

# METABOLIC CASCADES: AN EVOLUTIONARY STRATEGY FOR AN INTEGRATED AND SENSITIVE RESPONSE TO MULTIPLE SIGNALS

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Eduard Buchner (1897) is rightly regarded as the founder of modern biochemistry, because his observation that alcoholic fermentation could proceed in a cell-free extract of yeast supplied the final nail in the coffin of the vitalism that dominated physiological thinking in the 19th Century. His work can also be regarded as the beginning of enzymology, but although he regarded his “zymase” as a single enzyme it became clear in the years that followed his discovery that it is really a mixture of several enzymes, all of them necessary if fermentation is to proceed. His experiments can equally well, therefore, be regarded as the first study of the behaviour of a multi-enzyme system, a topic that has taken nearly a century to be taken up again seriously. In this chapter I shall consider one particular type of multi-enzyme system, in which the combined effects of at least three different enzymes allow regulatory properties that would not be possible with just one.

The modern study of metabolic regulation can be regarded as having started in the 1950s with the discovery of feedback inhibition (Yates and Pardee, 1956; Umbarger, 1956) and the recognition, a little later, that cooperativity is not just a special property of haemoglobin but is crucial in the regulatory design of many pathways (Monod *et al.*, 1963). Cooperativity, or unusually high sensitivity, often occurs together with inhibition or activation by a molecule that does not bear

any necessary structural similarity to the substrate and products of the reaction catalysed, which is called allosteric inhibition or activation. The importance of cooperativity is that it allows a much more sensitive response to a signal than is possible with an enzyme obeying Michaelis–Menten kinetics: in the latter case an 81-fold change in inhibitor concentration is necessary to bring the reaction rate from 90% to 10% of its uninhibited value, but a cooperative enzyme can manage the same transformation with a much smaller ratio of concentrations, though rarely smaller than three-fold. A cooperative response often has its origin in cooperative binding, and the degree of cooperativity (degree of sensitivity) is then a function of the number of binding sites and of the strength of interaction between the sites. As the interaction is never infinitely strong the degree of cooperativity expressed as the Hill coefficient is usually smaller than the number of sites, and no higher than four. This is well illustrated by haemoglobin, which has four binding sites for oxygen and a Hill coefficient of 2.8; the case of CTP synthetase, with four binding sites and a Hill coefficient of 3.8, is rather exceptional.

Probably because of structural constraints, it appears to have been impossible to evolve enzymes offering much more sensitivity than this. Certain oxygen binding proteins from invertebrates have a very large Hill coefficient in the middle of the range, but they are unable to maintain this over a wide range, and on average are no more sensitive than haemoglobin. Moreover, they require interactions between very large numbers of binding sites, and are in consequence very large proteins.

Kinetic factors may also contribute to generate responses more sensitive than is provided by Michaelis–Menten kinetics. They are at the origin of cooperativity in monomeric enzymes (Cornish-Bowden and Cárdenas, 1987), though in these cases the Hill coefficient is normally lower than 2.0. If binding and kinetic cooperativity both operate in a multimeric protein the joint contribution of both could generate responses of higher sensitivity than is expected from the number of sites. This appears to be rare, however, though it probably explains why phosphofructokinase shows a Hill coefficient of the order of 6 to 8, greater than 4.0, the number of binding sites.

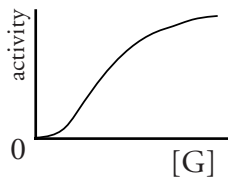
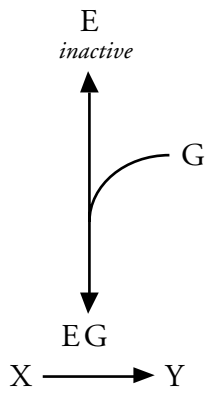
In practice, therefore, a three-fold change in stimulus to span the middle 80% range of responses is about the best that a single enzyme can do (corresponding to a Hill coefficient of 4). Yet a three-fold change in inhibitor (or activator) concentration is by no means negligible, and the concentrations of many metabolites need to be maintained within much narrower limits; it would thus be an exaggeration to claim that a

cooperative enzyme allows a system to respond as if to a switch. Clearly, therefore, in some circumstances living organisms require a greater degree of sensitivity than any single enzyme can offer, and they obtain this by use of energy-consuming systems of interconvertible enzymes.

Such systems have been known for more than half a century, since Cori and Green (1943) discovered that glycogen phosphorylase exists in two forms, a catalytically active phosphorylated form, phosphorylase a, and an inactive dephosphorylated form, phosphorylase b. Since then a great many other similar cases have been found, with functions ranging from regulation of metabolic pathways to control of the cell cycle (Chock *et al.*, 1980; Cohen, 1982; Boyer and Krebs, 1986). Their contribution to metabolic regulation was studied quantitatively and in detail by Chock and Stadtman (1977), Stadtman and Chock (1977) and Goldbeter and Koshland (1982). The latter authors discovered that when the enzymes responsible for catalysing the conversions between inactive and active forms operated close to saturation, a condition they called “zero-order ultrasensitivity”, the cycle as a whole is capable of providing an output response with much more sensitivity to a change in stimulus than the hyperbolic (Michaelis–Menten) equation. Thus, even though none of the enzymes in the cycle may show cooperative behaviour the cycle can generate a more sensitive response than a single cooperative enzyme can offer. The high sensitivity comes at a price, however, because unlike a cooperative enzyme the cycle requires continuous hydrolysis of ATP (or a similar process) to maintain itself in a steady state (Goldbeter and Koshland, 1987). The comparison of an effector acting on a single allosteric and cooperative enzyme with one acting on the simplest kind of cycle of interconvertible enzymes is illustrated in Fig. 1, for the typical case where one reaction is a phosphorylation, catalysed by a protein kinase, and the other is a hydrolysis (dephosphorylation), catalysed by a protein phosphatase.

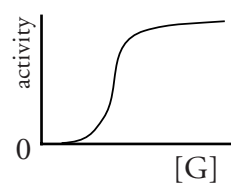
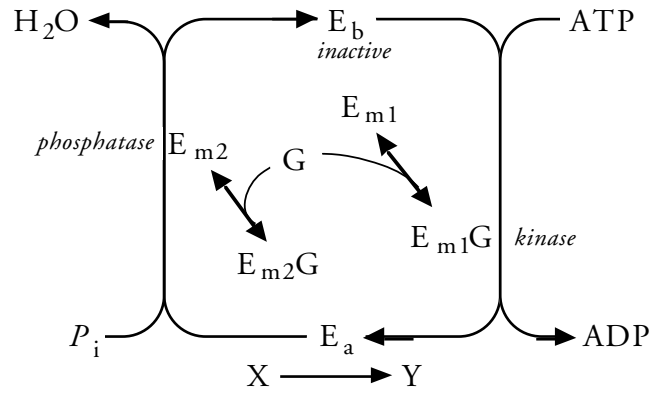
It is sometimes implied that high sensitivity is an automatic consequence of the existence of a cycle such as that illustrated in Fig. 1, but this is not correct. The mere existence of a cycle does not guarantee high sensitivity, and if a number of conditions are not met the cycle may provide no more sensitivity than can be obtained from a Michaelis–Menten enzyme obeying the classical equations for inhibition or activation, and may even be less (Cárdenas and Cornish-Bowden, 1989). The first condition was discovered by Goldbeter and Koshland (1981, 1982), and has been noted already: the two modifier (or converter) enzymes,  $E_{m1}$  and  $E_{m2}$  in the symbols of Fig. 1, must

Allosteric enzyme



No energy consumption

Cycle of interconvertible enzymes



Continuous energy consumption



Fig. 1. Comparison between direct allosteric action of an effector G on a target enzyme that catalyses the conversion of a metabolite X to a metabolite Y (*left*), and indirect action through inhibitory and activatory effects on the converter enzymes  $E_{m1}$  and  $E_{m2}$  that catalyse the reactions between the active and inactive forms of the target enzyme (*right*). For purposes of illustration  $E_{m1}$  and  $E_{m2}$  are assumed to be a protein phosphatase and a protein kinase respectively, but other possibilities exist. The allosteric enzyme is less complicated, requiring only one protein rather than three (*top row*); even at optimum sensitivity it offers only a mildly sigmoid dependence of activity on effector concentration whereas the interconvertible enzymes offer virtually unlimited “switch-like” sensitivity (*middle row*); however, the allosteric enzyme consumes no energy whereas maintaining the cycle in operation requires continuous hydrolysis of ATP (*bottom row*).

bind their substrates,  $E_b$  and  $E_a$  respectively, so tightly that at the concentrations of these substrates in the cell they are close to saturation at all times. This condition appears to be satisfied in some systems, such as phosphorylase b kinase and phosphorylase a phosphatase from muscle (Meinke *et al.*, 1986), but not in others. In any case, we have shown that if other conditions are satisfied, about 30% saturation may be sufficient for a highly sensitive response. Two other conditions appear equally important (Cárdenas and Cornish-Bowden, 1989): the allosteric effector G should act on the limiting rates of the converter enzymes rather than on their specificity constants, and do so with

different affinity: the modifier enzyme that is activated should require much higher concentrations of the effector than the enzyme that is inhibited. If the effector acts on only one of the converter enzymes, is still possible to have a highly sensitive response if the catalytic constant is affected (Cárdenas and Cornish-Bowden, 1989). If it acts on both converter enzymes, affecting only the specificity constant and not the limiting rate, there can be no higher sensitivity than that given by the Michaelis–Menten equation. Action of an effector at several points in the pathway, for example on both converter enzymes, has been called “multistep effects” (Goldbeter and Koshland 1984), and it contributes to an increase in sensitivity, which can be amplified in certain conditions if there is more than one cycle.

A cascade with a single cycle can generate responses with extremely high Hill coefficients (about 800) if the three conditions for high sensitivity are satisfied: (i) modifier enzymes near saturation; (ii) catalytic rather than specific effects; and (iii) inhibition stronger than activation (Cárdenas and Cornish-Bowden, 1989, 1990). Furthermore, analysis according to the methods of metabolic control analysis give very high response coefficients (Szedlacsek *et al.*, 1992). Even if the conditions are only partially satisfied the monocyclic system can still generate very sensitive responses.

These requirements for high sensitivity are perhaps unintuitive. It is often assumed (albeit with little direct evidence) that most enzymes operate *in vivo* at about half-saturation, i.e. that physiological substrate concentrations are similar to the relevant Michaelis constants, and saturation is likely to be rare for an enzyme involved in intermediary metabolism. Likewise, specific (competitive) inhibition and activation appear to be much more common than their catalytic (uncompetitive) counterparts, and in any case in normal laboratory conditions are not very different from one another (and hence are often not clearly distinguished). However, enzymes are usually studied under conditions where the concentrations are determined by the experimenter and the rates that result are measured, whereas in the cell most enzymes operate under more complex conditions that are closer to constant-flux than to constant-concentration. This is important, because at constant flux specific and catalytic effects become drastically different from one another: in the one case a slight adjustment of the system is sufficient to counteract even quite a large concentration of inhibitor; in the other a small amount of inhibitor is sufficient to raise the substrate concentration without limit, converting an apparently stable steady state into a state where no steady state is possible (Cornish-Bowden, 1986).

Finally, the need for the activated modifier enzyme to respond to

higher effector concentrations than the inhibited enzyme can often lead to the unwarranted conclusion that even if such activation occurs it has no physiological significance. A clear example came to light when we studied data for the response of muscle fibre tension to the toxin okadaic acid (Takai *et al.*, 1987): myosin phosphatase is strongly inhibited by okadaic acid, with a mixed type of inhibition (catalytic and specific effects), the concentration for half-inhibition is around 0.01  $\mu\text{M}$ , but the concentration for half-maximal muscle tension is very much higher, around 0.5  $\mu\text{M}$ ; moreover, the curve for the effect on muscle tension is much steeper (more cooperative) than that for the effect on myosin phosphatase. Although these discrepancies could easily be regarded as anomalous, they are better interpreted as evidence that the effect on muscle tension derives not solely from the effect on myosin phosphatase but also from activation of the corresponding kinase at much higher and supposedly unphysiological concentrations. We have discussed this example in more detail elsewhere (Cárdenas and Cornish-Bowden, 1990).

Much of the discussion to this point is based on theoretical analysis, and Newsholme and Walsh (1992) pointed out that our suggestion (Cárdenas and Cornish-Bowden, 1989) that regulation of kinase-phosphatase cycles ought to involve catalytic effects did not correspond to what were thought to be the experimental facts in relation to glycogen phosphorylase kinase, that effectors such as calcium ions acted only on binding, with no effects on limiting rates. However, they did not simply dismiss our analysis as the typically unreal ideas of theoretically inclined biochemists, but undertook the necessary experiments to determine the truth; we were gratified to read their conclusion that our predictions were correct and that the “facts” were not.

For a cascade mechanism to be of physiological importance the transition from one steady state to another in response to an effector must occur within a reasonable interval of time. This appears to be the case, as analysis of the kinetics have shown that the switch from one activity level to the other can occur within physiologically significant time intervals matching those observed experimentally for some systems (Goldbeter and Koshland, 1981). For example, when the known experimental constants are inserted into a model for glycogen synthesis it appears that about 3–4 minutes are sufficient for phosphorylase a to be completely inactivated and glycogen synthase a to be more than 50% activated by an increase in glucose concentration from 5 mM to 60 mM (Cárdenas and Goldbeter, 1996), corresponding

## INTEGRATED AND SENSITIVE RESPONSE TO SIGNALS

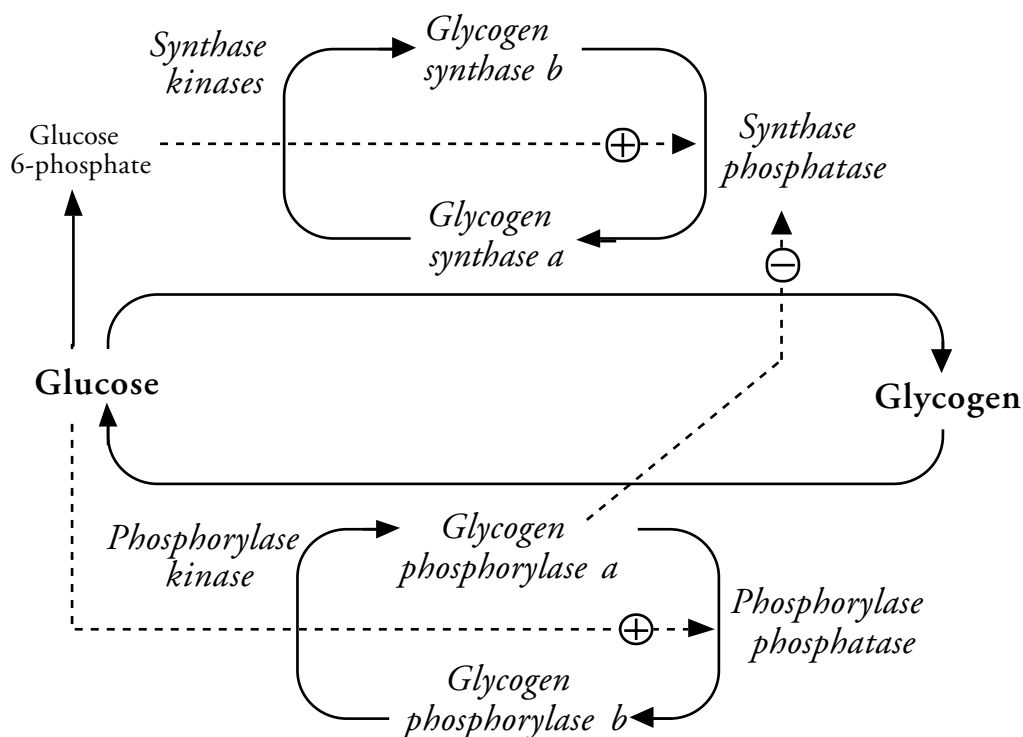


Fig. 2. Glycogen synthesis and degradation. The figure shows in a simplified form the system of three interconnected cycles that regulate the synthesis and degradation of glycogen in mammalian liver. Catalytically active species are shown in *italics*, others in roman type; enzymes or effects that increase glycogen synthesis or decrease glycogen degradation are shown with seriffed type, those that act in the opposite direction are shown with sanserif type.

almost exactly with the experimental observations (Stalmans *et al.*, 1974).

Given the importance of glycogen metabolism, both in biochemistry in general and in the history of interconvertible enzymes in particular, we have recently started to make a detailed study of the kinetic information that exists about the enzymes involved in glycogen metabolism (Cárdenas and Goldbeter, 1996). The system is much more complex than the single cycle illustrated in Fig. 1, and even the scheme shown in Fig. 2, involving three different cycles, is considerably simplified and does not include all of the known experimental information.

The essential role of the system is to control the balance between glycogen synthesis, catalysed by glycogen synthase, and degradation, catalysed by glycogen phosphorylase; both of these two enzymes exist in active and inactive states interconverted by different protein

phosphatases and kinases (Fig. 2). The relative degrees of phosphorylation of glycogen synthase and glycogen phosphorylase are under hormonal control via the activation of protein kinases by cyclic AMP and cytosolic calcium ions. In the liver the switch between glycogen synthase and glycogen phosphorylase can also be controlled by glucose, and Fig. 2 deals mainly with this aspect of their regulation.

Glucose activates glycogen phosphorylase phosphatase directly, and glycogen synthase phosphatase indirectly through the action of its metabolic product glucose 6-phosphate. Both effects tend to tip the balance from glycogen degradation towards glycogen synthesis when the concentration of glucose is high, as one would expect, and in fed animals the switch between the two enzymes is abrupt: addition of sufficient glucose causes the activity of glycogen phosphorylase to decrease as a result of the activation of glycogen phosphorylase phosphatase, and when the fraction of activity has fallen below 10% glycogen synthase begins to be activated (Stalmans *et al.*, 1974). This observation, which implies that at no time do both glycogen synthase and glycogen phosphorylase have high activity, has become a textbook example of metabolic regulation (e.g. Stryer, 1995), and has recently been confirmed and amplified with data for animals in a variety of different metabolic states (Massillon *et al.*, 1995).

In the model of these effects proposed by Stalmans *et al.* (1987) and Bollen and Stalmans (1992), the lag in the activation of glycogen synthase is explained in terms of the removal of inhibition of glycogen synthase phosphatase by glycogen phosphorylase a. However, Guinovart's group (Carabaza *et al.*, 1992) found that removal of this inhibition is not sufficient to activate glycogen synthase, and concluded that the activation by glucose 6-phosphate is also required. This requirement now appears to be a well established fact (Massillon *et al.*, 1995; Cadefau *et al.*, 1997).

The model as shown in Fig. 2 thus incorporates the properties that need to be taken into account in any attempt to explain the effects of glucose on glycogen metabolism in the liver. It does not allow for the known effects of cyclic AMP and calcium, but they appear not to vary in relation to glucose concentration and as long as the concentrations of these effectors are constant their effects can be taken as subsumed in the kinetic constants of the different enzymes. The essential properties that remain are activation of glycogen phosphorylase phosphatase by glucose, inhibition of glycogen synthase phosphatase by phosphorylase a, and activation of glycogen synthase phosphatase by glucose 6-phosphate. An additional complication is that phosphorylation of glycogen synthase occurs at multiple sites (Roach, 1986), but we have

considered a two-state model for this enzyme. This is a point that ought to be borne in mind in future studies, as it has been proposed that if dual phosphorylation resulted from two separate enzyme-catalysed events this could contribute to increased the sensitivity of the response (Huang and Ferrell, 1996; Ferrell, 1996). Similarly, the recent observations of the glucose-induced translocation of glycogen synthase and hexokinase D between nucleus and cytoplasm (Agius *et al.*, 1996; Fernández-Novell *et al.*, 1997) need to be considered in future models, but it would be premature to attempt this now.

Despite its simplified character, this model proved to give a good description of the main observations (Cárdenas and Goldbeter, 1996), and it suggests that the different sets of observations complement one another, so that the effects of both increased glucose 6-phosphate and decreased phosphorylase a are needed to explain the activation of glycogen synthase to significant levels.

In fasted animals and in the presence of AMP there is no inhibition of synthase phosphatase by phosphorylase a, and in these conditions both our model and the experimental observations (Stalmans *et al.*, 1987) agree that the rise in the glucose 6-phosphate is sufficient by itself to bring about the increased activity of glycogen synthase. There is then no lag in the activation, apart from any that may derive from a delay in the production of glucose 6-phosphate after administration of glucose to previously fasted animals.

Certain experimental facts, such as the sharp threshold in the curve comparing glycogen synthase and glycogen phosphorylase activities after administration of glucose to fed animals, remain to be studied in detail. Nonetheless, it is already clear that a theoretical model of this kind is of great value in understanding the complex web of regulatory processes that exist in glycogen metabolism, that it can make predictions, and that it can help to reconcile experimental observations that appear at first sight to be in conflict.

The metabolism of glycogen also illustrates another characteristic of interconvertible enzymes, that often there is not just one cycle, but several. There are many examples of this, such as the mitogen-activated protein (MAP) kinase cascade, which consists of three protein kinases: a MAP kinase (MAPK), a kinase that phosphorylates MAPK (MAPKK) and a third that phosphorylates MAPKK (MAPKKK). As with glycogen the obvious questions are why are there several kinases and not just one, and why are there so many intermediates. Many reasons could be advanced (Ferrell, 1996) as the degree of sensitivity and the amplification of the signal increase with the number of cycles (Chock and Stadtman, 1977; Goldbeter and Koshland, 1984), but the

questions remain pertinent when one recalls that just one cycle has the potential to respond to a stimulus with as much sensitivity as could be required (Cárdenas and Cornish-Bowden, 1989, 1990; Szedlacsek *et al.*, 1992). That is, if the initial stimulus activated MAPK by acting directly to activate MAPKK or inhibit MAPKase, or both, (instead of activating MAPKKK and requiring the whole of the rest of the cascade to act on MAP) a Hill coefficient as large as the experimental value of 4.9 could have been obtained, provided that (i) the signal effector increased the catalytic constant of MAPKK and decreased that of MAPKase, (ii) the inhibition occurred at a lower concentration of effector than the activation, and (iii) MAPKK and MAPKase acted at more than 30% saturation. As the experimental value is not very high there is no doubt that a single-cycle cascade system could fulfil the role even if the constraints were only partially satisfied.

Previous quantitative studies of multicyclic cascades (for example Chock and Stadtman, 1977; Goldbeter and Koshland, 1984), lead one to expect that the total sensitivity will be the product of the sensitivities at each level, and this has recently been reconfirmed by Kholodenko *et al.* (1997). However, this leaves unanswered the essential question of why three cycles are needed when evolution could easily have selected kinetic parameters and enzyme concentrations in the MAP kinase cascade to provide the same modest degree of sensitivity with just one cycle. Why three? Probably the answer is that cascades are not merely a mechanism of increasing sensitivity but also a way of allowing an integrated response to multiple signals, as Chock *et al.* (1990) and Fell (1997) have indicated.

The regulation of metabolism is concerned primarily with modulation of reaction rates, and it is mainly achieved by controlling the activities and concentrations of enzymes. Enzyme-catalysed reactions are not regulated in isolation, but as parts of metabolic pathways; it is the whole pathway that is regulated. In the same way, each metabolic pathway needs to be regulated in tune with the rest of metabolism, to satisfy the particular requirements of the cell and of the whole organism. The result is a very complex net of interactions where a great many signals need to be taken into account and integrated. Moreover, a satisfactory integration will require a respect of hierarchy, as some signals must have priority over others.

Regulation of a metabolic pathway in which a single enzyme has most of the control could be achieved by the regulation of that enzyme by allosteric interaction or by the action of a monocyclic cascade, the latter giving more sensitivity than the former, as discussed above. However, a problem is to integrate multiple signals, as allosteric

interaction requires allosteric sites on the target enzyme, and it appears that on the whole a single enzyme cannot have many different allosteric effectors. Probably the same type of structural constraints that prevent Hill coefficients from being much greater than four also prevent a single enzyme from responding directly to many different effectors; the only exceptions are likely to be very large proteins like glutamine synthetase from *E. coli*. A cascade thus represents a mechanism with the potential not only for providing a high sensitivity but also, and possibly more important, for integrating more signals than direct allosteric interaction allows. This is because each level of the cascade offers the possibility of additional sites at which different signals can act. If there is only one cycle all the different signals must act on just three enzymes: the target enzyme (without very much sensitivity) and the two modifier enzymes. In addition, covalent modification allows the response of the enzyme to particular effectors to vary, increasing flexibility in responding to fluctuations in the concentrations of many different metabolites.

As soon as one recognizes that the cascade system has the potential to integrate signals it becomes evident that the more cycles there are the more signals can be integrated. Evolution has surely optimized not only sensitivity but also the integration of signals, and this could explain why there are multiple cycles. There is probably a correlation between the number of different signals acting and the number of cycles. When there are many different signals, for example the concentrations of calcium, cAMP, glucose, glucose 6-phosphate, AMP, ADP and glycogen in the case of glycogen degradation, we are inevitably faced with a system that requires many allosteric sites. The strategy has been to have these signals act on different enzymes organized as a multicyclic cascade.

Cascade systems, even ones of a single cycle, clearly have all the potential to act as switches between one steady state to another, as they can trigger a response to a very small change in a signal or stimulus, variations in concentration that occur outside a certain range being ignored. Equally, however, cascades not only have the potential to function as *on/off* mechanisms for metabolic pathways that require such switching, but also for allowing the fractional activities of the interconvertible enzymes to be varied progressively over a wide range, as appears to be the case of the adenylation of glutamine synthetase and the phosphorylation of the mammalian pyruvate dehydrogenase complex (Chock *et al.*, 1990). Nevertheless, an adequate response to many different signals requires not just sensitivity or a progressive change, but integration and this also becomes possible if there are

several interacting cycles.

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