-actin could be a regulatory mechanism to control glycolytic rate. However, this study was based solely on the equilibrium distribution of free and bound protein species. This is far from the reality of muscle cells where the glycolytic enzymes are responsible for very high net fluxes. Also not considered in this analysis was the effect of the intermediate metabolites, which is very important, as shown for example in the studies of Smolen and Keizer (1990; Keizer and Smolen, 1992)—the association constant for an E1–E2 enzyme complex is certain to be different from that when the intermediate is part of the complex (E1–M–E2).

Sauro and Kacser (1990), from a theoretical metabolic control analysis of a model of static channeling, predicted that the increase of the logarithmic flux of a binary–enzyme complex when the logarithmic concentration of both enzymes is increased simultaneously is not linear. Westerhoff and Kell (1988), Kell and Westerhoff (1990) and Welch and Keleti (1990) detail other properties of metabolic channels for which metabolic control analysis may be used to distinguish channeling from pool behavior.

All the above studies were based on catalysis in homogeneous solution, which we know is irrelevant for cellular compartments *in vivo* (Porter and Tucker, 1981; Clegg, 1984; Srere, 1987). Many of the supposed consequences of channeling through multienzyme complexes are specifically dependent on this feature. Perhaps the most frequently cited consequence of channeling is the reduction of the transient time of a reaction sequence, numerically equal to the sum of the metabolite concentrations divided by the pathway flux. A lower bound on the value of the transient time, τ, for the enzyme reaction (viewed as an intermediary metabolic process) can, under simplified (viz. pseudo-first order) steady-state conditions, be expressed as follows:

$$\tau = \frac{1}{\Omega \cdot D_{ES} \cdot R_{ES} \cdot f_e \cdot f_g\,[E]_T \cdot N_0} + \frac{k_{-s} + k_{cat}}{k_{+s} \cdot k_{cat} \cdot [E]_T} \qquad (1)$$

$$= \tau_d + \tau_r$$

where Ω is a steric-orientation factor (e.g. a geometric solid angle within the range $0 < \Omega \leq 4\pi$) relating to the approach of the substrate to the enzyme active site, D_{ES} is the sum of the diffusion coefficients of E and S (where usually $D_{ES} \approx D_S$), R_{ES} is the "reaction distance" (viz. the sum of the radii of the substrate molecule and of the "recognition volume" of the enzyme active site), f_e is an electrostatic term arising from the possible influence of the net charge of the globular protein on an approaching, charged substrate molecule (with typical values of f_e ranging from 0.1 to 10, depending on whether the algebraic product of the net charge of the globular

protein and that of the substrate molecule is positive or negative, respectively), f_g is a "gating" term due to the potential effect of local motions of proteinaceous lobes surrounding the active site (where typically $f_g \leq 0.5$ if slow protein-dynamical gating is identifiable), $[E]_T$ is the total enzyme concentration, N_0 is Avogadro's number, k_{+s}, k_{-s} and k_{cat} are the (intrinsic) unitary rate constants for the binding of substrate to the enzyme, the release of substrate, and catalytic turnover respectively. (For details of the derivation, see Westerhoff and Welch, 1992.)

Such effects can only be investigated if the spatial dimension is explicitly part of the model. Reaction–diffusion models are unfortunately more difficult to simulate than normal kinetic models and there are no software packages available for this purpose. We suggest that only when such studies are properly performed can the "physiological significance" of metabolic channeling be understood.

Marmillot et al. (1992) have recently studied the spatiotemporal organization of the reaction catalyzed by phosphofructokinase (PFK). They extended a model of Goldbeter and Lefever (1972) by allowing PFK to exist in both free and bound (to subcellular structures) forms. They observed sustained unidirectional wave propagation, a consequence of the nonuniform distribution of oscillation periods in the soluble phase (Marmillot et al., 1992). In this case, the segregation of a metabolite in more than one pool *in the aqueous phase* is temporal as well as spatial (see also Friedrich, 1984; 1985). In parallel with the arguments for doing enzymology *in vivo* rather than *in vitro*, this type of spatiotemporal modeling (as in Goodwin and Trainor, 1985) must become widely used to account for the inhomogeneity of the cellular compartments, and indeed of populations of cells generally (Kell, 1988a; Kell et al., 1991). Model studies of "well-stirred" reactions can give insight only into experiments *in vitro*. This is even more important in the case of metabolic channeling and only then can we really start discussing the consequences of channeling in cells. We can only be tempted to call this type of analysis "*in vivo* modeling".

VI. CONCLUDING REMARKS

Biology seems beset today with the same *fin de siècle* euphoria which affects the science of physics. Many sages are hailing the "end of physics," what with the apparent explanatory successes of modern-day relativistic quantum field theory, "string" theory, etc., leading to what some are calling a "Theory of Everything." Likewise, the discovery of the molecular basis of genetics in the 1950s (what many biologists have dubbed the "Secret of Life"), has sometimes seemed to have cast an air of finality on the study of living systems. As the 20th century comes to a close, the subject of "metabolism" has become rather *passé*. The great focus is on the isolation, cloning, sequencing and cutting/splicing of genetic elements. As we enter the 21st century, the United States government (with some contributions from other countries) stands poised to spend billions of dollars on the singular task of

identifying all the loci within the human genome. One may argue that the science of biology has lost its philosophical view of life as a *process*, in favor of the perspective of *substance*. Hopefully, the import of a book with the title *Enzymology in vivo* will assert to the readership that the subject of metabolism is, in fact, far from being "solved."

The Humpty Dumpty effect (Kell and Welch, 1991) might appear to throw a shadow of nihilism on any analytical reductionist effort to understand the living state. In actuality, Humpty Dumpty serves as an abiding reminder that in hierarchically ordered systems one must seek to analyze the "parts" within the context of the "whole." It is only thus that an understanding of the emergent properties at each level of organization is attainable. In today's utilitarian science, the "understanding" of Nature has come to mean the "control" of Nature; in this vein one may indeed conclude that the organizational properties of the cellular metabolic machinery are crucial to (our understanding of) its control.

NOTE ADDED IN PROOF

H-K. Luh and S. L. Pimm (The assembly of ecological communities: a minimalist approach, J. Animal Ecol. 62, 749–765, 1993) have also used the phrase "The Humpty Dumpty Effect" to account for the inability to reassemble structured ecosystems from mixtures of their components.

Cornish-Bowden and Cárdenas (Channeling can affect concentrations of metabolic intermediates at constant net flux: artefact or reality? Eur. J. Biochem. 213, 87–92, 1993; hereafter CBC) have claimed that simulation results previously published by us (Mendes, P., Kell, D.B., and Westerhoff, H.V. Channeling can decrease pool size, Eur. J. Biochem. 204, 255–266, 1992) which had demonstrated that large reductions of intermediate pool sizes could be accompanied by increasing channel flux in a model metabolic pathway, were an artefact of changes in the pathway's overall flux of the order of 0.0075%, or of inappropriate alterations of enzyme activities. They also claimed to prove that "channeling of an intermediate cannot affect its free concentration at constant net flux".

In recent work (Mendes, P., Kell, D.B., & Westerhoff, H.V., in preparation) we consider the co-response of the intermediate metabolite concentration ("pool") and the channel flux to changes in kinetic (or thermodynamic) parameters. Both by analytical proofs and by numerical examples we show that this co-response can be positive, negative or null, depending on the parameter change. In particular we prove that there is always a number of ways of changing parameters such that the intermediate metabolite concentration decreases with increasing channel flux, whether the total flux varies or is constant. We also show that increased stability of the (dynamic) enzyme-intermediate-enzyme complex, as well as a single parameter change that similarly displays no cross-over effects, can lead to decreased intermediate metabolite concentration and increased channel flux at constant total flux.

More specifically:

(1) the algebraic analysis ("general proof") given in CBC contains the constraint that the elasticities of various steps to the modulation parameters which were used to vary the channel flux at constant net flux were unity. This is an unfortunate and unnecessary constraint which when lifted means that the concentration of the pool in the general case can indeed change at constant net flux. A "simplified proof" given in CBC also fails, due in addition to the consequent failure to include mass conservation relations for some of the enzymes.
(2) in the systems studied by CBC, flux is properly to be considered as a variable (since it varies during the transition to the steady state), and not a parameter, and as such cannot per se affect the magnitude of other variables in the steady state;
(3) by relaxing the constraint referred to in (1), above, and by making dual modulations (i.e. of more than on parameter at once) which are different from those carried out in CBC we find many instances in which channeling (described by a parameter p) does significantly affect the concentration of the pool intermediate C at constant total flux.
(4) in the same pathways, but in which the flux is held constant by setting it via a zero-order flux-generating reaction, the addition of a channel is also able significantly to modulate the size of the pool at constant total flux.

These results show that the effectiveness of channeling in decreasing a pool, even at constant flux, is very much a reality.

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