Chapter 1

THE IMPACT OF RETROACTIVITY ON THE BEHAVIOR OF BIO-MOLECULAR SYSTEMS

A Review of Recent Results

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Abstract Modularity is a powerful property for analyzing the behavior of a system on the basis of the behavior of its components. According to this property, any two components maintain their behavior unchanged upon interconnection. Is modularity a natural property of bio-molecular networks? In this review, we summarize recent theoretical and experimental results that demonstrate that the answer to this question is negative. Just as in many electrical, mechanical, and hydraulic systems, impedance-like effects, called retroactivity, arise at the interconnection of bio-molecular systems and alter the behavior of connected components. Here, we illustrate the effects of retroactivity on the static characteristics and on the dynamic input/output response of bio-molecular systems by employing a mixture of control theoretic tools, mathematical biology, and experimental techniques on reconstituted systems.

Keywords: Modularity, retroactivity, insulation, transcriptional networks, signaling cascades.

Introduction

A common approach to either designing or analyzing a complex system is to decompose it into smaller components, or modules, whose functions are well isolated by those of the neighboring components. This approach has been employed for long time in engineering disciplines, such as electrical engineering and computer science and, more recently, it has been proposed also for the analysis of bio-molecular systems [mod-

ules; alon-book; kirschner-gerhart]. Specifically, scientists have been advocating for the recognition of functional modules, which include signaling systems such as MAPK cascades and covalent modification cycles, machinery for protein synthesis, and DNA replication [lauffenburger-modules; asthagiri-lauffenburger2000]. However, whether modular organization is a general property of bio-molecular systems is still subject of debate. The need for understanding the extent of modularity in bio-molecular systems has become particularly pressing when designing synthetic circuits. In synthetic biology, in fact, a number of simple functional modules, such as oscillators, toggles, and inverters, are available, but connecting these "modules" together to engineer complex functionalities is still out of reach [WeissEMBO2006; Elowitz; Ninfa; Collins].

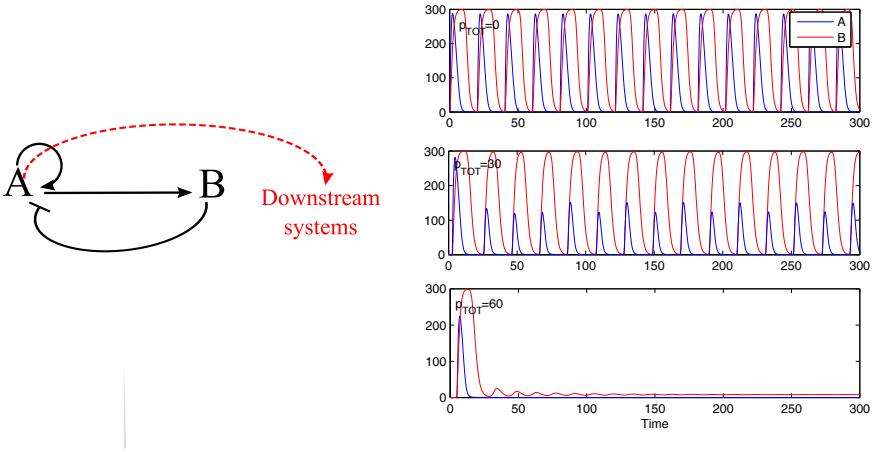

Figure 1.1. (Left) Diagram representing the activator-repressor clock of [Ninfa]. This clock is composed of two proteins, A and B, in which A activates its own production and the production of B through transcriptional activation, while B represses the production of A through transcriptional repression. The downstream systems represent transcriptional components that take protein A as an input. (Right) In the case in which A is taken as an input to downstream systems through the binding with DNA promoter sites in total amount p_{TOT}, the behavior of the clock changes and is disrupted for high enough load.

The fundamental assumption made when analyzing or designing a system modularly is that the behavior of each component does not change upon interconnection. However, as it occurs in several engineering systems such as electrical, mechanical, and hydraulic systems, this assump-

tion does not generally hold in biological systems. Upon interconnection, the behavior of an "upstream" component (the one that sends the signal) is affected by the presence of the "downstream" component (the one that receives the signal). Consider for example the oscillator of [Ninfa] as a source generator to be employed to synchronize a number of downstream transcriptional processes (Figure 1.1). The oscillator is connected to these downstream processes by having one of the proteins of the oscillator, say the activator A, serve as a transcription factor for the downstream systems by binding to promoter sites in amounts p_{TOT}. These downstream processes in turn act as a load on the oscillator by using up its output protein and by thus affecting its dynamics (right-side plot of Figure 1.1). We broadly call *retroactivity* the phenomenon by which the behavior of an upstream component changes upon connection to a downstream client.

These considerations strongly motivate the need for a novel theoretical framework to formally define and quantify retroactivity effects. In this chapter, we review a recently proposed framework for studying systems with retroactivity along with theoretical and experimental findings on the effects of retroactivity on bio-molecular systems [ddv-MSB; ddv-ACC2008; ddv-CDC2008; ddv-jayanthiCDC2009; ddv-PNAS-ss; ddv-DSCC2010]. We illustrate this framework through a simple transcriptional system example and we then review theoretical and experimental results on the effects of retroactivity on the steady state and dynamic response of a signaling system.

This chapter is organized as follows. In Section 1, we discuss the concept of retroactivity and its general modeling. In Section 2, we illustrate the modeling and quantification of retroactivity on a transcriptional system example. In Section 3, we describe in detail the static and dynamic effects of retroactivity in signaling systems along with experimental validation on a reconstituted system. Section 4 concludes the chapter with a short discussion.

1. Modeling Retroactivity

The principle of studying complex systems through decomposition and interconnection techniques is central in control theory. Approaches based on this general principle range from passivity and more generally dissipativity-based analysis [Wil72; Wil721; vidyasagar; MoyHill78; vds], to the derivation of stability properties of large interconnected systems from the graph-theoretic properties of interconnections and stability of individual systems [michel; siljak], to the use of backstepping feedback approaches [kkk; sepulchre] based on input to state stability

[04cime]. The work we describe here complements, but differs from, problems of optimally partitioning large networks into "modules" for which retroactivity-like effects are minimized, which typically employ graph theoretic and statistical approaches [papin-reed-palsson04; survey-graph-networks; WeissEMBO2006; Gilles2004; functional-modules-genes; kremling-saez-rodriguez07]. The contribution by Saez-Rodriguez et al. in this book focuses on these problems. In contrast, and similar to the work in [Sauro04], we are not concerned with network topology but with the understanding of dynamical behavior. Our ultimate goal is not top-down partitioning or to necessarily minimize retroactivity, but to formally define and characterize these effects especially in view of enabling modular assembly of synthetic bio-molecular networks.

The standard model, used in any control and systems theory mathematical and engineering textbook since the 1950s, e.g. [mct], is based on the view of devices described solely in terms of input channels, output channels, and state (internal, non-shared) variables. A notable exception to this standard model is found in the work of Willems [willems-book]. Willems has emphasized the fact that, for many physical situations, directionality of signals is an artificial, and technically wrong, assumption. While agreeing with this general point of view, we argue that, in certain circumstances such as those illustrated in this work, it is appropriate to distinguish between input and output channels. Thus, instead of blurring the distinction between inputs, states, and outputs, we keep these three distinct entities but augment the model with two additional signals, namely the retroactivities to inputs and to outputs, respectively (Figure 1.2).

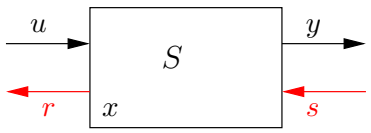

Figure 1.2. A system model S with retroactivity. The red signals originate from retroactivity upon interconnection.

Specifically, we add an additional input, called s to the system to model any change in its dynamics that may occur upon interconnection with a downstream system. Similarly, we add to a system a signal r as another output to model the fact that when such a system is connected downstream of another system, it sends upstream a signal that alters the dynamics of the upstream system. More generally, we define a system S to have internal state x, two types of inputs (I), and two types of

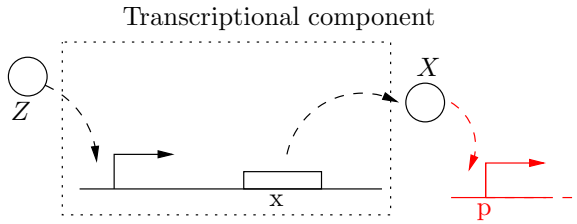

Figure 1.3. A transcriptional component takes as input u protein concentration Z and gives as output y protein concentration X.

outputs (O): an input "u" (I), an output "y" (O), a *retroactivity to the input* "r" (O), and a *retroactivity to the output* "s" (I) (Figure 1.2). We thus represent a system S by the equations

$$\dot{x} \quad = \quad f(x,u,s),\ y = Y(x,u),\ r = R(x,u), \tag{1.1}$$

in which f, Y, R are arbitrary functions and the signals x, u, s, r, y may be scalars or vectors. In such a formalism, we define the input/output model of the isolated system as the one in equations (1.1) without r in which we have also set $s = 0$. In practice, it is simpler to model the isolated system first, and only later model the interconnection mechanism to obtain model (1.1). Let S_i be a system with inputs u_i and s_i and with outputs y_i and r_i. Let S_1 and S_2 be two systems with disjoint sets of internal states. We define the interconnection of an upstream system S_1 with a downstream system S_2 by simply setting $y_1 = u_2$ and $s_1 = r_2$. For interconnecting two systems, we require that the two systems do not have internal states in common. For example, in the case of transcriptional components, this would mean that the two transcriptional components express different protein species; in the case of electrical circuits, this would mean that the two circuits do not share common electrical parts except for the ones that establish the interconnection mechanism.

2. Example: A Transcriptional System

Transcriptional networks are usually viewed as the input/output interconnection of transcriptional components, which take transcription factors as inputs and produce transcription factors as outputs [alon-book]. We showed in [ddv-MSB] that the behavior of a transcriptional component in isolation differs from that of the same component when connected in the network. Specifically, consider a transcriptional component whose output is connected to downstream processes (Figure 1.3). The activity of the promoter controlling gene x depends on the amount of Z bound to the promoter. For any species X, we denote by X (*italics*)

its concentration. If $Z = Z(t)$, such an activity changes with time. We denote it by $k(t)$. By neglecting the mRNA dynamics, which are not relevant to the current discussion, we can write the dynamics of X as

$$\frac{dX}{dt} = k(t) - \delta X, \tag{1.2}$$

in which δ is the decay rate of the protein. Equation (1.2) models the *isolated system* dynamics. Now, assume that X drives a downstream transcriptional system by binding to a promoter p with concentration p (1.3). The reversible binding reaction of X with p is given by X+p $\underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}}$ C, in which C is the complex protein-promoter and k_{on} and k_{off} are the binding and dissociation rates of the protein X to promoter site p. Since the promoter is not subject to decay, its total concentration p_{TOT} is conserved so that we can write $p + C = p_{TOT}$. Therefore, the new dynamics of X are governed by the equations

$$\begin{aligned} \frac{dX}{dt} &= k(t) - \delta X + \boxed{k_{off}C - k_{on}(p_{TOT} - C)X} \\ \frac{dC}{dt} &= -k_{off}C + k_{on}(p_{TOT} - C)X, \end{aligned} \tag{1.3}$$

in which $s = k_{off}C - k_{on}(p_{TOT} - C)X$ is the *retroactivity to the output*. Here, we can interpret s as being a "flow" between the upstream and the downstream system. Equations (1.3) model the *connected system* dynamics. When $s = 0$, the first of equations (1.3) reduces to the dynamics of the isolated system given in equation (1.2).

The effect of the retroactivity s on the behavior of X can be very large (Figure 1.4). This is undesirable in a number of situations in which we would like an upstream system to "drive" a downstream one as is the case, for example, when a biological oscillator has to time a number of downstream processes. We next focus on quantifying the retroactivity to the output s as function of measurable parameters (the quantification of r is similar).

Quantification of the retroactivity to the output

We quantify the difference between the dynamics of X in the isolated system (equation (1.2)) and the dynamics of X in the connected system (equations (1.3)) by establishing conditions on the biological parameters that make the two dynamics close to each other. This is achieved by exploiting the difference of time scales between the protein production and decay processes and the binding/unbinding process to promoter p [alon-book]. By virtue of this separation of time scales, we can approximate system (1.3) by a one dimensional system describing the evolution

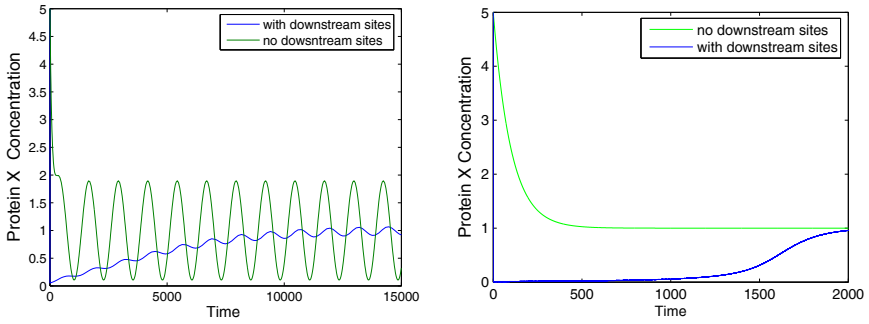

Figure 1.4. The dramatic effect of an interconnection. Simulation results for the system in equations (1.3). The green line represents $X(t)$ originating by equation (1.2), while the blue line represents $X(t)$ obtained by equations (1.3). Both transient and permanent behaviors are different. Here, $k(t) = 0.01(1 + sin(\omega t))$ with $\omega = 0.005$ in the left side plots and $\omega = 0$ in the right side plots, $k_{on} = 10$, $k_{off} = 10$, $\delta = 0.01$, $p_{TOT} = 100$, $X(0) = 5$. The choice of protein decay rate (in min^{-1}) corresponds to a half life of about one hour. The frequency of oscillations is chosen to have a period of about 12 times the protein half life in accordance to what is experimentally observed in the synthetic clock of [Ninfa].

of X on the slow manifold [kokotovic]. This reduced system takes the form $\frac{d\bar{X}}{dt} = k(t) - \delta\bar{X} + \bar{s}$, where $\bar{X}$ is an approximation of X and $\bar{s}$ is an approximation of s, which can be written as $\bar{s} = -\mathcal{R}(\bar{X})(k(t) - \delta\bar{X})$ with (see [ddv-MSB; ddv-ACC2008] for details)

$$\mathcal{R}(\bar{X}) = \frac{1}{1 + \frac{(1+\bar{X}/k_D)^2}{p_{TOT}/k_D}}, \tag{1.4}$$

in which $k_D = k_{off}/k_{on}$ is the dissociation constant. The expression $\mathcal{R}(\bar{X})$ quantifies the retroactivity to the output after a fast transient when $X(t) \approx \bar{X}(t)$. Retroactivity is thus low if the affinity of the binding sites p is small (k_D large) or if the signal $X(t)$ is large enough compared to p_{TOT}. Thus, the expression of $\mathcal{R}(\bar{X})$ provides an operative quantification of retroactivity as a function of the concentration of the binding sites p_{TOT}, the dissociation constant k_D, and the range of $\bar{X}(t)$, which are all directly measurable.

Retroactivity and Noise

It is well known that biological processes are intrinsically stochastic [Elowitz-noise; Thattai-PNAS; Paulsson-Nature2004]. Since retroactiv-

ity alters the dynamics of a bio-molecular system, it may also alter its noise properties. Here, we summarize some results that appeared in [ddv-jayanthiCDC2009] about the interplay between retroactivity and biological noise. One of the traditional metrics used to assess noise in many electrical engineering applications is the signal-to-noise ratio. This quantity is usually defined by taking the ratio between the power of the signal and the power of the noise. Specifically, consider periodic input signals of the form $k(t) = \bar{k} + \tilde{k}(t)$, in which $\bar{k}$ is a constant bias and $\tilde{k}(t) = A_0 sin(\omega t)$ is a periodic signal with amplitude $A_0 < \bar{k}$ and frequency ω. We assume that all the information transmitted is contained in the signal $\tilde{k}(t)$. To obtain a signal-to-noise figure of merit, the power of a signal is taken to be the square of its amplitude. The power of the noise is quantified by the steady-state variance calculated when the input is a constant and equals the bias, that is, $k(t) = \bar{k}$. Denoting A the amplitude of a signal and $\bar{\sigma}^2$ the steady-state variance, the signal-to-noise ratio is given by

$$SNR := \frac{A^2}{\bar{\sigma}^2}. \tag{1.5}$$

To calculate the value of $\bar{\sigma}^2$, we set $k(t) = \bar{k}$ and calculate the first and second order moments from the master equation by employing the linear noise approximation [vankampen; gardiner]. For calculating the amplitude A, we use the small signal approximation and calculate the frequency response (see [ddv-jayanthiCDC2009] for details of the derivations). This leads to the signal-to-noise ratio for X given by

$$SNR(\omega) = \frac{\Omega}{k\delta} \frac{1}{1 + \frac{\omega^2}{\delta^2}(1 + R_l)^2} A_0^2, \tag{1.6}$$

in which $R_l = \frac{k_D p_{TOT}}{(k/\delta + k_D)^2}$ and Ω is the volume. Expression (1.6) shows that for a signal with non-zero frequency retroactivity leads to a lower value of SNR. This is mainly due to the fact that while the amplitude of response decreases in the presence of retroactivity, the steady state variance does not depend on retroactivity. Notice that the higher the frequency, the more sensitive SNR is to retroactivity.

3. Retroactivity Effects in Signaling Systems

Cellular signaling systems cover a central role in a cell ability to respond to both internal and external input stimuli. These stimuli (often time-varying) include the transient presence of nutrients, hormonal and morphogenic signals, and the periodic excitation of cellular

clocks. Numerous signaling systems consist of cycles of protein covalent modification, such as phosphorylation, and in several cases multiple cycles of covalent modification are linked to form cascade systems [MAPK1; MAPK2]. The importance of these signaling systems has long been realized, and a wealth of theoretical work has established the potential behaviors of such systems and the mechanisms by which parameters and circuitry affect system behavior [Stadtman1977; Goldbeter; Goldbeter-2; Cardenas89]. These milestone works described how covalent modification cycles would behave in the absence of any loading caused by interconnection with downstream systems, that is, how the cycle would behave as an *isolated* signaling module. But, of course signaling systems are usually connected to the downstream targets they regulate. It is thus important to determine the effect of retroactivity by these targets on the static and dynamic response of the upstream system.

Here, we summarize the results of [ddv-PNAS-ss; ddv-DSCC2010], which explicitly quantify the effect of retroactivity on the shape of the input-output static response of a covalent modification cycle and on the frequency response.

Model

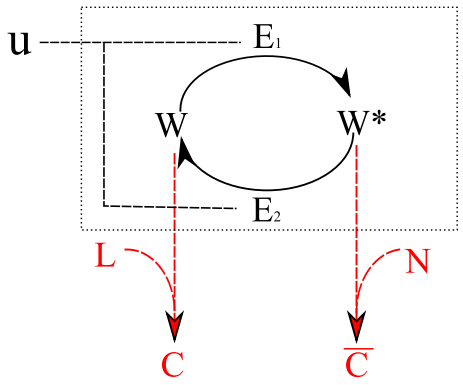

Figure 1.5. Covalent modification cycle subject to loading due to downstream target sites N and L for the active and inactive protein species, respectively.

Covalent modification cycles can be depicted according to the general scheme of Figure 1.5, in which a signaling protein is converted from its inactive form W to its active form W* by enzyme E_1 and back to its inactive form by enzyme E_2. The converting enzymes activities can be in turn controlled by an effector through allosteric modification [Fell].

Here, we have denoted the effector by u and have left unspecified in the diagram whether it is an activator or a repressor of enzyme activity. The results obtained here are independent of the details of enzyme modification and we will consider different cases to ease presentation. Usually, the active protein W* transmits the signal to downstream systems (for example, other signaling targets or DNA binding sites) by binding with appropriate targets [alon-book; Kholo2006-review; KoloPNAS]. However, some signal transduction systems display downstream targets both for the active and inactive protein [PII-2; PII-1; Ninfa2005]. Hence, we analyze both cases and consider downstream targets L for the inactive protein and downstream targets N for the active protein.

Let C_1 denote the complex of E_1 with W and C_2 be the complex of E_2 with W*. The standard two-step reaction model for the enzymatic reactions is given by

$$\mathrm{W} + \mathrm{E}_1 \underset{d_1}{\overset{a_1}{\rightleftharpoons}} \mathrm{C}_1 \xrightarrow{k_1} \mathrm{W}^* + \mathrm{E}_1 \text{ and } \mathrm{W}^* + \mathrm{E}_2 \underset{d_2}{\overset{a_2}{\rightleftharpoons}} \mathrm{C}_2 \xrightarrow{k_2} \mathrm{W} + \mathrm{E}_2,$$

to which we add the binding reaction of W with its downstream targets L in total amount L_T and the binding of W* with downstream targets N in total amounts N_T:

$$\mathrm{W} + \mathrm{L} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \mathrm{C} \text{ and } \mathrm{W}^* + \mathrm{N} \underset{\bar{k}_{off}}{\overset{\bar{k}_{on}}{\rightleftharpoons}} \bar{C}.$$

The kinetic equations governing the system are given by

$$\begin{aligned}
\frac{dW}{dt} &= -a_1 W E_1 + d_1 C_1 + k_2 C_2 - k_{on} N W + k_{off} C \\
\frac{dC_1}{dt} &= a_1 W E_1 - (d_1 + k_1) C_1 \\
\frac{dW^*}{dt} &= -a_2 W^* E_2 + d_2 C_2 + k_1 C_1 - \bar{k}_{on} N W^* + \bar{k}_{off} \bar{C} \\
\frac{dC_2}{dt} &= a_2 W^* E_2 - (d_2 + k_2) C_2 \\
\frac{dC}{dt} &= k_{on} L W - k_{off} C \\
\frac{d\bar{C}}{dt} &= \bar{k}_{on} N W^* - \bar{k}_{off} \bar{C}. \qquad (1.7)
\end{aligned}$$

To this differential equations, we add the algebraic equations expressing the conservation laws for the protein and the enzymes: $W_T = W + W^* + C_1 + C_2 + C + \bar{C}$, $E^*_{1T} = E_1 + C_1$, $E^*_{2T} = E_2 + C_2$, $N_T = N + \bar{C}$, $L_T = L + C$, in which we have denoted by E^*_{1T} and E^*_{2T} the total active enzyme

amounts. If we assume that the allosteric effector u acts, for example, as an absolute activator for E_2 and a non-competitive inhibitor for E_1, we have that $E^*_{1T} = \frac{E_{1T}}{1+u/k'_D}$ and $E^*_{2T} = \frac{E_{2T}u}{u+k'_D}$, in which k'_D and $\bar{k}'_D$ are the dissociation constants for the binding of u with E_1 and E_2, respectively, and E_{1T} and E_{2T} are the total amounts of enzymes [SysBio]. This specific choice of allosteric modification does not alter the results that follow and well represents the experimental system used to test these predictions.

Steady State Effects

In order to quantify the effect of retroactivity on the static input/output characteristics of the system, we solve system (1.7) for the steady state and determine the values of W^* and W as functions of the input u, and the amount of loads L_T and N_T. Letting $k_D := k_{off}/k_{on}$ and $\bar{k}_D := \bar{k}_{off}/\bar{k}_{on}$ and assuming that $k_D \gg W$ and that $\bar{k}_D \gg W^*$, the steady state value of C and $\bar{C}$ satisfy

$$C = \lambda W \text{ and } \bar{C} = \alpha W^*, \text{ with } \lambda = \frac{L_T}{k_D} \text{ and } \alpha = \frac{N_T}{\bar{k}_D}.$$

Note that in the case in which $\alpha = 0$, we have that $\bar{C} = 0$ and we obtain as a special case of our derivations the situation in which the load is applied only on W.

From the conservation law for W in which we have neglected the complexes C_1 and C_2 (in analogy to what is performed in [Goldbeter]), we obtain that

$$W_T = W(1+\lambda) + W^*(1+\alpha). \tag{1.8}$$

Further, from setting $\frac{dC_1}{dt} = 0$ and $\frac{dC_2}{dt} = 0$, we obtain

$$C_1 = \frac{E^*_{1T} w}{K_1 + w} \text{ and } C_2 = \frac{E^*_{2T} w^*}{K_2 + w^*},$$

in which we have employed the normalized quantities

$$w^* := \frac{W^*}{W_T},\ w := \frac{W}{W_T},\ K_1 := \frac{d_1 + k_1}{a_1 W_T},\ K_2 := \frac{d_2 + k_2}{d_2 W_T}.$$

From the equilibrium equation $k_1 C_1 = k_2 C_2$ and the conservation law $1 = w(1+\lambda) + w^*(1+\alpha)$ with

$$S := \frac{E^*_{2T} k_2}{E^*_{1T} k_1} \text{ and } \bar{w}^* = w^*(1+\alpha)$$

we obtain that $\bar{w}^*$ satisfies the equation

$$S = \frac{(1-\bar{w}^*)(K_2(1+\alpha) + \bar{w}^*)}{\bar{w}^*(K_1(1+\lambda) + 1 - \bar{w}^*)}, \tag{1.9}$$

in which, we have that S is monotonically increasing with the input u. We have chosen to study the effects of retroactivity on the steady state value of $\bar{w}^*$ as opposed to consider w^* because in the experimental system we will illustrate, only the total modified protein $W^* + \bar{C}$ can be measured.

From expression (1.9), it is apparent that the net effect of a load on the steady state response is to increase the "effective" normalized Michaelis-Menten constants K_1 and K_2 by factors of $(1+\lambda)$ and $(1+\alpha)$, respectively. It is well known, in turn, that the values of these constants establish the steepness of the steady state response of the cycle to the input stimulus S and that their relative values establish the point of half maximal induction [Goldbeter]. We next mathematically quantify the steepness, through the response coefficient, and the point of half maximal induction, called S_{50}.

Effect of retroactivity on response coefficient and S_{50} The steepness of the characteristics and the point of half maximal induction are physiologically relevant quantities in signaling systems as they determine how linear versus ultrasensitive, i.e., switch-like, the response to input stimuli is [Goldbeter; Goldbeter-2]. We thus mathematically define the steepness and the point of half maximal induction and analytically determine how they are affected by retroactivity.

Since $\bar{w}^*$ is a decreasing function of S, the response coefficient is defined as the ratio between the value of S corresponding to 10% of the maximal value of $\bar{w}^*$, denoted S_{10}, and the value of S corresponding to 90% of the maximal value of $\bar{w}^*$, denoted S_{90}, that is,

$$R := \frac{S_{10}}{S_{90}}.$$

For a Hill equation with Hill coefficient n_H, we have that

$$R = (81)^{1/n_H},$$

that is, R decreases as the Hill coefficient n_H increases. Therefore, we can also take R as a measure of the effective Hill coefficient of a steady state response.

The maximal value of $\bar{w}^*$ corresponds to when $w = 0$ and is obtained from $1 = w(1+\lambda) + w^*(1+\alpha)$ as $\bar{w}_{max} = 1$. As a consequence, we have that

$$R = \frac{S_{10}}{S_{90}} = \frac{81(K_2(1+\alpha)+0.1)(K_1(1+\lambda)+0.1)}{(K_1(1+\lambda)+0.9)(K_2(1+\alpha)+0.9)},$$

which is a monotonically increasing function of α and λ. As a consequence, independently of where the load is applied, the steepness of the

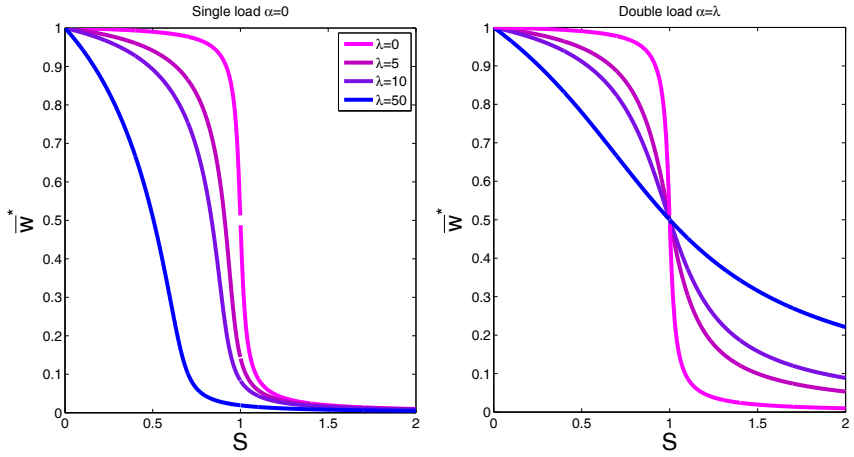

Figure 1.6. (Left) Effect of the load on the steady state response of $\bar{w}^*$ to S when the load is applied only to W, that is, $\alpha = 0$. (Right) Effect of the load on the steady state response of $\bar{w}^*$ to S when the load is applied to both W and W*.

response decreases. For the case of no load, i.e., $\alpha = \lambda = 0$, the expression of R reduces to the same expression obtained by [Goldbeter], while when both α and λ tend to infinity we have that $R = 81$, corresponding to Hill coefficient $n_H = 1$. That is, the response becomes hyperbolic (Michaelis-Menten type of response). In the case in which the load is applied only on W, that is, $\alpha = 0$, we obtain the same behavior for R. However, while with load applied on both W and W^* we have that R tends to 81 for large α and λ *independently* of the parameters K_1 and K_2, when the load is applied to W only, we have that $R = 81\frac{K_2+0.1}{K_2+0.9}$ for $\lambda \to \infty$, which depends on K_2 and tends to 81 only when K_2 is sufficiently large.

The expression of the half maximal induction point S_{50} is given by

$$S_{50} = \frac{(K_2(1+\alpha)+0.5)}{(K_1(1+\lambda)+0.5)},$$

which is an increasing function of α and a decreasing function of λ. In the case in which the load is applied only on W, that is, $\alpha = 0$, we obtain that S_{50} is a monotonically decreasing function of the load.

These results are summarized in Figure 1.6. With the load applied to W only, the effect of the load is mostly to shift the point of half maximal induction to the left. When the load is applied to both W

and W* in comparable amounts, the effect of the load is mostly on reducing the steepness of the response. Hence, retroactivity from large enough loads transforms an ultrasensitive response into a more graded Michaelis-Menten type response.

Finally, we directly study the behavior of the steady state value of $\bar{w}^*$ when α and λ are varied. We thus solve equation (1.9) for $\bar{w}^*$, obtaining as the only root between 0 and 1 the expression

$$\bar{w}^* = \frac{(1-\bar{K}_2 - S(\bar{K}_1+1)) + \sqrt{(1-\bar{K}_2 - S(\bar{K}_1+1))^2 + 4(1-S)\bar{K}_2}}{2(1-S)}, \tag{1.10}$$

in which we have denoted $\bar{K}_2 := K_2(1+\alpha)$ and $\bar{K}_1 := K_1(1+\lambda)$. By computing the derivative of this expression with respect to α and λ, we have that when the load is applied to W only the steady state always decreases with the load for all values of S. By contrast, when the load is applied on both active and inactive species, the effect of the load depends on the input stimulation. Specifically, the steady state increases for large input stimulations, while it decreases for small input stimulations. This is depicted in the left plot of Figure 1.6.

Dynamic Effects

To study the effects of retroactivity on the dynamics of the signaling system of Figure 1.5, we consider a one-step model for the enzymatic reactions as found, for example, in [Heinrich]. Also, we assume that u is an absolute activator for E_1, while it does not regulate the activity of E_2. This substantially simplifies the analysis without affecting the end result. In this model, we neglect the complexes formed between W and E_1 and between W* and E_2:

$$W + E_1 \xrightarrow{k_1} W^* + E_1 \text{ and } W^* + E_2 \xrightarrow{k_2} W + E_2.$$

Therefore, the new ODE model describing the covalent modification cycle is given by

$$\frac{dW^*}{dt} = k_1 \frac{E_{1T}u(t)}{k'_D + u(t)}(W_T - W^*) - k_2 E_{2T} W^*, \tag{1.11}$$

in which now $u(t)$ is a time-varying input for our study. We will refer to the ODE system model (1.11) as the *isolated system*. For shortening notation, we denote $V_1(t) := k_1 \frac{E_{1T}u(t)}{k'_D+u(t)}$ and $V_2 := k_2 E_{2T}$.

When the covalent modification cycle transmits its signal through W^* to the downstream system, we add to the isolated system model the reversible binding reaction of W* with downstream target sites denoted

p. These sites can either belong to a substrate that is modified by X_B through another covalent modification cycle as it occurs in the MAPK cascades [MAPK2; MAPK1], or they can belong to promoter regions on the DNA if W* is an active transcription factor [alon-book]. We model this additional binding reaction as $\mathrm{W}^* + \mathrm{p} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \mathrm{C}$, with $p + C = p_{TOT}$, in which C denotes the complex of W* with p. The conservation law for W thus modifies to $W + W^* + C = W_T$. The new ODE model describing the covalent modification system with its downstream system is thus given by

$$\begin{aligned} \frac{dW^*}{dt} &= k_1 \frac{E_{1T}u(t)}{k'_D + u(t)}(W_T - W^* - \boxed{\mathrm{C}}) - k_2 E_{2T} W^* \\ &\quad \boxed{-k_{on}W^*(p_{TOT} - C) + k_{off}C} \\ \frac{dC}{dt} &= k_{on}W^*(p_{TOT} - C) - k_{off}C, \end{aligned} \tag{1.12}$$

which we refer to as the *connected system*. Retroactivity enters the dynamics of the covalent modification cycle in two places indicated by the boxes. Specifically, the term in the small box causes an effect on the steady state response of the system, which we have analyzed in detail in the previous section, while the term in the large box does not have any effect on the steady state and it affects the dynamics only.

In order to precisely quantify how the dynamic response of the system is affected by retroactivity, we linearize the system about its steady state and compute the transfer function for both the isolated and connected systems. Linearization is a good approximation of the system dynamics for sufficiently small amplitudes of the input stimulus. A study on how large the amplitude of the input can be for maintaining a good approximation can be found in [Uribe].

Isolated system. For the isolated system, let $(\bar{u}, \bar{W}^*)$ be the equilibrium point and let $\tilde{u}(t) = u(t) - \bar{u}$ and $\tilde{W}^*(t) = W^*(t) - \bar{W}^*$ denote the variations about the equilibrium value. The linearized dynamics are given by

$$\dot{\tilde{W}}^* = \beta\tilde{u} - \alpha\tilde{W}^*, \tag{1.13}$$

in which we have defined

$$\beta := k_1(W_T - \bar{W}^*)\frac{E_{1T}k'_D}{(k'_D + \bar{u})^2}, \quad \alpha := \left(k_1 \frac{E_{1T}\bar{u}}{k'_D + \bar{u}} + k_2 E_{2T}\right). \tag{1.14}$$

Direct integration of system (1.13) starting from zero initial condition and with input $\tilde{u}(t) = 1$ leads to the time response to constant input

stimuli as

$$\tilde{W}^*(t) = \frac{\beta}{\alpha}(1 - e^{-\alpha t}). \tag{1.15}$$

The *response time*, that is, the time the signal takes to rise from 10% of its final value to 90% of its final value is equal to $t_{response} = 2/\alpha$. The transfer function from $\tilde{u}$ to $\tilde{W}^*$ is given by $T(s) = \frac{\beta}{s+\alpha}$, in which $T(s) := \tilde{W}^*(s)/\tilde{u}(s)$, so that amplitude and phase lag are given by

$$\begin{aligned} A(\omega) &= \sqrt{T(j\omega)T(-j\omega)} = \frac{\beta}{\sqrt{\omega^2 + \alpha^2}} \\ \phi(\omega) &= \arctan\left(\frac{\mathrm{Im}(T(j\omega))}{\mathrm{Re}(T(j\omega))}\right) = \arctan(-\omega/\alpha). \end{aligned} \tag{1.16}$$

The frequency *bandwidth*, corresponding to the value of ω such that $A(\omega) = \frac{1}{\sqrt{2}}A(0)$, is given by $\omega_{bandwidth} = \alpha$.

Connected system. For the connected system, let the equilibrium point be given by $(\bar{u}, \bar{W}_c^*, \bar{C})$ and the variations about this equilibrium be denoted by $\tilde{u}(t) = u(t) - \bar{u}$, $\tilde{W}^*(t) = W^* - \bar{W}_c^*$, and $\tilde{C}(t) = C(t) - \bar{C}$. The linearized system is thus given by

$$\begin{aligned} \dot{\tilde{W}}^* &= \bar{\beta}\tilde{u} - (\alpha + \gamma)\tilde{W}^* - (\sigma + \eta)\,\tilde{C} \\ \dot{\tilde{C}} &= \gamma\tilde{W}^* - \eta\tilde{C}, \end{aligned} \tag{1.17}$$

in which we have denoted

$$\bar{\beta} := k_1(W_T - \bar{W}_c^* - \bar{C})\frac{E_{1T}k'_D}{(k'_D + \bar{u})^2},\ \sigma := k_1\frac{E_{1T}\bar{u}}{k'_D + \bar{u}},$$

$$\gamma := k_{on}(p_{TOT} - \bar{C}),\ \ \eta := k_{on}\bar{W}_c^* + k_{off}.$$

The transfer function $T_c(s) := \tilde{W}^*(s)/\tilde{u}(s)$ is given by

$$T_c(s) = \frac{\bar{\beta}(s + \eta)}{s^2 + s(\eta + \alpha + \gamma) + \eta\alpha + \sigma\gamma}.$$

Exploiting the fact that the binding and unbinding process of a protein to binding sites is usually much faster than covalent modification reactions [Fell], we set $\eta = \bar{\eta}/\epsilon$ and $\gamma = \bar{\gamma}/\epsilon$, in which $\epsilon \ll 1$ and $\bar{\gamma}$ and $\bar{\eta}$ are of the same order as k_1 and k_2. By using the expressions of $\bar{\eta}$ and $\bar{\gamma}$ and setting $\epsilon = 0$, we obtain the reduced transfer function for the connected system as

$$T_c(s) = \frac{\bar{\beta}}{s(1 + \mu) + \alpha + \sigma\mu},\ \text{with}\ \mu = \frac{p_{TOT}k_D}{(\bar{W}_c^* + k_D)^2}.$$

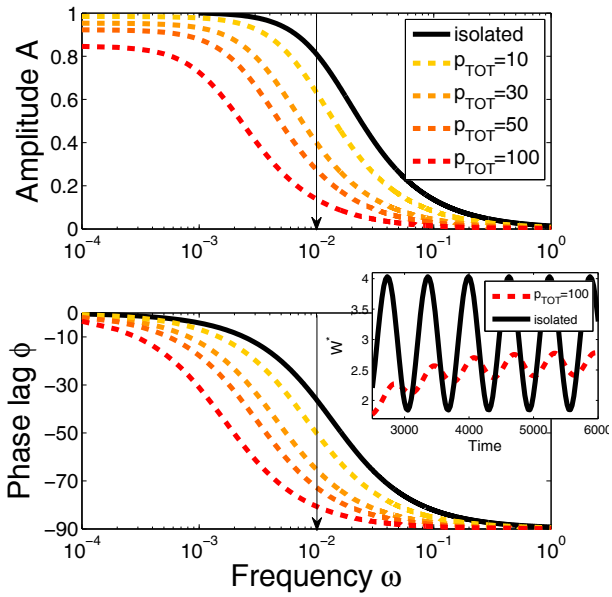

Figure 1.7. Effect of increasing the amount of p_{TOT} on the frequency response of the system. The parameters are $k_1 = k_2 = 0.01$, $E_{1T} = 0.075$, $W_T = 600$, $E_{2T} = 1.36$, $k'_D = 100$, $k_{on} = 50$, and $k_{off} = 50$. The small panel shows simulation results for the input frequency as indicated by the arrow in the left plots for the value $p_{TOT} = 100$.

Therefore, the response of $\tilde{W}^*$ to a constant input stimulus $\tilde{u}(t) = 1$ is given by (computing the inverse Laplace transform of $T_c(s)\frac{1}{s}$)

$$\tilde{W}^*(t) = \frac{\bar{\beta}}{\alpha + \sigma\mu}(1 - e^{-(\alpha+\sigma\mu)/(1+\mu)t}). \tag{1.18}$$

The response time is thus given by $t_{response,c} = \frac{2}{\alpha}\left(\frac{1+\mu}{1+\mu(\sigma/\alpha)}\right)$, which is larger than $t_{response}$ for the isolated system as $\sigma < \alpha$. Also, it is monotonically increasing with μ: for $\mu = 0$ it is equal to the response time of the isolated system while for $\mu \to \infty$ it tends to $2/\sigma$. In turn, μ monotonically increases with p_{TOT} and (for k_D sufficiently large) it also increases with $1/k_D$ (the affinity of W* to sites p). For values of k_D close to zero, the value of μ is not informative as the linear approximation does not hold. Furthermore, since $\bar{\beta} < \beta$ the amplitude of the response is also reduced for the connected system. The difference $\bar{\beta} - \beta$ is proportional to $\bar{C}$, so that the difference between the amplitude of the responses increases as p_{TOT} increases and/or k_D decreases. The amplitude and

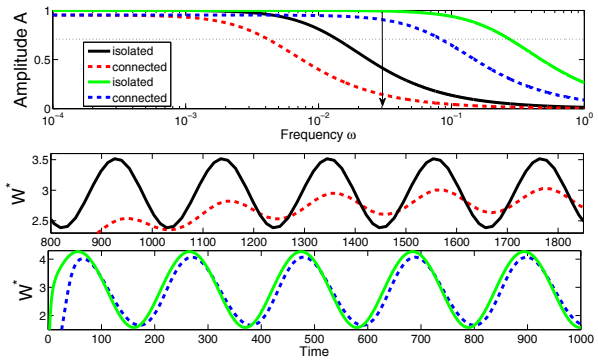

Figure 1.8. Increasing the values of the enzymes E_{1T} and E_{2T} increases the bandwidth of the covalent modification cycle. As a result, the response of the connected system becomes closer to the one of the isolated system. The solid black and dashed red plots correspond to the isolated and connected system behavior, respectively, while the solid green and dashed blue plots correspond to the isolated and connected system behavior, respectively, when the modification rates are increased by setting $E_{2T} = 30$ and $E_{1T} = 1.5$.

phase lag corresponding to $T_c(s)$ are given by

$$\begin{aligned} A_c(\omega) &= \sqrt{T_c(j\omega)T_c(-j\omega)} = \frac{\bar{\beta}}{\sqrt{\omega^2(1+\mu)^2 + (\alpha+\sigma\mu)^2}} \\ \phi_c(\omega) &= \arctan\left(\frac{\mathrm{Im}(T_c(j\omega))}{\mathrm{Re}(T_c(j\omega))}\right) = \arctan\left(\frac{-\omega(1+\mu)}{\alpha+\sigma\mu}\right), \end{aligned} \quad (1.19)$$

so that the bandwidth of the connected system is given by $\omega_{bandwidth,c} = \alpha\frac{1+\mu(\sigma/\alpha)}{1+\mu}$. Therefore, $\omega_{bandwidth,c} < \omega_{bandwidth}$, that is, the bandwidth of the connected system is strictly smaller than the bandwidth of the isolated system and the connected system displays a phase lag with respect to the isolated system. This is illustrated in Figure 1.7. We thus conclude that the larger the value of μ the larger the effect of retroactivity on the dynamical properties of the cycle, that is, the larger the response time, the phase lag, and the smaller the frequency bandwidth.

The bandwidth $\omega_{bandwidth,c}$ of the connected system can be increased by increasing α. One way to increase α is to equally (so not to alter the equilibrium of the system) increase the values of both E_{1T} and E_{2T}. The result is that the behavior of the connected system becomes closer to the one of the isolated system (Figure 1.8). In the limit in which $A_c(0) = A(0)$, the behavior of the connected system approaches the one of the isolated system when both E_{1T} and E_{2T} are increased. That is,

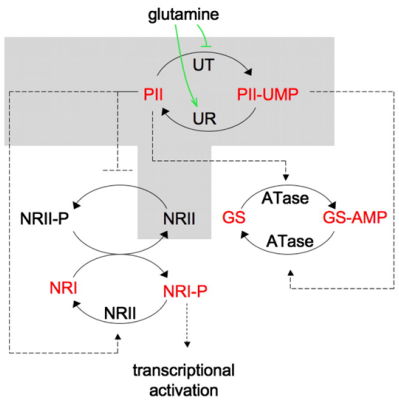

Figure 1.9. Experimental system employed in [ddv-PNAS-ss]. The part used in the experiments is highlighted in Grey. **[Permission Pending]**

the system becomes *insulated* from retroactivity. This is in accordance with the principle for insulation based on time-scale separation, according to which faster system time-scales contribute to better retroactivity attenuation [ddv-JayanthiTAC10]. Note that if $\bar{\beta}$ is much smaller than β, that is, $A_c(0) \ll A(0)$, the dominant effect of retroactivity is on the steady state. In fact, increasing the frequency of the input stimulation will not result in a dramatic decrease of the connected system response compared to the isolated system response as these two responses are apart from each other already at zero frequency.

Experimental Results

The prediction that retroactivity makes an ultrasensitive response into a graded one has been experimentally validated on a covalent modification cycle extracted from the nitrogen assimilation control system of *E. coli* and reconstituted *in vitro* [ddv-PNAS-ss]. Here, we briefly summarize these experimental results.

The instance of the covalent modification cycle of Figure 1.5 employed in the experiments is highlighted in Grey in Figure 1.9 [Ninfa2005; PII-0; Fell]. This system was reconstituted *in vitro* to allow well controlled experimental conditions. Referring to Figure 1.5, protein W is the PII signal transduction protein, protein W* is the active (uridylylated) protein PII-UMP, active enzyme E_1 is the UT activity of the UTase/UR bifunctional enzyme, while the active E_2 enzyme is the UR activity of the UTase/UR enzyme. The allosteric effector u is glutamine, which

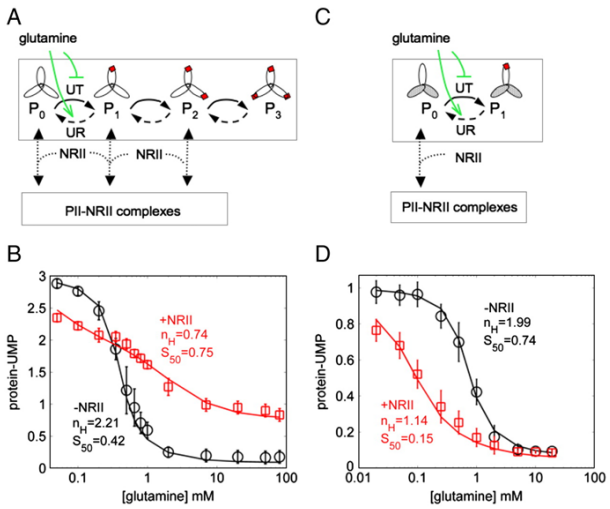

Figure 1.10. Experimental results from [ddv-PNAS-ss]. (Left) Using the trimeric PII protein. (Right) Using a monovalent version of the PII protein.

regulates both UT and UR activities by binding to a regulatory domain of the UTase/UR. The protein PII has one downstream signaling target, NRII.

The PII protein is a homotrimer, and can be uridylylated on each of its subunits (Figure 1.10(A)). Hence, comparing Figures 1.5 and Figure 1.10(A), we have that the modified protein W* comprises all of the modified forms of PII (P_1, P_2, and P_3 of Figure 1.10(A)). Also, partially modified forms of PII (P_1 and P_2, 1.10(A)) can bind to NRII. As a consequence, we have that the downstream targets L and N are the same and are given by the NRII protein. Thus, the use of the trimeric PII protein results in a cycle with "double load" as depicted in Figure 1.5, in which both the active and inactive protein species have downstream targets. In order to study the effects of applying the load on one side only of the cycle, which is a configuration often found in natural systems, we employed a monovalent version of the PII protein (Figure 1.10 (C)), which is obtained by proper mutation of two PII subunits.

Figure 1.10 illustrates how retroactivity makes an ultrasensitive input/output static response into a more graded response independently of where the load is applied. Also, it illustrates how the value of S_{50} decreases when the load is applied only on the inactive protein.

4. Discussion and Conclusion

In this work, we have summarized some recent results that illustrate how retroactivity impacts the behavior of bio-molecular systems. Retroactivity by downstream targets slows down the dynamic response by decreasing the effective bandwidth and reduces the sensitivity of the steady state input/output characteristics. These effects, which are more dramatic as the amounts and affinity of downstream targets increase, indicate that the behavior of a bio-molecular system cannot be understood in isolation. This is especially the case in signaling systems, in which covalent modification cycles have several downstream targets. What is the role of retroactivity in these systems? Signaling systems have been selected by nature for effective signal transduction. Hence, retroactivity must have a clear evolutionary advantage, or there must be insulation mechanisms to attenuate undesirable retroactivity effects. From a design point of view, the results summarized in this chapter indicate that retroactivity must be taken into account when engineering bio-molecular circuits and that suitable insulation mechanisms should be designed in order to buffer connected components from each other [ddv-MSB; ddv-CDC2008; ddv-JayanthiTAC10].

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