ELSEVIER

Progress in Biophysics & Molecular Biology 86 (2004) 5–43

Progress in Biophysics & Molecular Biology

www.elsevier.com/locate/pbiomolbio

# Quantitative analysis of signaling networks

Herbert M. Sauro[a,b,*], Boris N. Kholodenko[c]

[a] *Computational Biology, Keck Graduate Institute, 535 Watson Drive, Claremont, CA 91711, USA*
[b] *Control and Dynamical Systems, California Institute of Technology, Pasadena, CA 91125, USA*
[c] *Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, 1020 Locust Street, Philadelphia, PA 19107, USA*

**Abstract**

The response of biological cells to environmental change is coordinated by protein-based signaling networks. These networks are to be found in both prokaryotes and eukaryotes. In eukaryotes, the signaling networks can be highly complex, some networks comprising of 60 or more proteins. The fundamental motif that has been found in all signaling networks is the protein phosphorylation/dephosphorylation cycle—the cascade cycle. At this time, the computational function of many of the signaling networks is poorly understood. However, it is clear that it is possible to construct a huge variety of control and computational circuits, both analog and digital from combinations of the cascade cycle. In this review, we will summarize the great versatility of the simple cascade cycle as a computational unit and towards the end give two examples, one prokaryotic chemotaxis circuit and the other, the eukaryotic MAPK cascade.


*Keywords:* Biological signal transduction; Control systems; Quantitative; Simulation; Engineering; Systems biology

## 1. Introduction

It is probably reasonable to speculate that the earliest living cells had little capacity to sense their environment and presumably lived in rich surroundings which did not require even basic sensory devices to locate resources. However, once the most easily accessible sources of energy had been exploited, evolutionary pressure must have forced the development of the first rudimentary sensory apparatus. Today, the ability of biological cells to respond to signals from

*Corresponding author. Computational Biology, Keck Graduate Institute, 535 Watson Drive, Claremont, CA 91711, USA. Tel.: +1-909-607-0377; fax: +1-909-607-8598.
*E-mail address:* herbert_sauro@kgi.edu (H.M. Sauro).

0079-6107/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.pbiomolbio.2004.03.002

the external environment is so ubiquitous that the property is considered by many to be a fundamental characteristic of life.

Modern living systems receive a multitude of signals, ranging from environmental conditions, mating potential from nearby partners to developmental signals in a multi-cellular organism. In multi-cellular organisms, cells receive a flood of signals aimed at regulating their behavior. It is not surprising therefore to discover that cells have evolved highly elaborate and complex networks of 'signaling' pathways whose role is to coordinate and integrate an appropriate response.

In recent years, it has become apparent that signaling pathways are not simply concerned with processing external signals but are also used to integrate internal signaling. In prokaryotes, there are many cases where simple signaling pathways are employed to monitor and make decisions based on the internal state of the cell. Traditionally, we have tended to separate gene network regulation, metabolic regulation and signal transduction systems as separate control systems; however, research on prokaryotic control systems has shown that the division is much more blurred. Indeed, there is a very welcome trend to integrate all control systems, including metabolic, signal and gene networks into one systems approach (Rao and Arkin, 2001; Wolf and Arkin, 2002). Thus, although this review is concerned with protein/protein signaling networks, the reader should bear in mind that these networks integrate into a much larger network of gene and metabolic subsystems (Arkin et al., 1998; Ryan and Shapiro, 2003) and many of the ideas discussed here are also directly applicable to these other networks.

This review will be concerned with work that has been focused on interpreting signaling networks as control and computational systems. Since these networks apparently serve to integrate and interpret external and internal signals such an approach seems most natural. We will not be concerned here with the variety of specific simulation models that have been published (Kholodenko et al., 1999; Brightman and Fell, 2000; Asthagiri and Lauffenburger, 2001; Schoeberl et al., 2002; Moehren et al., 2002; Shvartsman et al., 2002; Wiley et al., 2003), instead we will focus on ideas, basic properties and towards the end select two particular examples for study, bacterial chemotaxis and the MAPK pathway as examples of signaling control systems. Moreover, we will not be concerned with the large body of literature that now exists on the connectivity properties of networks as exemplified by small world studies. Interested readers are asked to consult (Solée et al., 2002) for more details.

## 2. The basic motif of signaling networks

One of the central mechanisms used in signaling networks is protein phosphorylation and dephosphorylation via kinases and phosphatases, respectively. In eukaryotes, kinases phosphorylate at specific Ser, Thr or Tyr residues thereby altering protein activities. In prokaryotic systems, a somewhat similar mechanism exists although phosphorylation occurs at Asp and His residues instead, and the kinases are referred to as histidine protein kinases (West and Stock, 2001). In some 'lower' organisms such as yeast and the slime mold Dictyostelium, one finds a small number of histidine protein kinases in addition to the Ser/Thr kinases. Additionally, plants have also been found to employ some histidine protein kinases (The-Arabidopsis-Initiative, 2000). Animals, such as human, fly or worm do not appear to encode any histidine protein kinases (Manning et al., 2002).

The most common motif found in signaling networks is the cycle formed by a kinase and an opposing phosphatase (Fig. 1). These cycles form the backbone of most if not all signaling networks so far studied and appear to exist in both prokaryotes and eukaryotes. The cycles themselves are often linked forming multiple layers of cycles, the so-called cascades. Interestingly, the cascades found in prokaryotes tend to be much shorter than those found in eukaryotes. In addition, the cascades, particularly in eukaryotic systems, will often cross-link with other cascades forming a complex web of inter-connections. In addition to the layers of kinase/phosphatase cycles, there are also positive and negative feedback loops, that criss-cross, within and between the layers further complicating the system. To appreciate the complexity of signaling networks in eukaryotes, the book by Gomperts et al. (2002) offers an excellent overview of the different kinds of networks, ranging from the reception of signal, through to the resulting changes in gene expression.

One of the key issues which confronts signaling research today is what is all the complexity for? What are the networks attempting to achieve, what is the data integration that is occurring and what parallel, if any, can we find between natural signaling networks and man-made networks. There is a growing appreciation that many of the designs and strategies that man has developed to manipulate information, particularly within the electronics world, are present in biological networks. The excellent reviews by Tyson et al. (2003) and Wolf and Arkin (2003) offer an exciting glimpse into the parallels between natural and man-made signaling and control systems.

A notable example of a relatively well studied signaling network is the MAP kinase family of proteins (Kyriakis and Avruch, 2002). The MAP kinase family of proteins, besides being highly conserved, are common components in signal transduction pathways and are probably the most widespread mechanisms of eukaryotic cell regulation (Chang and Karin, 2001). All eukaryotic cells that have been examined (ranging from yeast to man) possess multiple MAPK pathways each of which respond coordinately to multiple inputs. In mammalian systems, the MAPK pathways are activated by a wide range of input signals, including a large variety of growth factors and environmental stresses such as osmotic shock and ischemic injury (Kyriakis and Avruch, 2002). In yeast, physiological processes regulated by MAP kinases include mating, sporulation, cell wall integrity and many other processes. In Drosophila, MAP kinase is a regulator of the immune response and embryonic development. Once the MAPK pathways have integrated these signals, they coordinately activate gene expression with resulting changes in protein expression, cell cycling, cell death and cell differentiation (Gomperts et al., 2002).

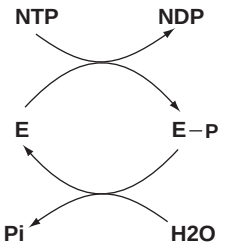


Fig. 1. Basic motif used in signaling networks. E represents unphosphorylated enzyme, E-P phosphorylated enzyme. The upper arm is catalyzed by a kinase and the lower arm a phosphatase.

In prokaryotes, the histidine protein kinases are also involved in a wide variety of processes, including chemotaxis, osmoregulation, and sporulation (West and Stock, 2001). Given the widespread occurrence of protein kinases and their involvement in a huge variety of cellular processes, it should come as no surprise to learn that they comprise from 1.5% to 2.5% of all proteins in almost all genomes that have been looked at. Table 1 indicates the abundance of kinases in different organisms.

### 2.1. Device analogs

One of the most well-known properties of the single cascade cycle is ultrasensitivity (Chock and Stadtman, 1977a; Goldbeter and Koshland, 1981; Fell, 1997), that is, the property of both species in the cycle to switch rapidly in opposite directions in response to a change in the input signal (Fig. 2). The graph in Fig. 3 illustrates the steady-state behavior of the two cycle components, $E_1$ and $E_2$, as the activity of the forward cycle arm is increased. Note the rapid change in concentrations around the central portion of the curves. This switching has the character of a sigmoid curve. The degree of sigmoidicity or switch behavior can be easily modified by changing the *Km*'s of the catalyzing enzymes (the kinase and phosphatase). Thus, the behavior can range from simple hyperbolic to extremely steep sigmoidicity (Goldbeter and Koshland, 1984). This behavior will be reviewed in more detail in the next section.

What is remarkable about this behavior is the resemblance to a man-made device, the transistor. Although not often expressed as such, a plot showing the collector current versus the base current is remarkably similar to the behavior to a cascade cycle undergoing an ultrasensitive

Table 1
Abundance of kinases in various organisms: taken from Manning et al. (2000)

| Organism | Number of putative kinases |
|---|---|
| *Escherichia coli* | 29 |
| *Saccharomyces cerevisiae* | 130 |
| *Drosophila melanogaster* | 239 |
| *Caenorhabditis elegans* | 454 |
| Human | 518 |

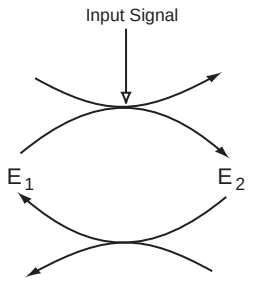


Fig. 2. Cascade cycle model, note that cycle has the property $E_1 + E_2 = T$, where $T$ is the total amount of enzyme mass in the cycle.

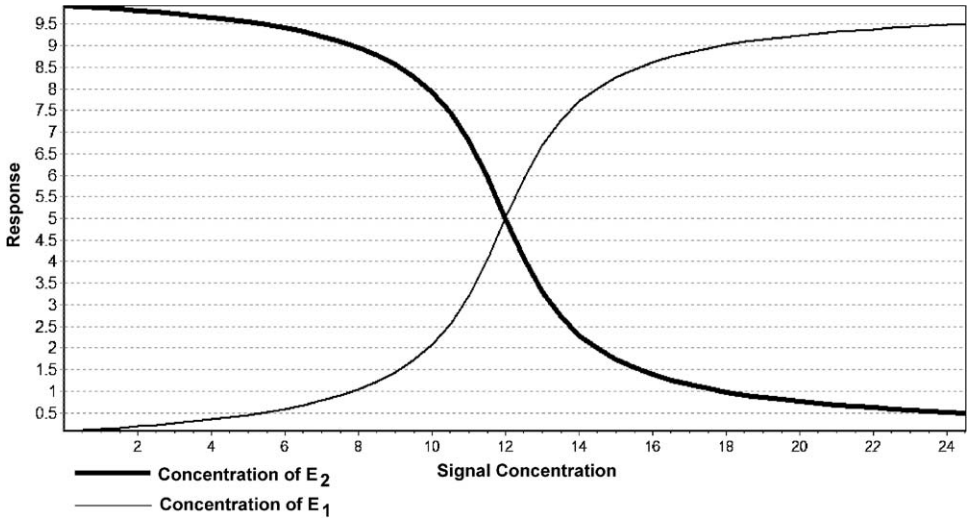


Fig. 3. Response of cascade cycle to changes in input.

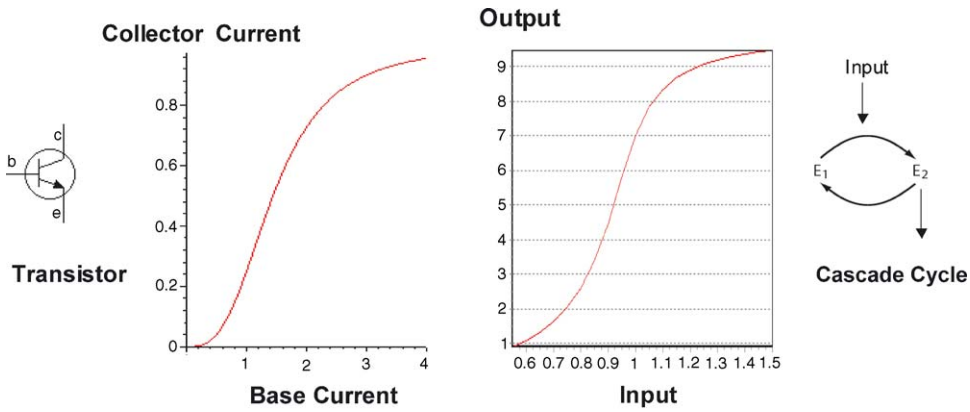


Fig. 4. Comparison of transistor to cascade response.

response (see Fig. 4). The cascade cycle is thus behaving in a very similar manner to an electronic transistor. This observation obviously opens up a whole range of possibilities.

### 2.1.1. *Circuit complexity*

Transistors, in one form or another, are the basic components used to build highly complex electronic circuits. The latest Pentium 4 from Intel, for example, is said to contain 43 million transistors (Intel, 2003), and the new AMD Hammer microprocessor is reported to include 100 million transistors (AMD, 2003). How is such enormous complexity managed and how is it possible to design such complexity? The answer to this lies partly with the use of modularity. Without a modular approach, and in particular a hierarchy of functional units, such circuits could not be so easily designed. A very simple example of this approach can be seen in the design of the electronic square root circuit.

Fig. 5 illustrates, how, staring with the square root unit, we break it up into a circuit made up of a multiplier unit and an operational amplifier. These units are in turn broken down further into simpler units (Wong and Ott, 1976).

At this point, we may wonder whether biological signaling networks are similarly structured. Of course the main difference between electronic and biological signaling networks is that one is man-made while the other is a product of evolution. Thus, the question arises whether evolution would naturally generate a modular and hierarchical network. Unfortunately, the question still remains to be answered although attempts have been made using network analysis (Ravasz et al., 2002) and more significantly metabolic control analysis (Kahn and Westerhoff, 1991; Rohwer et al., 1996; Hofmeyr and Westerhoff, 2001; Bruggeman et al., 2002). See also Alm and Arkin (2003) for a more general discussion.

Some interesting empirical research that is related to the question of evolved modularity is the work presented by Koza (Koza et al., 1999) using genetic programming. This research involved using artificial evolution to evolve electronic circuits. Often, the results of the evolution generated circuits that any competent engineer would recognize; however, more worrying from our perspective was that the technique also generated dense circuitry which proved very difficult to understand. A striking example of this is the evolution of the square root circuit (Fig. 6). This circuit does not appear to have very much resemblance to the man-made equivalent and in fact the operation of this circuit of poorly understood. A readable account of this work can be found in Koza et al. (2003).

The question thus remains, how has evolution fashioned biological networks. The hope is that biological networks are structured in a modular fashion, if not then we will be confronted with trying to understand dense, functionally overlapping circuitry, a daunting task. The most likely scenario is that nature has done both and tentative studies by Alon and coworkers (Shen-Orr et al., 2002) suggest that only a limited number of topological structures exist that are combined in different ways to achieve different outcomes. This study, however, was only concerned with network topology whereas much of the richness of biological behavior originates from kinetic

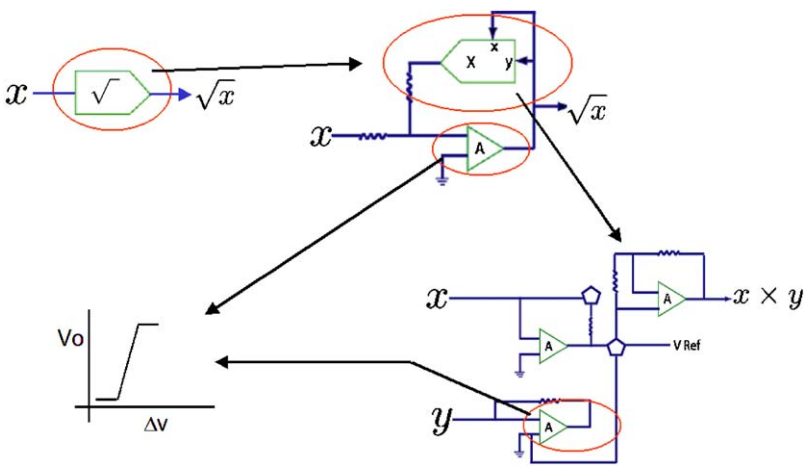


Fig. 5. Modularity in the square root circuit.

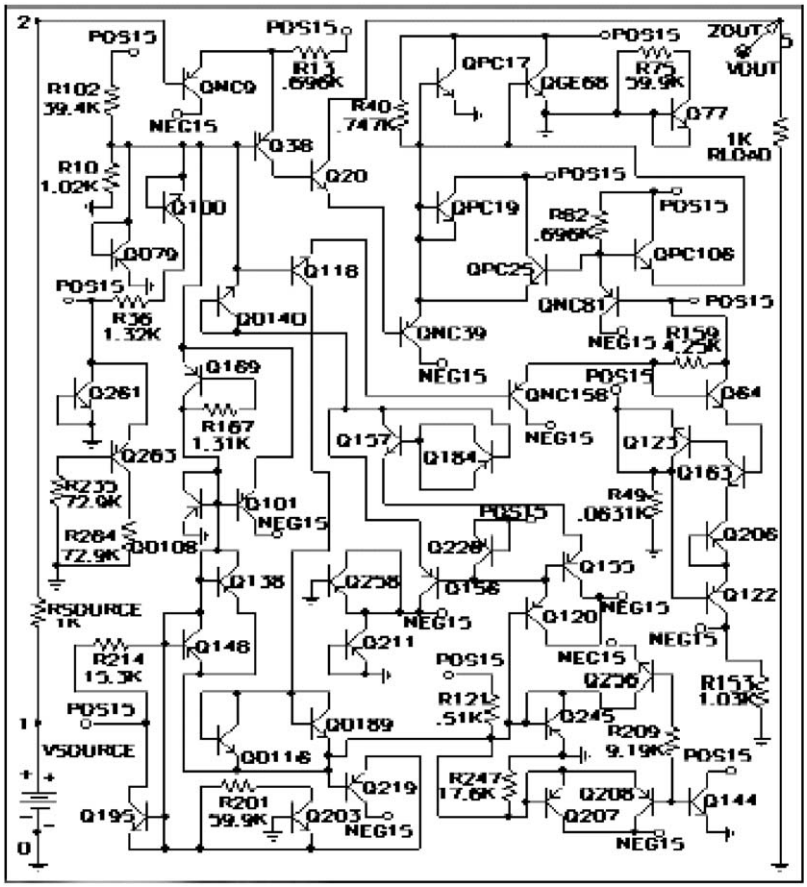


Fig. 6. Evolved square root circuit from Koza et al. (1999).

properties coupled to topology. In a later section, we will discuss an alternative view to generating modular structures through the use of feedback.

## 3. Quantitative approaches to analyzing signaling networks

There are a number of complementary approaches that one can use to analyze signaling networks quantitatively. Probably the most obvious is simulation, this involves building a kinetic model of the network and solving the resulting differential equations numerically. There are many tools available to assist in this process, including Cellerator (Shapiro et al., 2003), E-Cell (Tomita et al., 1999), Gepasi (Mendes, 1993), Jarnac (Sauro, 2000), JDesigner (Sauro et al., 2003), SCAMP (Sauro and Fell, 1991), VCell (Loew and Schaff, 2001), to name but a few. In recent years, there has also been a growing list of signaling network models, including Kholodenko et al. (1999), Brightman and Fell (2000), Asthagiri and Lauffenburger (2001), Schoeberl et al. (2002), Moehren et al. (2002), Shvartsman et al. (2002), and Wiley et al. (2003). Such models offer an ideal

environment to test and raise new hypotheses, in addition they are excellent at determining the limits of our knowledge for a particular pathway, since in the process of model building, one has to collect extensive information on the network's configuration, values for kinetic parameters and so on. However, many signaling models are complex, with up to 60 or 70 differential equations; as a result, the behavior of a model can be difficult to interpret in terms of the model's component parts. It is particularly difficult to try and relate the behavior to any 'design' features of the network. A complimentary approach to simulation is to look at signaling networks from a more theoretical point of view. Thus, rather than building numerical models of complex networks, we instead analyze much smaller networks algebraically. The aim of such an analysis is to gain an understanding of the general principles by which signaling networks operate. By combining such theoretical studies with modelling, it becomes much easier to interrogate the kinetic model and gain a clearer insight into the operation and 'design' of the network. If we used simulation as our only approach, it would be like an electronic engineer attempting to understand a complex electrical network without understanding basic concepts such as Kirchoffs laws, the properties of the unit processes, such as capacitors, transistors, etc., or higher-level concepts such as signal processing, amplifiers, comparators and so on.

The theoretical analysis of signaling networks, in particular, the properties of the cascade cycle, has a relatively long history. The notion that a protein cascade could amplify signals was understood at least as far back as the 1960s (Wald, 1965; Levine, 1966) particularly in relation to blood clotting. However, it was Savageau (1972), who first began a systematic investigation into the properties of cascade networks with further work published in his book Savageau (1976) in 1976. Savageau was also probably the first to investigate the effect of feedback on a cascade system which lead to some interesting conclusions regarding the effect of feedback (to be discussed more thoroughly in a subsequent section).

Two groups who were particularly prominent in the late 1970s and early 1980s were Stadtman and Chock (Chock and Stadtman, 1977a, b) and Goldbeter and Koshland (1981, 1984). It was Goldbeter and Koshland who probably first introduced the idea of zero-order ultrasensitivity, that is the notion that small changes in the activity of the catalyzing enzymes when operating near saturation can lead to large concentration changes. The book by Fell (1997) discusses at length the early work by both Stadtman and Goldbeter.

Both Stadtman and Goldbeter derived their conclusions from using explicit mechanistic models; however with the advent of metabolic control analysis (MCA) (Kacser and Burns, 1973; Savageau, 1972) it became possible to investigate the properties of biochemical networks without having to consider detailed kinetic mechanisms. To those unfamiliar with MCA, there is an extensive literature covering many different aspects. For newcomers, the book by Fell (1997) is highly recommended, for the more adventurous, the book by Heinrich and Schuster (1996) is excellent.

MCA and the very closely related biochemical systems theory (BST) is a sophisticated method for investigating the effect of perturbations on a biochemical network. Both approaches can be used to analyze metabolic, genetic, signal, or a mixture of all three network types. Both methods linearize the network equations around a convenient operating point, for example the steady state, and study the behavior of the network when small perturbations are applied. Depending on the strengths of the causal links within the network, a given perturbation may grow or decay as it propagates from its point of origin to other parts of the network. The presence of feedback loops

as well as the general topological structure of the network will influence how perturbations make their way across the network. By quantifying the effect of the perturbation we can gain insight into the role and operation of the different parts of a network as they react.

Savageau (1972) was the first to apply perturbation analysis to signaling cascades; however, the model that he chose did not include the conservation of cycle species. The ability to deal with the sensitivity analysis of conserved cycles had to await the development by Fell and Sauro (1985) and Hofmeyr (Hofmeyr et al., 1986) of the necessary theoretical tools. On the basis of these theoretical developments, a full analysis which included the effect of conservation was possible and in the 1980s. Small and Fell (Fell and Small, 1986; Small, 1988; Small and Fell, 1990) were the first to show the emergence of ultrasensitivity without recourse to detailed molecular mechanisms. This work stimulated a wide range of analyses of signaling networks using MCA, most notable being the work by Cárdenas and Cornish-Bowden (1989) who made a close study of how different types of effector mechanism influenced the response characteristics of the cascade, the work by Kahn and Westerhoff (1991), who developed a modular method for the analysis of complex signaling networks and subsequently greatly enhanced by Hofmeyr and Westerhoff (2001) and by Bruggeman (Bruggeman et al., 2002). Further important work was carried out by Kholodenko (Kholodenko et al., 1997), Brown (Brown et al., 1997b) and Ferrell (1997) who considered the effect of multiple cascade cycles on the degree of amplification using MCA.

### 3.1. Control analysis of a single cascade cycle

Many of the initial analyses on cascade networks relied on detailed mechanistic assumptions to draw conclusions. However, it is possible to carry out analyses without such consideration by using metabolic control analysis. Small and Fell (Fell and Small, 1986; Small, 1988; Small and Fell, 1990) were one of the first to apply MCA to investigate the properties of cascade cycles. One of the advantages of this approach is that during the linearization we can eliminate detailed mechanistic considerations.

MCA permits global properties of networks to be expressed in terms of local properties. The local properties are termed elasticities and measure the sensitivity of individual reactions to perturbations in their local environment. For example, consider the simple irreversible Michaelis–Menton rate law

$$v = \frac{VmS}{Km + S}.$$

Given that the elasticity is defined as

$$\varepsilon_S^v = \frac{\partial v}{\partial S}\frac{S}{v},$$

we can derive the elasticity for the Michaelis–Menton rate law to be

$$\varepsilon_S^v = \frac{Km}{Km + S}.$$

Elasticities are closely related to the kinetic order of a reaction, thus an elasticity of one can be interpreted as indicating first-order kinetics, and an elasticity of zero, zero-order kinetics.

From the elasticity equation for the Michaelis–Menton rate law, we see that at low substrate levels, the elasticity is approximately 1; this indicates first-order kinetics. In contrast, as the substrate concentration increases, thus saturating the enzyme, the elasticity tends to zero; zero-order kinetics.

With this information we can examine the properties of a simple, single-stage cascade cycle. Following, Fell and Sauro and Hofmeyr (Fell and Sauro, 1985; Hofmeyr et al., 1986) we can derive the connectivity theorem for the cycle shown in Fig. 2 as follows where $S_1$ and $S_2$ refer to $E_1$ and $E_2$ in Fig. 1:

$$C_{v_1}^{S_1}\left(\varepsilon_1^1\frac{1}{S_1}-\varepsilon_2^1\frac{1}{S_2}\right)+C_{v_2}^{S_1}\left(\varepsilon_1^2\frac{1}{S_1}-\varepsilon_2^2\frac{1}{S_2}\right)=-\frac{1}{S_1}.$$

In conjunction with the summation theorem

$$C_{v_1}^{S_1}+C_{v_2}^{S_1}=0$$

and assuming irreversible kinetics in order to make the analysis more transparent, we can use the connectivity and summation theorem to solve for $C_{v_1}^{S_1}$:

$$C_{v_1}^{S_1}=\frac{M_2}{\varepsilon_2^1 M_1+\varepsilon_1^2 M_2}, \tag{1}$$

where $M_2$ is the mole fraction of $S_2$ and $M_1$ the mole fraction of $S_1$. This result is the same as was published by Small and Fell (1990).

Let us now consider two cases, one where the cycle enzymes are unsaturated, and a second where the cycle enzymes are saturated.

In the unsaturated case, $\varepsilon_2^1=1$ and $\varepsilon_1^2=1$, and noting that $M_1+M_2=1$ the equation above can be simplified to

$$C_{v_1}^{S_1}=M_2,$$

that is the sensitivity of $S_1$ to changes in $v_1$ is simply equal to the mole fraction of $S_2$. Since the mole fraction is bounded between 0 and 1, we can state that the sensitivity is also bounded between 0 and 1.

If we now assume that the cycle enzymes are operating near saturation, that is both elasticities are very small, the denominator of the sensitivity equation is also small, hence $C_{v_1}^{S_1}$ can acquire values in excess of 1. This is the case that is frequently referred in the literature as zero-order ultrasensitivity (Goldbeter and Koshland, 1984). That is, a change in the activity of one of the cycle limbs, leads to a disproportionate change in the distribution of $S_1$ and $S_2$ in the cycle.

If we plot the steady-state concentration of $S_1$ against the activity of $v_1$ over a small range of enzyme *Km*'s we can see the effect of ultrasensitivity clearly. At low *Km*'s, when saturation is more likely, we see switching-like behavior in the cycle. At high *Km*'s, the behavior reverts to a hyperbolic-like behavior since the sensitivity is simply proportional to the mole fraction.

Note that the mechanism used to change the activity of $v_1$ is not specified in the equations, the equation simply states what would happen if the activity were changed. Activity changes could be the result of inhibitors or activators or more appropriately for cascading cycles, changes in protein activities upstream brought about by phosphorylation.

### 3.2. Multiple layered cascades

The analysis of sensitivity to more than one layer follows naturally from a single cascade. Following Small and Kholodenko (Small, 1988; Brown et al., 1997b; Kholodenko et al., 1997), the response of a cascade of $n$ cycles to a change in the input is simply the product of the sensitivities of the individual cycles.

Thus

$$R_S^T = r_S^1 r_1^2 r_2^3 \ldots r_{n-1}^T.$$

What this means is that the overall gain exhibited by the cascade depends on the gains of the individual cycles. If each cycle has a gain greater than unity, that is it exhibits some ultrasensitivity, then the over gain is amplified at each stage.

The ability of layered cascades to increase the overall amplification of the network has been proposed to be one possible reason why we see multiple layers in real cascade networks (Ferrell, 1996, 1997; Brown et al., 1997a).

### 3.3. Multi-site cascades

A number of cascades use multi-site phosphorylation, for example the MAPK family (Gomperts et al., 2002) (Fig. 7). The most well-known examples include Raf phosphorylating two serine residues on MEK, and MEK in turn phosphorylating a tyrosine and threonine on ERK. Why nature should have chosen to implement a cascade using double modulation is still an open question since, theoretically, amplification can be just as easily achieved with a single phosphorylation site. There are some indications that double phosphorylation may provide more robust amplification, the potential for bistability and hysteresis (Markevich et al., 2004) and possibly resistance to the effect of protein sequestration (Nils Bluethgen and Sauro, unpublished).

We can write down the response of a multi-site cycle to perturbations in the kinase steps. Fig. 8 illustrates the model we are considering. The input signal is usually another kinase which phosphorylates both forward arms in the multi-site cascade. The individual responses are given by

$$C_{v_1}^{S_3} = \frac{\varepsilon_2^3 S_1}{\varepsilon_2^2 \varepsilon_3^4 S_1 + \varepsilon_1^1 \varepsilon_3^4 S_2 + \varepsilon_1^1 \varepsilon_3^3 S_3},$$

$$C_{v_3}^{S_3} = \frac{\varepsilon_1^1 S_2 + \varepsilon_2^2 S_1}{\varepsilon_2^2 \varepsilon_3^4 S_1 + \varepsilon_1^1 \varepsilon_3^4 S_2 + \varepsilon_1^1 \varepsilon_3^3 S_3}.$$

However, since both kinases are activated by a kinase in the preceding layer, the overall response of the multi-site cascade is the sum of the two responses, that is

$$C_{v_3+v_1}^{S_3} = \frac{S_1(\varepsilon_2^3 + \varepsilon_2^2) + S_2 \varepsilon_1^1}{\varepsilon_2^2 \varepsilon_3^4 S_1 + \varepsilon_1^1 \varepsilon_3^4 S_2 + \varepsilon_1^1 \varepsilon_3^3 S_3}.$$

Inspection of the equations reveals that since the 'zero-order' elasticities are product terms in the denominator, the value of the denominator will be smaller than the equivalent denominator in the

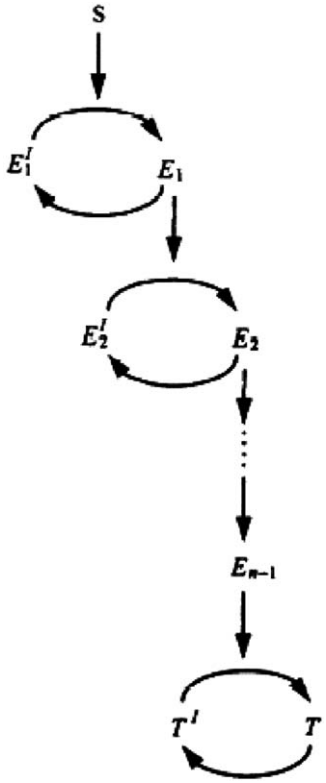


Fig. 7. Multiple cascade cycles forming a layered structure.

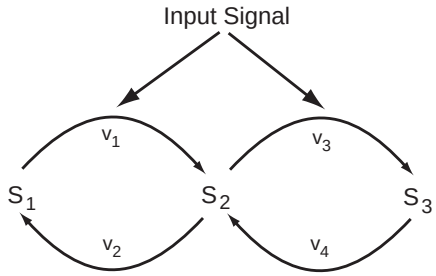


Fig. 8. Multi-site cascade cycle.

single-site cycle equation (1). Therefore, we can suppose that the potential to amplify in a multi-site cascade is even greater.

There is therefore no question that cascades, both single and multi-site have the potential to amplify signals, in fact gain factors of the order of 100,000 are possible. This has important implications when we consider the addition of feedback to cascades. For more details on the control analysis of multi-site systems, the reader is referred to a paper by Kholodenko et al. (1998).

## 4. Network motifs

As previously discussed, electronic engineering employs a modular strategy to design electronic networks. The complexity of modern devices is simply too overwhelming to employ any other strategy. As part of the strategy, electronic engineers have accumulated over the years a library of basic 'network motifs', that is, well understood sub-networks, which perform specific functions, such as switches, amplifiers, oscillators, filters and so on. The question which was asked in the previous section was whether evolution has followed a similar approach. The answer is, perhaps, or at least it appears so in a number of cases. This is of course very fortunate as it enables us to simplify the descriptions of cellular networks into manageable and understandable subsystems. Two recent and excellent reviews (Tyson et al., 2003; Wolf and Arkin, 2003) discuss the notion of biological motifs and cite a number of examples. These reviews cover a broader range of motifs than will be discussed here. We will focus on a few motifs and discuss them in greater depth.

The following sections will be divided into two broad categories, digital and analog. Whether evolution has fashioned digital devices on a large scale is still a matter of debate, but considering that our current technological mind set is digital, we may be inadvertently focusing too much attention on the possibility of a digitally driven biological cell. As a result, we may overlook the fact that not so long ago, analog was a critical aspect of man-made computational devices in the form of analog computers (Soroka, 1954). Given the flexibility of analog and its inherent ability to condense data handling to a far greater degree than its digital counterpart,[1] we think that the argument that evolution has selected largely for analog-based signaling networks is a strong one. Clearly, there are cases when on/off decision making is crucial, for example, the most obvious being cell division (Tyson et al., 2001, 2002) and bacterial sporulation (Grossman, 1995; Hecker and Volker, 2001) being another example. Boolean-based digital circuits may be employed by gene regulatory networks; however, even here the case is not certain (Smolen et al., 2000; Bolouri and Davidson, 2002; Rao and Arkin, 2001).

Since analog is not as well known to the molecular biology community as the digital world, the section on analog devices will discuss in some detail one of the fundamental motifs used by analog devices, namely feedback (positive and negative).

### 4.1. Digital circuits

#### 4.1.1. Basic logic gates

Digital circuits generated from reaction networks have been studied theoretically for a number of years (Okamoto et al., 1980, 1987, 1988, 1989; Hjelmfelt et al., 1991, 1992; Sauro, 1993; Arkin and Ross, 1994). For a lighter discussion on building logic gates using genetic circuits, the reader is directed to Hayes (2001).

The essential requirement for a logic gate is of a fast changing concentration that can exist in one of two states. Switching from one state to another is accomplished by a threshold level on an input. Cascade cycles, when operating in ultrasensitive mode have the property to change from one state to another when an input variable passes a threshold, and thus offer an obvious means

[1] Note that it requires a single transistor to store one bit in a digital device, whereas if the transistor were used as an analog storage device it could represent a value to an arbitrary high number base.

to implement Boolean functions in a reaction system. The easiest gate to construct from a simple cascade cycle is a NOT gate, that is, a positive input generates a zero output and a zero input generates a positive output (Fig. 9).

### 4.1.2. Ring oscillator

One of the easiest nontrivial circuits to construct using simple logic gates is the ring oscillator (Fig. 10). This is a well-known device in electronics and includes of an odd number of NOT gates connected to each other in series, with the last gate connected to the first, thus forming a ring. Provided the number of gates is odd, this system will oscillate. This type of circuit has been genetically engineered in vivo using gene regulatory components in *E. coli* (Elowitz and Leibler, 2000). Theoretically, it should also be possible to construct a ring oscillator from cascade cycles (Sauro, 1993). Fig. 11 illustrates such an arrangement with accompanying simulation curves in Fig. 12. A more detailed analysis of related circuits based on cascade cycles can be found in Gonze and Goldbeter (2001).

### 4.1.3. NAND gate

The logical NAND gate is also easily constructed from a cascade cycle (Fig. 13). Since the cascade cycle is a threshold device, a NAND gate can be constructed by simply applying two inputs to the kinase step. It can be arranged so that one input alone is not sufficient to cause the cascade cycle to switch, but two will. Obviously, each input must have an upper limit to prevent a single input from exceeding the threshold, however, this can be easily arranged since the concentration of the cycle species are limited by the cycle mass conservation law.

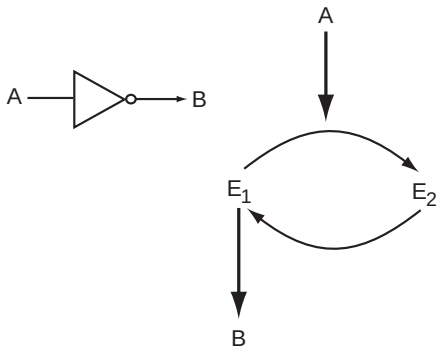


Fig. 9. Simple NOT gate constructed from a single cascade cycle.

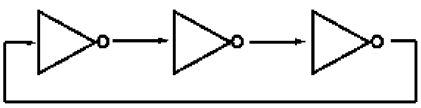

Fig. 10. Oscillator based on NOT gates.

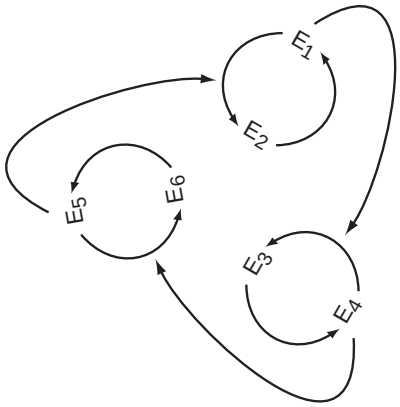


Fig. 11. Ring oscillator architecture.

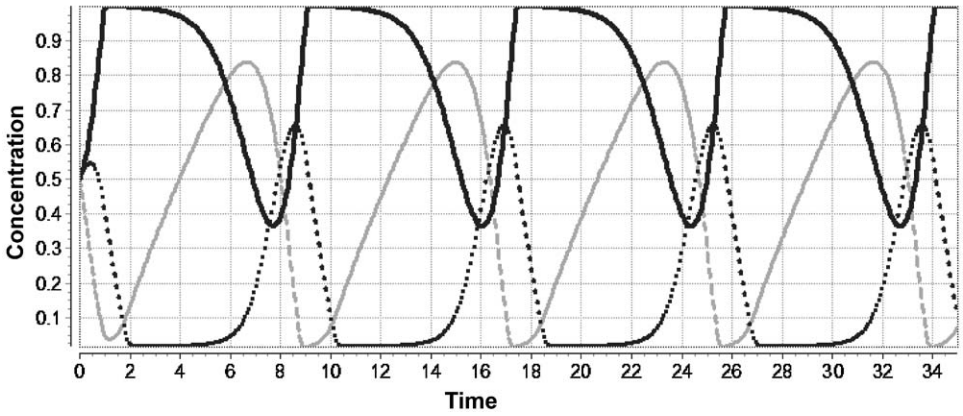


Fig. 12. Time course behavior of the ring oscillator shown in Fig. 11.

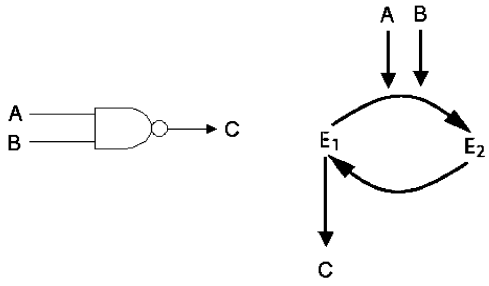


Fig. 13. Simple NAND gate.

### 4.1.4. *Memory units, counters and arithmetic*

Once a NAND gate can be constructed it is possible to devise any logical circuit. Three examples are illustrated here, a basic 1-bit memory unit, the so-called flip-flop, a 4-bit binary

counter constructed from four flip-flops and finally a half-adder which can sum two input bits and output the sum and a carry bit. Descriptions of these circuits and numerous others can be found in the TTL cookbook (Lancaster and Lancaster, 1980) (Figs. 14–16).

It should be clear from this brief description that in principle, it should be possible to construct any digital device from cascade elements. Whether biological cells actually employ such 'designs' in signaling pathways is still a matter of debate.

### 4.2. Analog circuits

Although modern computers are almost exclusively digital, it was not long ago that the bulk of simulation and general computation was performed on analog computers. These computers represented quantities not by a binary number but by some continuously variable physical

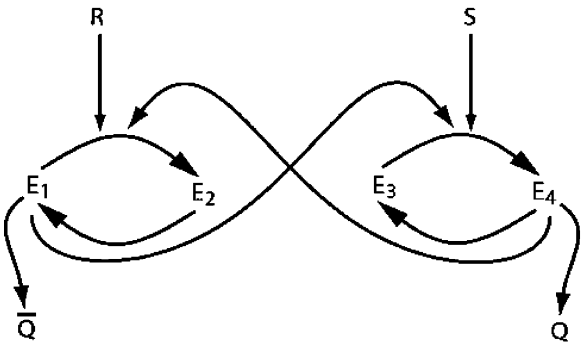


Fig. 14. Simple memory unit.

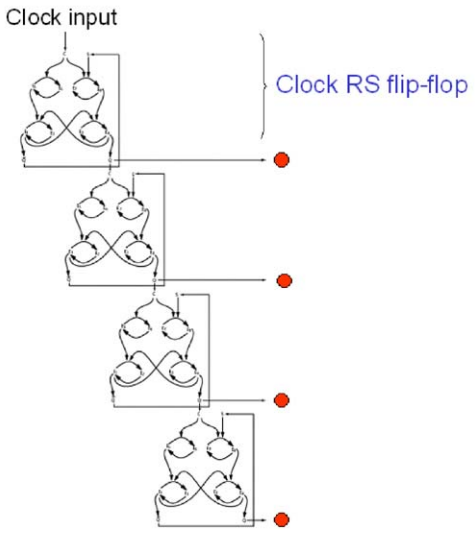


Fig. 15. 4-bit binary counter.

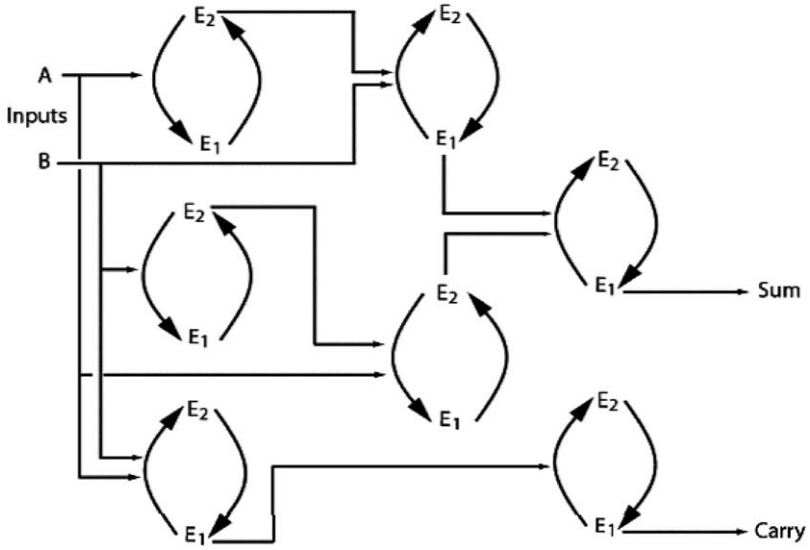


Fig. 16. Binary half-adder.

quantity, such as voltage, the rotational speed of a shaft or the level of fluid in a tank. In addition, unlike digital computers which compute sequentially, analog computers had the great advantage of being able to compute in parallel; thus, in an analog computer all computations occur simultaneously. The fundamental processing unit of an analog computer was the operational amplifier (Jung, 1986). These amplifiers were extremely versatile and could be coerced to perform a great variety of different computations, ranging from normal arithmetic to differential calculus, including the all important operation of numerical integration. Analog computers could therefore be 'programmed' to perform any desired computation, the most common being the solution of sets of nonlinear differential equations.

An analog computer is programmed, literally to be an analog of the physical problem in question. The technology used to construct the analog computer must be able to faithfully mimic the behavior of the physical system. In the past, the technologies used to build analog computers have been quite varied, including mechanical components, hydraulic devices and of course electronic components. It should come as no surprise therefore that we can also use reaction networks to construct analog computers. This begs the question whether nature has employed the analog approach in the evolution of signaling pathways. The answer to this is a definite yes although only a few examples are currently known. A case in point is the suggested role of the MAPK pathway. The current hypothesis (Huang and Ferrell, 1996; Bagowski et al., 2003) is that MAPK behaves as a digital switch, either on or off. However, there are other possibilities which have been explored and will be discussed further in a later section.

### *4.2.1. Negative feedback*

One of the most fundamental concepts in control theory and analog computation is the idea of feedback. Put simply, feedback is when information about a process is used to change the process

itself. Examples include the control of water height in a household cistern, or the control of temperature via a thermostat.

Feedback has a huge range of applications both in man-made and natural systems. In many cases, feedback is used to achieve some sort of automatic control, maintaining the level of water in a tank, or controlling the temperature of a water bath. Since the beginning of the 20th century, feedback has also been employed to construct robust signal amplifiers, now probably one of the most important and common applications of feedback.

In natural systems, such as biological systems, feedback controls are very common, in metabolic pathways, signaling networks and gene regulatory networks, feedback controls abound. In many cases, however, the role of these feedback circuits, other than as a vague notion of control, is obscure and only recently have efforts being made to rationalize the existence of such circuits in biochemical networks.

### *4.2.2. History of feedback*

The concept of feedback control goes back at least as far as the ancient Greeks. Of some concern to the ancient Greeks was the need for accurate time keeping. In about 270 BC, the Greek Ktesibios invented a float regulator for a water clock. The role of the regulator was to keep the water level in a tank at a constant depth. This constant depth yielded a constant flow of water through a tube at the bottom of the tank which filled a second tank at a constant rate. The level of water in the second tank thus depended on time elapsed. Philon of Byzantium in 250 BC is known to have kept a constant level of oil in a lamp using a float regulator and in the first century AD Heron of Alexandria experimented with float regulators for water clocks.

It was not until the industrial revolution that feedback control, or devices for automatic control, became economically important. In 1788, Watt completed the design of the centrifugal flyball governor for regulating the speed of the rotary steam engine. This device employed two pivoted rotating flyballs which were flung outward by centrifugal force. As the speed of rotation increased, the flyweights swung further out and up, operating a steam flow throttling valve which slowed the engine down. Thus, a constant speed was achieved automatically. So popular was this innovation that by 1868 it is estimated that 75,000 governors were in operation in England. Many similar devices were subsequently invented to control a wide range of processes, include temperature control and pressure control.

During this period, devices for automatic control were designed through trial and error and little theory existed to understand the limits and behavior of feedback control systems. One of the difficulties with feedback control is the potential for instability. Too much feedback and a system can begin to oscillate wildly out of control.

Until the 20th century, feedback control was generally used as a means to achieve automatic control, that is to ensure that a variable, such as a temperature or a pressure was maintained at some set value. However, an entirely new application for feedback control was about to emerge with the advent of electronics in the early part of the 20th century.

*Feedback amplifiers*: Amplification is one of the most fundamental tasks one can demand of an electrical circuit. One of the challenges facing engineers in the 1920s was how to design amplifiers whose performance was robust with respect to the internal parameters of the system and which could overcome inherent nonlinearities in the implementation. This problem was especially critical to the effort to implement long distance telephone lines across the USA.

These difficulties were overcome by the introduction of the *feedback amplifier*, designed in 1927 by Harold S. Black (Mindell, 2000), who was an engineer for Western Electric (the forerunner of Bell Labs). The basic idea was to introduce a negative feedback loop from the output of the amplifier to its input. At first sight, the addition of negative feedback to an amplifier might seem counterproductive.[2] Indeed, Black had to contend with just such opinions when introducing the concept—his director at Western Electric dissuaded him from following up on the idea, and his patent applications were at first dismissed. In his own words 'our patent application was treated in the same manner as one for a perpetual motion machine' (Black, 1977).

While Black's detractors were correct in insisting that the negative feedback would reduce the gain of the amplifier, they failed to appreciate his key insight—that the reduction in gain is accompanied by increased robustness of the amplifier and improved fidelity of signal transfer. This trade-off between gain and system performance can be elegantly demonstrated by considering linear systems, to which we now turn.

#### 4.2.3. Analysis of feedback

There have been numerous discussions on the role of feedback in biochemical networks ever since the discovery by Umbarger (Umbarger, 1956) of feedback inhibition in the isoleucine biosynthesis pathway and the feedback inhibition of aspartate transcarbamylase in *E. coli* by Yates and Pardee (1956).

Probably, the most extensive mathematical analysis of biochemical feedback was conducted by Savageau (1972, 1974, 1976) and Burns and Kacser (Burns, 1971; Kacser and Burns, 1973) and Othmer and Tyson (Othmer, 1976; Tyson and Othmer, 1978) in the 1970s and Dibrov et al. (1982) in the early 1980s. More recently, Cinquin and Demongeot (2002) have published an interesting review on the roles of feedback in biological systems.

However, without having to get into any sophisticated arguments, there is a very simple yet insightful algebraic analysis of a simple feedback system. This analysis illustrates many of the key properties that feedback systems possess. Consider the block diagram in Fig. 17.

We will consider only the steady-state behavior of the system. We take the input $u$, the output $y$, and the error $e$ to be constant scalars. Assume (for now), that both the amplifier $A$ and the feedback $F$ act by multiplication (take $A$ and $F$ as nonnegative scalars). Then without feedback (i.e. $F = 0$), the system behavior is described by $y = Au$, which is an amplifier (with gain $A$) provided that $A > 1$. Assuming for now that the disturbance $d$ is zero, and if we now include feedback in our analysis, the behavior of the system is as follows. From the diagram, we have

$$y = Ae, \quad e = u - Fy.$$

Eliminating $e$, we find

$$y = \frac{Au}{1 + AF} \quad \text{or simply} \quad y = Gu,$$

where $G = A/(1 + AF)$ is the *system* (*or closed loop*) *gain*. Comparing $G$ with $A$, it is immediate that the feedback does indeed reduce the gain of the amplifier. Further, if the *loop*

[2] As Horowitz and Hill put it in Horowitz and Winfield (1990) 'Negative feedback is the process of coupling the output back in such a way as to cancel some of the input. You might think that this would only have the effect of reducing the amplifier's gain and would be a pretty stupid thing to do.'

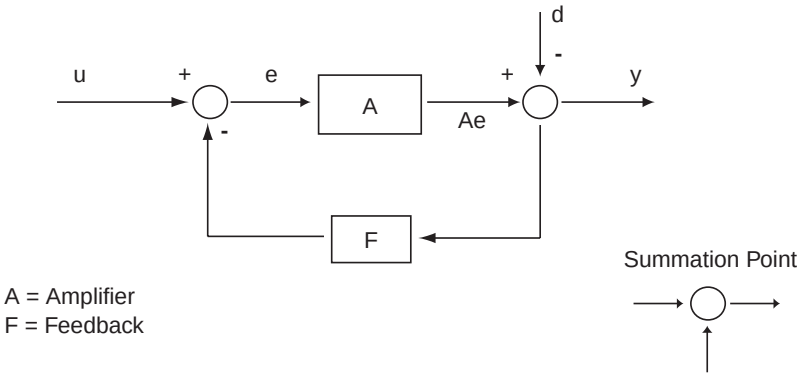


Fig. 17. Linear feedback system.

*gain* $AF$ is large ($AF \gg 1$), then

$$G \approx \frac{A}{AF} = \frac{1}{F}.$$

That is, as the gain $AF$ increases, the system behavior becomes more dependent on the feedback loop and less dependent on the rest of the system. We next indicate three specific consequences of this key insight.

*Resistance to internal parameter variation*: In all real amplifiers, both man-made and natural, there will be variation in the amplifier ($A$) characteristics, either as a result of the manufacturing process or internally generated thermal noise. We can study the effect of variation in the amplifier characteristics by investigating how $A$ causes variation in the gain $G$.

Considering the sensitivity of the system gain $G$ to variation in the parameter $A$, we find

$$\frac{\partial G}{\partial A} = \frac{\partial}{\partial A}\frac{A}{1+AF} = \frac{1}{(1+AF)^2}.$$

Clearly, this sensitivity decreases as $AF$ increases. It may be more telling to consider the relative sensitivity, in which case we find

$$\frac{\partial G}{\partial A}\frac{A}{G} = \frac{1}{1+AF},$$

so that for a small change $\Delta A$ in the gain of the amplifier, we find the resulting change $\Delta G$ in the system gain satisfies

$$\frac{\Delta G}{G} \approx \frac{1}{1+AF}\frac{\Delta A}{A}.$$

As the strength of the feedback ($F$) increases the influence of variation in $A$ decreases.

*Resistance to disturbances in the output*: Suppose now that a nonzero disturbance $d$ affects the output as in Fig. 17. The system behavior is then described by

$$y = Ae - d, \quad e = u - Fy.$$

Eliminating $e$, we find

$$y = \frac{Au - d}{1 + AF}.$$

The sensitivity of the output to the disturbance is then

$$\frac{\partial y}{\partial d} = -\frac{1}{1 + AF}.$$

Again, we see that the sensitivity decreases as the loop gain $AF$ is increased. In practical terms, this means that the imposition of a load on the output, for example, a current drain in an electronic circuit or protein sequestration on a signaling network, will have less of an effect on the amplifier as the feedback strength increases. In electronics, this property essentially modularizes the network into functional modules.

*Improved fidelity of response*: Consider now the case where the amplifier $A$ is nonlinear. For example a cascade pathway exhibiting a sigmoid response. Then the behavior of the system $G$ (now also nonlinear) is described by

$$G(u) = y = A(e), \quad e = u - Fy = u - FG(u).$$

Differentiating we find

$$G'(u) = A'(u)\frac{\mathrm{d}e}{\mathrm{d}u}, \quad \frac{\mathrm{d}e}{\mathrm{d}u} = 1 - FG'(u).$$

Eliminating $\mathrm{d}e/\mathrm{d}u$, we find

$$G'(u) = \frac{A'(u)}{1 + A'(u)F}.$$

We find then, that if $A'(u)F$ is large ($A'(u)F \gg 1$), then

$$G'(u) \approx \frac{1}{F},$$

so, in particular, $G$ is approximately linear. In this case, the feedback compensates for the nonlinearities $A(\cdot)$ and the system response is not distorted. (Another feature of this analysis is that the slope of $G(\cdot)$ is less than that of $A(\cdot)$, i.e. the response is 'stretched out'. For instance, if $A(\cdot)$ is saturated by inputs above and below a certain 'active range', then $G(\cdot)$ will exhibit the same saturation, but with a broader active range.)

A natural objection to the implementation of feedback as described above is that the system sensitivity is not actually reduced, but rather is shifted so that the response is more sensitive to the feedback $F$ and less sensitive to the amplifier $A$. However, in each of the cases described above, we see that it is the nature of the loop gain $AF$ (and not just the feedback $F$) which determines the extent to which the feedback affects the nature of the system. This suggests an obvious strategy. By designing a system which has a small 'clean' feedback gain and a large 'sloppy' amplifier, one ensures that the loop gain is large and the behavior of the system is satisfactory. Engineers employ precisely this strategy in the design of electrical feedback amplifiers, regularly making use of amplifiers with gains several orders of magnitude larger than the feedback gain (and the gain of the resulting system).

In summary, a feedback amplifier provides the following desirable characteristics:

1. amplification of signal;
2. robustness to internal component variation;
3. high fidelity of signal transfer;
4. low output impedance so that the load does not affect the amplifier.

These are only a few of the advantages to adding feedback to an amplifier. Additional advantages include improved frequency response—that is the circuit is able to respond to more rapidly changing signals—and lower input requirements, that is the circuit makes less demands (in terms of load) on the circuit that supplies the input signal.

*Experimental evidence*: There has been little experimental research in the biological community on the effect of feedback but the paper by Becskei and Serrano (2000) is instructive. Gardner and Collins (2000) discuss the paper in some detail, but essentially Becskei and Serrano construct an artificial gene circuit where they compare the circuit with and without feedback. They show that with feedback the effect of noise on the system, as indicated by the expression of green fluorescent protein (EGTP), is greatly reduced.

*Drawbacks of feedback*: The properties that feedback endows on a network are obviously very attractive. Indeed, the use of feedback in the electronics industry is extensive, however, there is at least one draw back.

Although negative feedback tends to be a stabilizing force, there are certain conditions when negative feedback can result in instability. In particular, if the feedback takes too long before it starts to correct a disturbance and/or it corrects too strongly, the network will begin to oscillate out of control. Such effects have been studied well in metabolic systems (Morales and McKay, 1967; Hunting, 1974; Othmer, 1976; Tyson and Othmer, 1978). In signaling networks, there has been less interest except for some efforts by Kholodenko (2000) and Bluethgen (Bluethgen and Herzel, 2001). Both authors discuss the possibility of oscillations in MAPK pathways with feedback. What is most remarkable about this is that such oscillations in a signaling network have now been observed experimentally (Ann E. Rundell, Purdue University, pers. comm.).

#### 4.2.4. Implications for drug targeting

The analysis of feedback illustrates an important principle for those engaged in finding new drug targets. The aim of a drug is to cause a disruption to the network in such a way that it restores the network to it 'healthy' wild-type state. Clearly, targets must be susceptible to disruption for the drug to have any effect. The analysis of feedback suggests that targets inside the feedback loop are not suitable because any attempt to disturb these targets will be resisted by the feedback loop. Conversely, targets upstream and particularly downstream are very susceptible to disturbance. Fig. 18 illustrates the effect of a 20 fold decrease in enzyme activity at two points in a simple reaction chain. In the first case, both disruption at the center or end of the network has a significant effect on the concentration of the last species, $S_3$. In the second panel, the same pathway is shown but with a negative feedback loop from $S_3$ to the first enzyme. The same activity reductions are also shown, but this time note the almost insignificant effect that modulating a step inside the loop has compared to modulating a step outside the loop.

Thus, the take-home message to pharmaceutical companies who are looking for suitable targets is to avoid targeting reaction steps inside feedback loops!

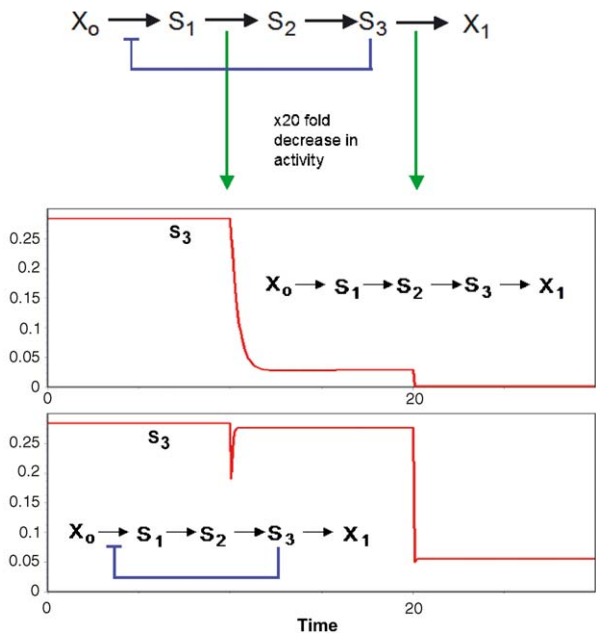


Fig. 18. Negative feedback and drug targets. See Section 4.2.4 for details.

### 4.2.5. *Positive feedback*

Whereas negative feedback tends to instill a stabilizing influence, positive feedback is inherently unstable. It might therefore come as some surprise to learn that positive feedback in biological networks is not so uncommon, perhaps more common than one might expect (McAdams and Arkin, 1998). Positive feedback circuits appear to be fairly common in bacterial gene regulatory networks and have been reported in a number of eukaryotic signaling pathways (Ferrell and Machleder, 1998; Ferrell, 2002). So why, given the instability inherent in a positive feedback, should evolution have selected for it? The answer is simple, positive feedback can be used to generate bistability, that is a device which can turn a graded signal into a all-or-nothing response (Edelstein, 1970). Positive feedback has also been shown to generate oscillatory dynamics (Seno et al., 1978) and chaotic dynamics (Decroly and Goldbeter, 1982; Goldbeter and Decroly, 1983) so it is quite a versatile control motif.

Bistability implies two states, usually a high state and a low state. At any one time, only one state can exist, although both states are accessible at the same parameter values (in contrast to the ultrasensitivity cascade, where the two states are only accessible by changing a parameter). Both states are generally very stable and movement from one state to the other tends to be difficult. There are many circumstances where the ability to move from one state to another is a useful property for a biological system. One of the most obvious is cell division.

Probably, the most familiar example of a bistable system is the common toggle light switch. This device clearly has two states, on and off, both of which are stable to small perturbations. One of the key characteristics of bistability is hysteresis, that is movement from one state to the other is not symmetric. There have been many recent reviews and comments on bistability, including a very readable account by Laurent and Kellershohn (1999).

Positive feedback circuits are well documented in the prokaryotes (Yildirim and Mackey, 2003; Hasty et al., 2001; Gardner et al., 2000; Arkin et al., 1998), and it is likely that they are fairly common in these organisms. In eukaryotes less is known, however a few examples are now emerging which suggests that positive feedback is also an important control motif in higher organisms (Sha et al., 2003; Bagowski et al., 2003; Bhalla et al., 2002). In addition, some very elegant in vitro studies have revealed bistability in yeast glycolytic reconstituted systems (Eschrich et al., 1990). There are thus a number of instances where positive feedback plays a critical role in the functioning of signaling pathways. For example, it is believed that the bistability of cyclin-dependent kinases are important for controlling cell progression through the cell cycle (Tyson et al., 2001, 2002; Thron, 1996). The role of bistability is to make sure that the cell cycle commits to mitosis and does not slip back to interphase. Bistability has been predicted for some time as the primary device for ensuring commitment through computer models. However, it is only very recently that experimental evidence has been obtained that convincingly shows hysteresis, the hall-mark of bistability (Sha et al., 2003; Pomerening et al., 2003).

Ferrell (1999) has argued convincingly that positive feedback is responsible for the maturation of Xenopus oocytes in response to the steroid hormone progesterone. Interestingly, the observation for all-or-nothing response in oocytes is not observed at the population level and it is only when individual cells are studied that bistability-like behavior is observed which clearly illustrates the danger in drawing conclusions from population studies.

Lastly, recent studies by Bhalla et al. (2002) have revealed what appears to be a positive feedback circuit in the MAPK pathway which responds to platelet-derived growth factor. In addition to a positive feedback, there is also a longer time scale negative feedback associated with this circuit. The rational here is that the positive feedback ensures a definite on state for the networks, but this is turned off by the slower acting negative feedback.

Bistablity has also proved to be a rich ground for theoretical studies, including early work by Edelstein (1970) and more recent work by Lisman (1985) on the suggested role of bistability as a means for implementing memory, and some interesting theoretical studies on bistability in cyclic structures by Hervagault et al. (Hervagault and Canu, 1987; Hervagault and Cimino, 1989) where bistability as well as the more exotic phenomenon of irreversible bistability (Tyson et al., 2003)—that is, it only possible to switch one way, switching the other way is impossible (or at least extremely difficult)—were investigated.

### *4.3. Neural nets*

Another form of analog computing is based on the idea of the artificial neural network. Although from its name it sounds like it should be closely related to biological neural networks, artificial neural networks do in fact more closely resemble standard numerical curve fitting. Artificial neural network models a complex mathematical function which by way of modifying weights (function coefficients) can be made to fit fairly complex data patterns.

Bray (1990, 1995) has made the case that reaction networks, in particular, signaling networks may in fact be modeling artificial neural networks. Given that the MAPK pathway is a three-layer structure, it is intriguing to speculate that MAPK might be operating as a neural network since the minimum number of layers required to compute an arbitrary function is three. In addition, some cross-talk has been suggested to exist between the different MAPK families which might

correspond to the weights in the neural network. However this is pure speculation at this point. Further discussion can be found in Bhalla (2003a).

## 5. Real networks

Having covered some theoretical aspects of signaling networks, we will now turn to discuss two real networks which illustrate some of the ideas that have been discussed. The first is the *E. coli* chemotaxis network which is probably the first example of an analog computer discovered in a cellular network. The second example is taken from the MAPK pathway family which is found in all eukaryotes. The example serves to illustrate how feedback control is employed in a signaling network to insulate the system from noise, stabilize the transfer signal from distortion and provide impedance matching for the downstream processes.

### 5.1. Chemotaxis

Probably one of the most understood signaling networks is the chemotaxis circuit in *E. coli* (Falke et al., 1997; Rao and Arkin, 2001). Chemotaxis, that is the ability to move towards food sources, was suspected as far back as 1676 with Leeuwenhoek's early work using a single-lens microscope (Ford, 1985). However, it is only in the last 30 years or so with the advent of modern biochemistry and molecular biology that significant advances have been made in our understanding of bacterial chemotaxis at the molecular level.

*E. coli* is attracted to organic molecules such as amino acids, sugars and dipeptides. The question arises, what are the mechanisms and processes that allow *E. coli* to accomplish this feat. It turns out that bacteria are too small to detect shallow concentration gradients (Berg and Purcell, 1977)—but see Thar and Kuhl (2003)—by say comparing the concentration of some attractant at the front and rear of the microbe. Instead, bacteria measure gradients by comparing concentrations at different times. It has been known for a long time that *E. coli* has two modes of swimming, so-called tumbling and running. Tumbling is a random process that allows the bacterium to reorient itself in some random direction and is caused by a reversal of the six flagella that *E. coli* uses to swim. When rotating counter clockwise, the flagella form a tight bundle so that the force they generate is directed, hence the bacterium tends to swim in a straight line (run). However, if the flagella rotate clockwise, they come apart and no net forward motion results, instead the organism tumbles randomly. Thus, by varying the ratio of tumble to run, *E. coli* can direct its movements in particular directions. Essentially, the organism exercises a biased random walk in three dimensions.

When *E. coli* tumbles, there is actually a slight forward bias, this ensures that it moves slightly forward. As a result, it will by chance eventually tumble into a region where the attractant concentration is slightly increased. At this point, the signaling networks kicks in and the probability of a run increases. However, the increased likelihood of a run does not last long because the signaling networks begins to adapt to the new concentration of attractant. The adaptation is very important because it allows *E. coli* to reset the signaling network so that it is ready to detect further changes in the attractant as it moves up the concentration gradient. The ability of the network to adapt is more remarkable for the fact that the adaptation appears to be

perfect, or at least within experimental error. The ability of the chemotaxis circuit to adapt in this way has been termed *perfect adaptation*.

The response of *E. coli* to changes in attractant thus operate on two time scales, a fast response to the immediate change in attractant ensures that it moves from its current position, and a second slower response which resets the signaling network back to its original state ready to detect additional changes in attractant. Note that when the signaling network is reset the organism also reverts to the original tumbling state. Studies indicate (Segall et al., 1986) that *E. coli* compares the current concentration over the proceeding one second to the concentration observed over the previous 3 s. The memory thus lasts approximately 3 s. If the gradient increases more rapidly than the adaptation circuitry can operate, the organism will tend to run for longer periods before reverting back to a tumbling state.

The molecular details of how the initial fast response and the slower adaptation response operate has been uncovered by detailed genetic, molecular and biochemical studies over the last 30 years or so. As a result, the chemotaxis network is probably one of the most understood signaling networks, both at the molecular and system level. Quite apart from the intrinsic fascination of how bacteria chemotax, the study of bacterial chemotaxis has acted as a role model for more complex systems, particular eukaryotic systems in multicellular systems. Even so, there is still considerable debate on the detailed aspects of the chemotactic response and comparative studies have shown that the chemotaxis circuit varies even among closely related organisms (Bourret and Stock, 2002; Rao and Arkin, 2001). Probably one of the most accessible descriptions of the network can be found in Rao and Arkin's (2001) review on Control Motifs.

In an attempt to understand one of the key properties of chemotaxis, namely the perfect adaptation, we will consider a highly simplified version of the network. This network is made up of only three proteins (and their phosphorylated or methylated forms), CheA, CheB and the aspartate receptor, indicated by the symbol R. Both CheA and CheB can be phosphorylated, CheB is phosphorylated by CheA-P and CheA autophosporylates itself when activated by the receptor complex. The receptor protein complex can also undergo covalent modification, however rather than phosphorylation, the receptor complex can undergo methylation. Moreover, the receptor complex can be methylated by up to four methyl groups. The methylated form of the receptor complex is responsible for activating CheA and causing autophosphorylation to CheA-P. The binding of aspartate to the receptor effectively inhibits this action.

There are a number of critical kinetic aspects to this pathway which enable it to display perfect adaptation to changes in ligand (Aspartate) concentrations. Experimental evidence suggests that the enzymes catalysing methylation (CheR) and demethylation (CheB-P) are saturated by methylated receptor and receptor, respectively. In terms of a kinetic model, methylation of receptor is at a constant rate *vm* and demethylation is a function only of the demethylation enzyme, CheB-P.

The chemotaxis circuit shown above will display the essential characteristics that are observed in live *E. coli*. Just as with *E. coli*, the circuits works on two time scales. When Aspartate is added to the model, the ability of the receptor (R-M) to stimulate autophosphorylation of CheA to CheA-P is diminished. CheA-P is the signal molecule that is responsible for signaling changes in the flagella motor via the relay protein Che-Y (not shown). CheA-P increases the likely-hood for *E. coli* to tumble (that is re-orientate itself). Since the addition of Aspartate causes CheA-P to decrease in level, this results in less tumbling and more running. These series of events take place

very quickly on the order of 1 s. The strategy that appears to be operating is if the concentration of aspartate increases then stop tumbling and move in a straight line, presumably towards a higher concentration of aspartate.

Once the organism begins to travel in a straight line, a second series of events begin to operate. From Fig. 19, one can see that CheA-P also stimulates the formation of phosphorylated CheB. CheB-P is responsible for demethylating the receptor complex. As the CheA-P level declines in response to an increase in aspartate, the level of phosporylated CheB also declines. This means that less demethylation takes place. As a result, more methylated receptor forms which in turn stimulates the formation of CheA-P, thus restoring the initial decline in CheA-P. As CheA-P rises, so does level of demethylation, until it balances *exactly* the demethylation rate. The only state in which this is stable is when CheA-P rises to exactly its original pre-stimulus level—this is what is termed perfect adaptation and is one of the key characteristics of the chemotaxis response.

Since CheA-P is restored to its pre-stimulus level, the organism reverts back to tumbling. Strategy wise, this serves two purposes. First, it prevents the organism from over-shooting the location of the higher concentration of aspartate which it originally detected in the first phase. But critically, it also enables the organism to reset its detection circuitry so that it can detect further rises in aspartate levels. This is one of the properties conferred by the adaptation circuitry and is believed to be responsible for enabling *E. coli* to respond to such a wide range in aspartate concentrations (over five orders of magnitude).

Since the property to adapt depends on the ability to change the ratio of methylated to demethylated receptor, there will clearly be a limit to the adaptation since only so much receptor can be methylated.

The graphs in Figs. 20 and 21, illustrate some simulations of the model depicted in Fig. 19. In the first figure, the level of aspartate is repeatedly raised and lowered. When the aspartate is initially raised, the level of CheA (or CheA-P) changes rapidly, at the same time the level of methylation begins to rise. This causes the level of CheA to slowly recover and continues to recover until it returns to its original prestimulus level, that is, it has perfectly adapted. In the process of adaptation, the level of methylation changes. The second set of simulations shows what happens as we repeatedly add more and more aspartate. In each case the level of CheA adapts to its prestimulus level. However, in each case, the level of methylation compensates, increasing at each addition of aspartate. Obviously there is a limit to how much methylation can be imposed since there is only a finite amount of receptor complex and eventually the system will saturate and perfect adaptation will no longer be possible.

### *5.1.1. Chemotaxis as an analog computer*

One of the most intriguing ideas to come out of the study of *E. coli* chemotaxis is the possibility that the methylation of the receptor is effectively acting as a numerical integrator.

When the aspartate level changes we note that CheA-P also changes. This change in CheA-P is recorded by the methylation of the receptor. The level of methylation is effectively the integral of CheA-P. The change in methylation is used in turn to modulate the level of CheA-P back to its pre-stimulus level. If we consider this change in CheA-P be a measure of the error from the pre-stimulus level, then from a control engineering perspective, what we have is termed an integral controller. That is, a control loop which, rather than feeding back the absolute error, feeds back the integral of the error (via the methylation level). Such controllers have a very special property,

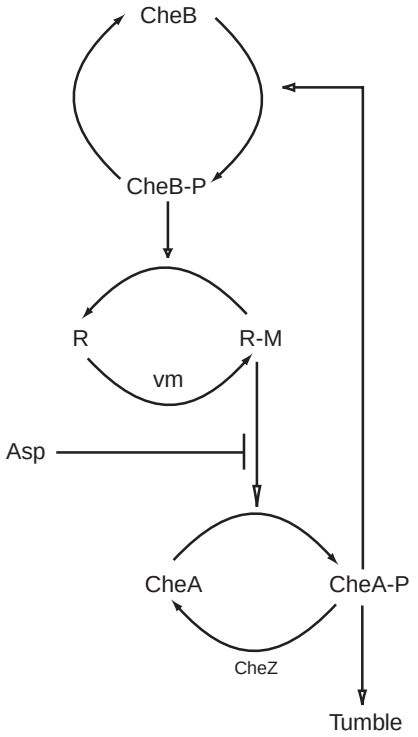


Fig. 19. Chemotaxis pathway. CheA-P and CheB-P are phorphorylated forms. R is receptor and R-M is methylated receptor.

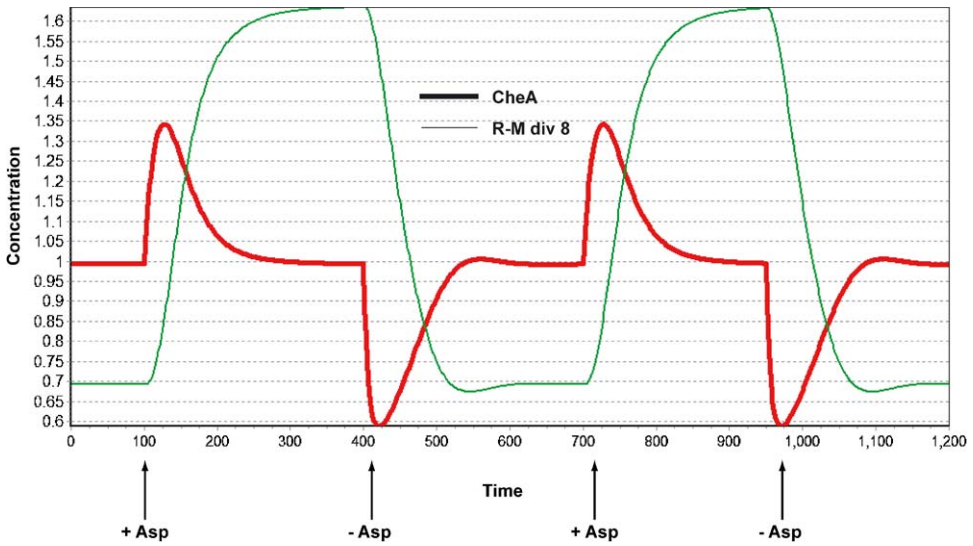


Fig. 20. Chemotaxis pathway simulation showing how CheA and methylation change and adapt when the level of attractant (Asp) is increased then decreased repeatedly. Note that R-M has been scaled by one-eighth of its actual simulation value and CheA has been scale upwards eight times and translated by five units.

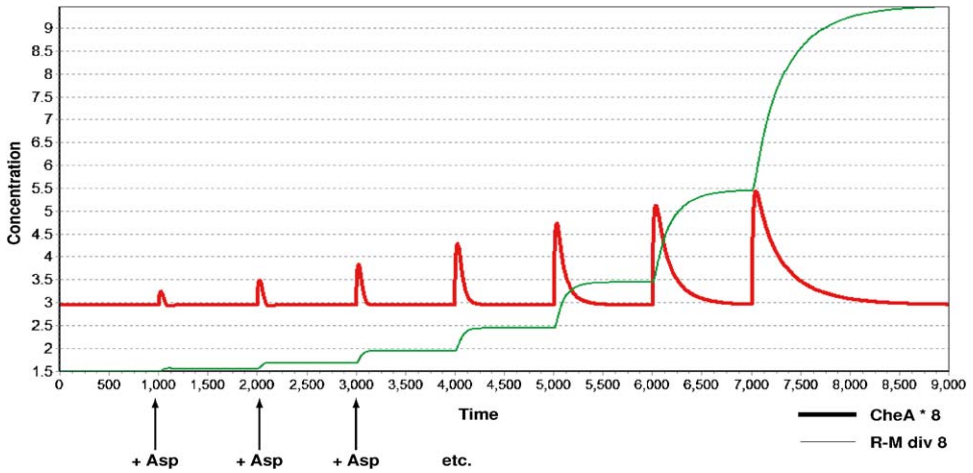


Fig. 21. Chemotaxis pathway simulation showing adaptation in CheA-P as the level of attractant (Asp) is increased. The graph also shows the effect on the methylation level which increases at each stimulus as the systems attempts (successfully) to adapt to the new conditions. Note how the system finds it more and more difficult to adapt (longer and higher transient times in CheA) as the attractant level increases, this is due to the limit on the amount of methylation that can be achieved. Eventually, the system will fail to adapt once all the receptors are fully methylated.

any disturbance is removed completely when they operate. This is in contrast to a proportional controller which feeds back the absolute error and is never able to restore a disturbance completely which results in a so-called off-set error. Yi et al. (2000) argued very convincingly that the chemotaxis circuit functions around an integral controller, without which perfect adaptation could never be achieved.

We thus have what is probably the first recorded instance of a cellular analog computer. It would not be surprising if we were to find many other forms of analog computation taking place in other signaling networks. It is interesting to note that the chemotaxis integrator used a protein with multiple sites for covalent modification so that the change in CheA-P could be recorded with some resolution. This might suggest that when we find other proteins which have multiple sites for modification, it might be indicative for the presence of an integral controller or at least it might suggest that some form of integration is taking place.

### 5.2. *MAPK pathway*

As discussed in the introduction, MAPK cascades are common components of extracellular signal-regulated pathways. Experimental results suggest that these cascades are highly conserved (Marshall, 1994) across a wide spectrum of organisms. In addition, the basic architecture of the pathway in each MAPK family is essentially the same. This implies that the functional roles played by each family is the same, or very similar. The basic architecture involves three protein kinases (See Fig. 22), each of which activates the kinase below only after being phosphorylated from above.

Recent studies have indicated the presence of one or more negative feedback loops surrounding the MAPK cascade (Kholodenko, 2000; Bhalla, 2003b; Asthagiri and Lauffenburger, 2001)—a

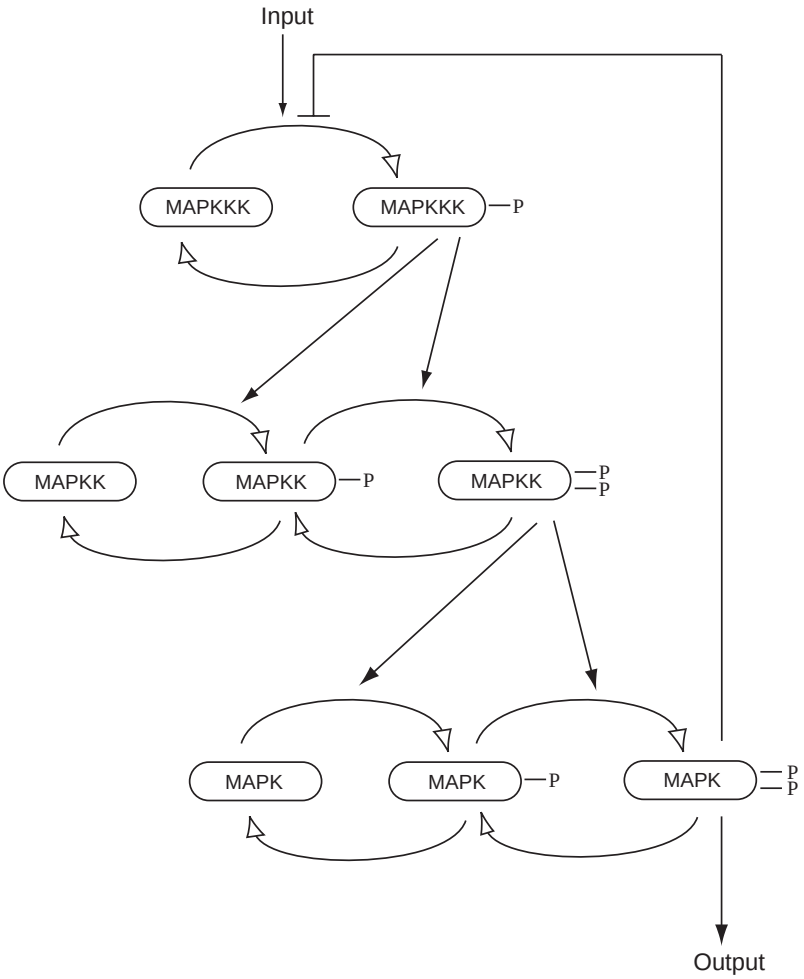


Fig. 22. Schematic of a generic MAPK pathway.

number of these studies center on the differential response of the p12 cell line's Ras/Ref/MEK/ERK MAPK pathway to stimulation by EGF and NGF. Several roles have been suggested for this putative negative feedback including enhanced deactivation (Brightman and Fell, 2000), adaptation to persistent signaling (Asthagiri and Lauffenburger, 2000), production of oscillations (Kholodenko, 2000), and flexibility of pathway design (Bhalla, 2003b). However, given the previous discussion on the properties of negative feedback, an attractive hypothesis is that the negative feedback loop, coupled with the high gain of the MAPK cascade is acting as a classic feedback amplifier.

Most of the previous studies on cascade cycles have centered on the compelling suggestion that cascades provide a switch-like response (Chock and Stadtman, 1977a; Goldbeter and Koshland, 1984). During a switching action, the concentration of activated kinase at the end of the cascade is switched from its basal, nonstimulated, level to saturation (i.e. 100% activation), as the ligand

level crosses a threshold value (Huang and Ferrell, 1996). This behavior is often described by the analogy to a cooperative enzyme—the cascade acts as a single enzyme with a large Hill coefficient. Along with its function as a switch, the cascades also appear to amplify ligand level, since the pool of MAPK typically has a concentration orders of magnitude higher than the ligand levels which activate the response. All amplifiers, man-made or natural, necessarily saturate above and below certain input levels. They are designed so that their 'active range' covers the levels of input of interest. When this active range is small, the result is switch-like behavior. Although many researches favor the switching hypothesis as an explanation for MAPK behavior, others have suggested a graded response as an alternative hypothesis. Hazzalin and Mahadevan (2002) have discussed at length the case for a graded response over a switch-like response. They provide evidence for the graded regulation of immediate-early genes in cell culture which are controlled via MAPK pathways. Furthermore, there is now clear evidence to support graded responses in the yeast mating pheromone pathway (Poritz et al., 2001; Ferrell, 2002) which is again based on an MAPK cascade.

One of the most compelling sets of data to support a graded response is that provided by the work of Bhalla et al. (2002). They discovered that under certain conditions, mouse NIH-3T3 fibroblasts, in response to platelet-derived growth factor (PDGF), showed a proportional response over a 10-fold concentration range of growth factor (PDGF). They reproduced this effect in a computational model, and showed both experimentally and computationally that an increase in a negative feedback modulator, MKP (MAPK phosphatase) was sufficient to elicit the proportional response. Although this feedback only operates on one level of the three cascade structure, it still induces the property of linearization due to the negative feedback. The work of Bhalla et al. provides strong evidence that part of the PDGF cascade operates as a feedback amplifier.

The primary improvement achieved with feedback is increased *robustness* of the amplifier to perturbations (both to internal variables and to the output level) and a linearization of the input/output response. In the electronics industry this property is used extensively to permit the mass production of sophisticated, cheap but relatively low tolerance amplifier components. To improve the performance of the amplifiers, end-users employ high tolerance, cheap, passive resistor components to implement feedback and thus significantly improve the characteristics of the amplifier. The idea that a cascade with negative feedback might be acting as a feedback amplifier has been suggested previously by Savageau (1976) and more recently by Sauro (2001).

In its active range, the MAPK cascade would act solely as an amplifier—providing a faithful amplification of the input presented to it from the mechanisms upstream. To these upstream mechanisms (which we do not address) would then be relegated the all-important task of integrating the myriad extracellular signals received by the cell. The MAPK cascade is then a 'plug-and-play' component whose role is to amplify the results of those biochemical calculations to the point where they can produce a response in the cell. (This is an obvious strategy, again exemplified in electrical engineering. Analog computers perform their calculations on tiny currents and voltages then, when finished, amplify the results so that they may be useful. The same energy-conserving principle may be used in the cell). Only after the computation is complete and the appropriate response has been determined is it necessary to amplify concentrations to the point where they will affect the activity of the cell. Fig. 23 illustrates a computer simulation of an MAPK-type model with and without negative feedback. The effect of feedback is clearly seen in

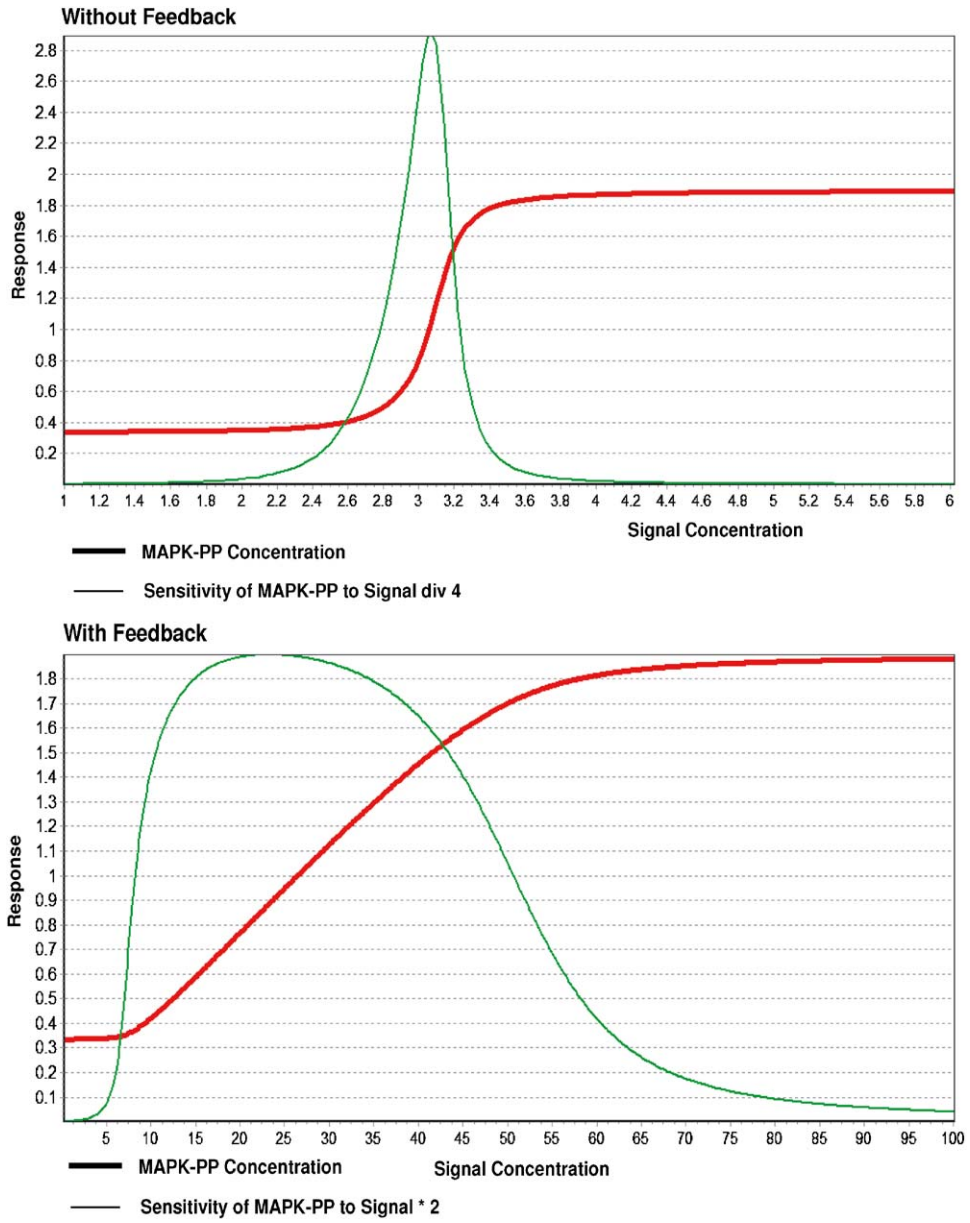


Fig. 23. Properties of an MAPK model with and without negative feedback. The response is measured as the change in MAPK-PP. The sensitivity curve measures the sensitivity of MAPK-PP to changes in the signal level.

the linearization of the response. In addition, the graphs also show the sensitivity of the system to changes in signal level, with feedback the sensitivity is roughly constant during the linear response.

One of the most attractive features of negative feedback is the effect it has on load disturbances. In relation to MAPK, this property is of particular interest because the last stage of MAPK, that is MAPK-PP (see Fig. 22) has to migrate to the nucleus in order to elicit a response. This diffusion

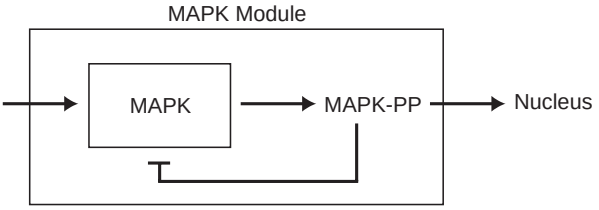


Fig. 24. Modularization as a result of feedback control.

is effectively a load on the MAPK circuit. Without feedback such a load would have a deleterious effect on the functioning of the MAPK pathway. As long as there is a pool of unphosphorylated MAPK, the feedback is able to compensate for this increased load. It is only when that pool dries up (as the amplifier is saturated) that the feedback is unable to provide any benefit (Fig. 24). As a result, feedback loops have the added property that they automatically modularize the network into functional units.

## 6. Conclusion

The engineering sciences, particularly electronic and control engineering are likely to have an ever increasing and pervasive impact on molecular biology. Not only will the concepts from control engineering give us new tools and concepts to understand cellular networks but they will also ultimately enable us to construct new networks with novel functionality—a discipline now called synthetic biology (Hasty et al., 2002; Hayes, 2001).

Signaling networks are probably the archetype system where more than anywhere there is a desperate need not only for quantification but for the infusion of ideas from control engineering. Much has been accomplished, including the development of ideas such as ultrasensitivity and the development of metabolic control analysis as a way of looking at the propagation of perturbations through a network. Initiatives such as the alliance for cellular signaling (http://www.cellularsignaling.org/) are specifically geared towards the quantification of signaling network dynamics.

In this short review, we have only covered some narrow aspects of signaling network analysis. In particular, we have not discussed any of the current dynamical models of signaling networks that have been published in recent years. In addition, there has been significant work carried out on the analysis of signaling networks using metabolic control analysis that is not discussed here (Heinrich and Schuster, 1996). In addition, the question of modeling and modeling tools has not been addressed, in particular the question of stochastic models versus deterministic models has not been mentioned at all.

Finally, we have not addressed one of the most acute problems in signaling networks, or in fact any cellular network analysis, which is knowledge pertaining to the structure of the network. One of the most frustrating aspects to understanding signaling networks is not knowing with certainty whether all the regulatory loops and reaction cycles have been accounted for. For example, whether the MAPK pathway (probably one of the most well-understood eukaryotic signaling

networks) possesses a negative feedback or not, what time scale does it operate on, etc are still open to debate. Clearly, the absence or presence of regulatory loops has a profound impact on the subsequent analysis. There thus need to be some systematic approaches to determine whether all interactions have been accounted for. In gene regulatory networks, there have been many attempts in trying to use gene microarray data to reverse engineer gene networks. However, aside from problems with the microarrays themselves, there is currently no theoretical justification at all that these approaches could ever work. More interesting is work carried out by Kholodenko (Kholodenko et al., 2002; Brazhnik et al., 2002). These authors have devised a method, based on sound theoretical arguments, for the determination of interactions in an arbitrary network given specific measurements, in particular specific perturbations. Kholodenko et al. in particular has indicated the minimum number of measurements required in order to fully determine a network's configuration. It is approaches like these that hopefully will help us determine, unambiguously the structure of signaling networks. Once the structure is known with a high degree if certainty, we can then begin the task of quantifying the dynamic response of the network. Coupling experimental work, with computation and theory should allow us to begin to unravel the logic behind the 'design' of signaling networks.

## Acknowledgements

We are very grateful to John Doyle, Hana El-Samad, Mustafa Khammash, Tau-Mu Yi and Chris Rao for useful and most simulating discussions. HMS acknowledges the support of the Japan Science and Technology Corporation's ERATO Kitano Systems Biology Project the Keck Graduate Institute and the DARPA BioComp Program: contract number MIPR 03-M296-01. Part of this work was carried out with the help of Brian Ingalls at the University of Waterloo. BNK acknowledges support from the NIH Grant GM59570.

## References

Alm, E., Arkin, A., 2003. Biological networks. Current Opin. Struct. Biol. 13, 193–202.

AMD, 2003. AMD corporation. URL: http://www.amd.com.

Arkin, A., Ross, J., 1994. Computational functions in biochemical reaction networks. Biophys. J. 67, 560–578.

Arkin, A., Ross, J., McAdams, H.H., 1998. Stochastic kinetic analysis of developmental pathway bifurcation in phage lambda-infected Escherichia coli cells. Genetics 149, 1633–1648.

Asthagiri, A.R., Lauffenburger, D.A., 2000. Bioengineering models of cell signaling. Annu. Rev. Biomed. Eng. 2, 31–53.

Asthagiri, A.R., Lauffenburger, D.A., 2001. A computational study of feedback effects on signal dynamics in a mitogen-activated protein kinase (mapk) pathway model. Biotechnol. Prog. 17, 227–239.

Bagowski, C.P., Besser, J., Frey, C.R., Ferrell, J.E., 2003. The jnk cascade as a biochemical switch in mammalian cells. Ultrasensitive and all-or-none responses. Curr. Biol. 13, 315–320.

Becskei, A., Serrano, L., 2000. Engineering stability in gene networks by autoregulation. Nature 405, 590–593.

Berg, H.C., Purcell, E.M., 1977. Physics of chemoreception. Biophys. J. 20, 193–219.

Bhalla, U.S., 2003a. Understanding complex signaling networks through models and metaphors. Prog. Biophys. Mol. Biol. 81, 45–65.

Bhalla, U.S., 2003b. Understanding complex signaling networks through models and metaphors. Prog. Biophys. Mol. Biol. 81, 45–65.

Bhalla, U., Ram, P., Iyengar, R., 2002. Map kinase phosphatase as a locus of flexibility in a mitogen-activated protein kinase signaling network. Science 297, 1018–1023.

Black, H.S., 1977. Inventing the negative feedback amplifier. IEEE Spectrum 14, 55–60.

Bluethgen, N., Herzel, H., 2001. Map-kinase-cascade: switch, amplifier or feedback controller. In: Gauges, R., van Gend, C. (Eds.), Second Workshop on Computation of Biochemical Pathways and Genetic Networks. Logos Verlag, Berlin, U.K., pp. 55–62.

Bolouri, H., Davidson, E.H., 2002. Modeling transcriptional regulatory networks. BioEssays. 24, 1118–1129.

Bourret, R.B., Stock, A.M., 2002. Molecular information processing: lessons from bacterial chemotaxis. J. Biol. Chem. 277, 9625–9628.

Bray, D., 1990. Intracellular signaling as a parallel distributed process. J. Theor. Biol. 143, 215–231.

Bray, D., 1995. Protein molecules as computational elements in living cells. Nature 376, 307–312.

Brazhnik, P., de la Fuente, A., Mendes, P., 2002. Gene networks: how to put the function in genomics. Trends Biotechnol. 20, 467–472.

Brightman, F.A., Fell, D.A., 2000. Differential feedback regulation of the mapk cascade underlies the quantitative differences in egf and ngf signaling in pc12 cells. FEBS Lett. 482, 169–174.

Brown, G., Hoek, J.B., Kholodenko, B.N., 1997a. Why do protein kinase cascades have more than one level? Trends Biochem. Sci. 22, 288–288.

Brown, G.C., Hoek, J.B., Kholodenko, B.N., 1997b. Why do protein kinase cascades have more than one level? Trends Biochem. Sci. 22, 288.

Bruggeman, F.J., Westerhoff, H.V., Hoek, J.B., Kholodenko, B.N., 2002. Modular response analysis of cellular regulatory networks. J. Theor. Biol. 218, 507–520.

Burns, J.A., 1971. Studies on complex enzyme systems. Ph.D. Thesis, University of Edinburgh. URL: http://www.cds.caltech.edu/hsauro/Burns/jimBurns.pdf.

Cárdenas, M.L., Cornish-Bowden, A., 1989. Characteristics necessary for an interconvertible enzyme cascade to generate a highly sensitive response to an effector. Biochem. J. 257, 339–345.

Chang, L., Karin, M., 2001. Mammalian map kinase signaling cascades. Nature 410, 37–40.

Chock, P.B., Stadtman, E.R., 1977a. Superiority of interconvertible enzyme cascades in metabolic regulation: analysis of monocyclic systems. Proc. Natl. Acad. Sci. USA 74, 2761–2765.

Chock, P.B., Stadtman, E.R., 1977b. Superiority of interconvertible enzyme cascades in metabolite regulation: analysis of multicyclic systems. Proc. Natl. Acad. Sci. USA 74, 2766–2770.

Cinquin, O., Demongeot, J., 2002. Roles of positive and negative feedback in biological systems. C.R. Biol. 325, 1085–1095.

Decroly, O., Goldbeter, A., 1982. Birhythmicity, chaos, and other patterns of temporal self-organization in a multiply regulated biochemical system. Proc. Natl. Acad. Sci. USA 79, 6917–6921.

Dibrov, B.F., Zhabotinsky, A.M., Kholodenko, B.N., 1982. Dynamic stability of steady states and static stabilization in unbranched metabolic pathways. J. Math. Biol. 15, 51–63.

Edelstein, B., 1970. A biochemical model with multiple steady states. J. Theor. Biol. 29, 57–62.

Elowitz, M.B., Leibler, S., 2000. A synthetic oscillatory network of transcriptional regulators. Nature 403, 335–338.

Eschrich, K., Schellenberger, W., Hofmann, E., 1990. A hysteretic cycle in glucose-6-phosphate metabolism observed in a cell-free yeast extract. Eur. J. Biochem. 188, 697–703.

Falke, J.J., Bass, R.B., Butler, S.L., Chervitz, S.A., Danielson, M.A., 1997. The two-component singaling pathway of batercial chemotaxis. Annu. Rev. Cell Dev. Biol. 13, 457–512.

Fell, D., 1997. Understanding the Control of Metabolism. Portland Press, London.

Fell, D., Small, J., 1986. Theoretical aspects of covalent modification in metabolic control. Biochem. Soc. Trans. 14, 623–624.

Fell, D.A., Sauro, H.M., 1985. Metabolic control analysis: additional relationships between elasticities and control coefficients. Eur. J. Biochem. 148, 555–561.

Ferrell, J.E., 1996. Tripping the switch fantastic: how a protein kinase cascade can convert graded inputs into switch-like outputs. Trends Biochem. Sci. 21, 460–466.

Ferrell, J.E., 1997. How responses get more switch-like as you move down a protein kinase cascade. Trends Biochem. Sci. 22, 288–289.

Ferrell, J.E., 2002. Self-perpetuating states in signal transduction: positive feedback, double-negative feedback and bistability. Curr. Opin. Cell Biol. 14, 140–148.
Ferrell, J.E.J., 1999. Building a cellular switch: more lessons from a good egg. BioEssays 21, 866–870.
Ferrell, J.E., Machleder, E.M., 1998. The biochemical basis of an all-or-none cell fate switch in xenopus oocytes. Science 280, 895–898.
Ford, B.J., 1985. Single Lens, The Story of the Simple Microscope. Harper & Row Publishers, New York.
Gardner, T.S., Collins, J.J., 2000. Neutralizing noise in gene networks. Nature 405, 520–521.
Gardner, T.S., Cantor, C.R., Collins, J.J., 2000. Construction of a genetic toggle switch in *Escherichia coli*. Nature 403, 339–342.
Goldbeter, A., Decroly, O., 1983. Temporal self-organization in biochemical systems: periodic behaviour vs. chaos. Am. J. Physiol. 245, R478–R483.
Goldbeter, A., Koshland, D.E., 1981. An amplified sensitivity arising from covalent modification in biological systems. Proc. Natl. Acad. Sci. 78, 6840–6844.
Goldbeter, A., Koshland, D.E., 1984. Ultrasensitivity in biochemical systems controlled by covalent modification. interplay between zero-order and multistep effects. J. Biol. Chem. 259, 14441–14447.
Gomperts, B.D., Mramer, I.M., Tatham, P.E.R., 2002. Signal Transduction. Academic Press, New York.
Gonze, D., Goldbeter, A., 2001. A model for a network of phosphorylation–dephosphorylation cycles displaying the dynamics of dominoes and clocks. J. Theor. Biol. 210, 167–186.
Grossman, A.D., 1995. Genetic networks controlling the initiation of sporulation and the development of genetic competence in *Bacillus subtilus*. Ann. Rev. Genet. 29, 477–508.
Hasty, J., McMillen, D., Isaacs, F., Collins, J.J., 2001. Computational studies of gene regulatory networks: in numero molecular biology. Nat. Rev. Genet. 2, 268–279.
Hasty, J., McMillen, D., Collins, J.J., 2002. Engineered Gene Circuits. Nature 420, 224–230.
Hayes, B., 2001. Computing omes to life. Am. Sci. 89, 204–208.
Hazzalin, C., Mahadevan, L.C., 2002. Mapk-regulated transcription: a continuously variable gene switch? Nat. Rev. Mol. Cell Biol. 3, 30–40.
Hecker, M., Volker, U., 2001. General stress response of *Bacillus subtilis* and other bacteria. Adv. Microb. Physiol. 44, 35–91.
Heinrich, R., Schuster, S., 1996. The Regulation of Cellular Systems. Chapman & Hall, London.
Hervagault, J.-F., Canu, S., 1987. Bistability and irreversible transitions in a simple substrate cycle. J. Theor. Biol. 127, 439–449.
Hervagault, J.-F., Cimino, A., 1989. Dynamic behaviours of an open substrate cycle: a graphical approach. J. Theor. Biol. 140, 399–416.
Hjelmfelt, A., Weinberger, E.D., Ross, J., 1991. Chemical implementation of neural networks and turing machines. Proc. Natl. Acad. Sci. 88, 10983–10987.
Hjelmfelt, A., Weinberger, E.D., Ross, J., 1992. Chemical implementation of finite-state machines. Proc. Natl. Acad. Sci. 89, 383–387.
Hofmeyr, J.-H., Westerhoff, H.V., 2001. Building the cellular puzzle: control in multi-level reaction networks. J. Theor. Biol. 208, 261–285.
Hofmeyr, J.-H.S., Kacser, H., van der Merwe, K.J., 1986. Metabolic control analysis of moiety-conserved cycles. Eur. J. Biochem. 155, 631–641.
Horowitz, P., Winfield, H., 1990. The Art of Electronics. Cambridge University Press, Cambridge.
Huang, C.F., Ferrell, J.E., 1996. Ultrasensitivity in the mitogen-activated protein kinase cascade. Proc. Natl. Acad. Sci. 93, 10078–10083.
Hunting, A., 1974. Limit-cycles in enzyme-systems with nonlinear negative feedback. Biophys. Struct. Mech. 1, 47–54.
Intel, 2003. Intel corporation. URL: http://www.intel.com.
Jung, W.G., 1986. IC Op-Amp Cookbook, 3rd Edition. Prentice-Hall PTR, Englewood Cliffs, NJ.
Kacser, H., Burns, J.A., 1973. The control of flux. In: Davies, D.D. (Ed.), Rate Control of Biological Processes, Symposium of the Society Exponential Biology, Vol. 27. Cambridge University Press, Cambridge, MA, pp. 65–104.
Kahn, D., Westerhoff, H.V., 1991. Control theory of regulatory cascades. J. Theor. Biol. 153, 255–285.

Kholodenko, B.N., 2000. Negative feedback and ultrasensitivity can bring about oscillations in the mitogen-activated protein kinase cascades. Eur. J. Biochem. 267, 1583–1588.

Kholodenko, B.N., Hoek, J.B., Westerhoff, H.V., Brown, G.C., 1997. Quantification of information transfer via cellular signal transduction pathways. FEBS Lett. 414, 430–434.

Kholodenko, B.N., Hoek, J., Westerhoff, H.W., Brown, G.C., 1998. Control analysis of cellular signal transduction pathways. In: Larsson, C., Pahlman, I.-L., Gustafsson, L. (Eds.), Proceedings of the Eigth International Meeting on BioThermoKinetics, BioThermoKinetics in the Post Genomic Era. Chalmers Reproservice, Göteborg, pp. 102–107.

Kholodenko, B.N., Demin, O.V., Moehren, G., Hoek, J.B., 1999. Quantification of short term signaling by the epidermal growth factor receptor. J. Biol. Chem. 274, 30169–30181.

Kholodenko, B.N., Kiyatkin, A., Bruggeman, F.J., Sontag, E., Westerhoff, H.V., Hoek, J.B., 2002. Untangling the wires: a strategy to trace functional interactions in signaling and gene networks. Proc. Natl. Acad. Sci. USA 99, 12841–12846.

Koza, III, J.R., F.H.B., Bennett, H.F., Andre, D., 1999. Genetic Programming III: Automatic Programming and Automatic Circuit Synthesis. Morgan Kaufmann, Los Altos, LA.

Koza, J.R., Keane, M.A., Streeter, M.J., 2003. Evolving inventions. Sci. Am. 288, 52–59.

Kyriakis, J.M., Avruch, J., 2002. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflamation. Physiol. Rev. 81, 807–869.

Lancaster, D., Lancaster, D., 1980. TTL Cookbook. Sams.

Laurent, M., Kellershohn, N., 1999. Multistability: a major means of differentiation and evolution in biological systems. Trends Biochem. Sci. 24, 418–422.

Levine, S.M., 1966. Enzyme amplifier kinetics. Science 152, 651–653.

Lisman, J.E., 1985. A mechanism for memory storage insensitive to molecular turnover: a bistable autophosphorylating kinase. Proc. Natl. Acad. Sci. 82, 3055–3057.

Loew, L.M., Schaff, J.C., 2001. The virtual cell: a software environment for computational cell biology. Trends Biotechnol. 19, 401–406.

Manning, G., Whyte, D.B., Martinez, R., Hunter, T., Sudarsanam, S., 2000. The protein kinase complement of the human genome. Science 298, 1912–1934.

Manning, G., Plowman, G.D., Hunter, T., Sudarsanam, S., 2002. Evolution of protein kinase signaling from yeast to man. Trends Biochem. Sci. 27, 514–520.

Markevich, N.I., Hoek, J.B., Kholodenko, B.N., 2004. Signaling switches and bistability arising from multisite phosphorylation in protein kinase cascades. J. Cell Biol. 164, 353–359.

Marshall, C.J., 1994. Map kinase kinase kinase, map kinase kinase and map kinase. Curr. Opin. Genet. Dev. 4, 82–89.

McAdams, H.H., Arkin, A., 1998. Simulation of prokaryotic genetic circuits. Annu. Rev. Biophys. Biomol. Struct. 27, 199–224.

Mendes, P., 1993. Gepasi: a software package for modelling the dynamics, steady states and control of biochemical and other systems. Comput. Appl. Biosci. 9, 563–571.

Mindell, D., 2000. Opening black's box. Technol. Culture 14, 405–434.

Moehren, G., Markevich, N., Demin, O., Kiyatkin, A., Goryanin, I., Hoek, J.B., Kholodenko, B.N., 2002. Temperature dependence of the epidermal growth factor receptor signaling network can be accounted for by a kinetic model. Biochemistry 41, 306–320.

Morales, M., McKay, D., 1967. Biochemical oscillations in controlled systems. Biophys. J. 7, 621–625.

Okamoto, M., Katsurayama, A., Tsukiji, M., Aso, Y., Hayashi, K., 1980. Dynamic behavior of enzymatic system realizing two-factor model. J. Theor. Biol. 83, 1–16.

Okamoto, M., Sakai, T., Hayashi, K., 1987. Switching mechanisms of a cyclic enzyme system: role as a "chemical diode". Biosystems 21, 1–11.

Okamoto, M., Sakai, T., Hayashi, K., 1988. Biochemical switching device—monocyclic enzyme-system. Biotechnol. Bioeng. 32, 527–537.

Okamoto, M., Sakai, T., Hayashi, K., 1989. Biochemical switching device—how to turn on (off) the switch. Biosystems. 22, 155–162.

Othmer, H.H., 1976. The quantitative dynamics of a class of biochemical control circuits. J. Math. Biol. 37, 53–78.

Pomerening, J.R., Sontag, E.D., Ferrell, J.E., 2003. Building a cell cycle oscillator: hysteresis and bistability in the activation of cdc2. Nat. Cell. Biol. 5, 346–351.

Poritz, M.A., Malmstrom, S., Kim, M.-H., Rossmeissl, P.J., Kamb, A., 2001. Graded mode of transcriptional induction in yeast pheromone signaling revealed by single-cell analysis. Yeast 18, 1331–1338.

Rao, C.V., Arkin, A., 2001. Control motifs for intracellular regulatory networks. Annu. Rev. Biomed. Eng. 3, 391–419.

Ravasz, E., Somera, A.L., Mongru, D.A., Oltvai, Z.N., Barabasi, A.-L., 2002. Hierarchical organization of modularity in metabolic networks. Science 297, 1551–1555.

Rohwer, J.M., Schuster, S., Westerhoff, H.V., 1996. How to recognize monofunctional units in a metabolic system. J. Theor. Biol. 179, 213–228.

Ryan, K.R., Shapiro, L., 2003. Temporal and spatial regulation in prokaryotic cell cycle progression and development. Annu. Rev. Biochem. 72, 367–394.

Sauro, H.M., 1993. A biochemical nand gate and assorted circuits. In: Schuster, S., Rigoulet, M., Ouhabi, R., Mazat, J.-P. (Eds.), Modern Trends in Biothermokinetics. Plenum Press, New York, London, pp. 133–140.

Sauro, H.M., 2000. Jarnac: a system for interactive metabolic analysis. In: Hofmeyr, J.-H.S., Rohwer, J.M., Snoep, J.L. (Eds.), Animating the Cellular Map: Proceedings of the Ninth International Meeting on BioThermoKinetics, Stellenbosch University Press.

Sauro, H.M., 2001. A rationale for the 'design' of the MAP kinase pathway. In: Yi, T.-M., Hucka, M.(Eds.), Second International Conference on Systems Biology. Caltech, Pasadena, pp. 13; http://www.sys–bio.org.

Sauro, H.M., Fell, D.A., 1991. Scamp: a metabolic simulator and control analysis program. Math. Comput. Model. 15, 15–28.

Sauro, H.M., Hucka, M., Finney, A., Wellock, C., Bolouri, H., Doyle, J., Kitano, H., 2003. Next generation simulation tools: the systems biology workbench and biospice integration. OMICS 7 (4), 355–372.

Savageau, M.A., 1972. The behaviour of intact biochemical control systems. Curr. Topics Cell. Reg. 6, 63–130.

Savageau, M.A., 1974. Optimal design of feedback control by inhibition: steady-state considerations. J. Mol. Evol. 4, 139–156.

Savageau, M.A., 1976. Biochemical Systems Analysis: A Study of Function and Design in Molecular Biology. Addison-Wesley, Reading, MA.

Schoeberl, B., Eichler-Jonsson, C., Gilles, E.D., Muller, G., 2002. Computational modeling of the dynamics of the map kinase cascade activated by surface and internalized egf receptors. Nat. Biotechnol. 20, 370–375.

Segall, J.E., Block, S.M., Berg, H.C., 1986. Temporal comparisons in bacterial chemotaxis. Proc. Natl. Acad. Sci. USA 83, 8987–8991.

Seno, M., Iwamoto, K., Sawada, K., 1978. Instability and oscillatory behavior of membrane-chemical reaction systems. J. Theor. Biol. 72, 577–588.

Sha, W., Moore, J., Chen, K., Lassaletta, A.D., Yi, C.-S., Tyson, J.J., Sible, J.C., 2003. From the cover: hysteresis drives cell-cycle transitions in *Xenopus laevis* egg extracts. Proc. Natl. Acad. Sci. USA 100, 975–980.

Shapiro, B.E., Levchenko, A., Meyerowitz, E.M., Wold, B.J., Mjolsness, E.D., 2003. Cellerator: extending a computer algebra system to include biochemical arrows for signal transduction simulations. Bioinformatics 19, 677–678.

Shen-Orr, S.S., Milo, R., Mangan, S., Alon, U., 2002. Network motifs in the transcriptional regulation network of *Escherichia coli*. Nat. Genet. 31, 64–68.

Shvartsman, S.Y., Muratov, C.B., Lauffenburger, D.A., 2002. Modeling and computational analysis of egf receptor-mediated cell communication in drosophila oogenesis. Development 129, 2577–2589.

Small, J.R., 1988. Theoretical aspects of metabolic control. Ph.D. Thesis, Oxford Polytechnic.

Small, J.R., Fell, D.A., 1990. Covalent modification and metabolic control analysis: modification to the theorems and their application to metabolic systems containing covalently-modified enzymes. Eur. J. Biochem. 191, 405–411.

Smolen, P., Baxter, D.A., Byrne, J.H., 2000. Modeling transcriptional control in gene networks–methods, recent results, and future directions. Bull. Math. Biol. 62, 247–292.

Solée, R.V., Cancho, R.F., Valverde, S., Montoya, J.M., 2002. Selection, tinkering, and emergence in complex networks. Complexity 8, 20–33.

Soroka, W.W., 1954. Analog Methods in Computation and Simulation. McGraw-Hill Book Company Inc., New York.

Thar, R., Kuhl, M., 2003. Bacteria are not too small for spatial sensing of chemical gradients: an experimental evidence. Proc. Acad. Natl. Sci. 100, 5748–5753.

The-Arabidopsis-Initiative, 2000. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408, 796–815.

Thron, C.D., 1996. A model for a bistable biochemical trigger of mitosis. Biophys. Chem. 57, 239–251.

Tomita, M., Hashimoto, K., Takahashi, K., Shimizu, T.S., Matsuzaki, Y., Miyoshi F, S.K., Tanida, S., Yugi, K., Venter, J.C., Hutchison, C.A., 1999. E-cell: software environment for whole-cell simulation. Bioinformatics 15, 72–84.

Tyson, J., Othmer, H.G., 1978. The dynamics of feedback control circuits in biochemical pathways. In: Rosen, R., Snell, F.M. (Eds.), Progress in Theoretical Biology, Vol. 5, pp. 1–62.

Tyson, J.J., Chen, K., Novak, B., 2001. Network dynamics and cell physiology. Nat. Rev. Mol. Cell Biol. 2, 908–916.

Tyson, J.J., Csikasz-Nagy, A., Novak, B., 2002. The dynamics of cell cycle regulation. BioEssays 24, 1095–1109.

Tyson, J.J., Chen, K.C., Novak, B., 2003. Sniffers, buzzers, toggles and blinkers: dynamics of regulatory and signaling pathways in the cell. Curr. Opin. Cell Biol. 15, 221–231.

Umbarger, H.E., 1956. Evidence for a negative-feedback mechanism in the biosynthesis of leucine. Science 123, 848.

Wald, G., 1965. Visual excitation and blodd clotting. Science 150, 1028–1030.

West, A.H., Stock, A.M., 2001. Histidine kinases and response regulator proteins in two-compartment signaling systems. Trends Biochem. Sci. 26, 369–376.

Wiley, H.S., Shvartsman, S.Y., Lauffenburger, D.A., 2003. Computational modeling of the egf-receptor system: a paradigm for systems biology. Trends Cell. Biol. 13, 43–50.

Wolf, D.M., Arkin, A.P., 2002. Fifteen minutes of fim: control of type 1 pili expression in *E. coli.*. OMICS 6, 91–114.

Wolf, D.M., Arkin, A.P., 2003. Motifs, modules and games in bacteria. Curr. Opin. Microbiol. 6, 125–134.

Wong, Y.J., Ott, W.E., 1976. Function Circuits: Design and Applications. McGraw-Hill Book Company, New York.

Yates, R.A., Pardee, A.B., 1956. Control of pyrimidine biosynthesis in *Escherichia coli* by a feed-back mechanism. J. Biol. Chem. 221, 757–770.

Yi, T., Huang, Y., Simon, M.I., Doyle, J., 2000. Robust perfect adaptation in bacterial chemotaxis through integral feedback control. Proc. Natl. Acad. Sci. USA 97, 4649–4653.

Yildirim, N., Mackey, M.C., 2003. Feedback regulation in the lactose operon: a mathematical modeling study and comparison with experimental data. Biophys. J. 84, 2841–2851.