# Chapter 19

# Computational Analysis of Sphingolipid Pathway Systems

**Eberhard O. Voit,* Fernando Alvarez-Vasquez and Yusuf A. Hannun**

## Abstract

Sphingolipid metabolism constitutes a complex pathway system that includes biosynthesis of different types of sphingosines and ceramides, the formation and recycling of complex sphingolipids and the supply of materials for remodeling. Many of the metabolites have several roles, for instance, as substrates and as modulators of reactions in other parts of the system. The large number of sphingolipid compounds and the different types of nonlinear interactions among them render it difficult to predict responses of the sphingolipid pathway system to perturbations, unless one utilizes mathematical models. The sphingolipid pathway system not only invites modeling as a useful tool, it is also a very suitable test bed for developing detailed modeling techniques and analyses, due to several features. First, the reaction network is relatively well understood and many of the steps have been characterized, at least in vitro. Second, sphingolipid metabolism constitutes a relatively closed system, such that most reactions occur within the system rather than between the system and other pathways. Third, the basic structure of the pathway is conserved throughout evolution, but some of the details vary among different species. This degree of similarity permits comparative analyses and may one day elucidate the gradual evolution toward superior system designs. We discuss here some reasons that make sphingolipid modeling an appealing companion to experimental research and sketch out applications of sphingolipid models that are different from typical model uses.

## Introduction

Most sphingolipid analyses over the past decades have in great detail characterized individual metabolites, genes, or enzymes along with the reactions they catalyze (for example see refs. 1-7). As other chapters in this book attest, these studies have greatly improved our understanding of the components of sphingolipid metabolism and we have by now assembled a fairly good impression of the functionality of biosynthesis, metabolic conversions within the pathway and the ultimate fates of the various sphingolipid compounds. While the detailed characterizations of the genomic, metabolic and regulatory components are of undisputed importance, they do not paint a complete picture of how the integrated metabolic pathway system responds to environmental challenges, such as heat stress.

There are many reasons for why characterizing solely the parts of the sphingolipid pathway system is insufficient for a full understanding. The most obvious is the sheer number of components. In yeast there are roughly twenty to thirty "base" metabolites like sphingosine and phytoceramide.[8] However, many of these may exist in variant forms that differ in the lengths of their fatty acid chains, thereby multiplying the number of possibly relevant "players" several fold. In mammals, many sphingolipids may furthermore bind to various carbohydrates, forming glycosphingolipids

*Corresponding Author: Eberhard O. Voit—Integrative BioSystems Institute and Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology, Atlanta, Georgia. Email: eberhard.voit@bme.gatech.edu

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such as glucosylceramide and lactosylceramide, so that the number of all theoretically possible combinations reaches into the thousands (for example, see ref. 9). It is presently unknown whether every different combination of chain length and carbohydrate group plays a unique role for the functioning of the organism, but it is to be expected that there are at least distinctive differences between sphingolipids with long, as opposed to very long, fatty acid chains. As soon as large numbers of metabolites with subtle differences in their roles and functions are to be considered, the unaided human mind quickly becomes overwhelmed.

A second reason for failure in our intuitive predictive ability of global systems responses is the nonlinear nature of the interactions between the contributing sphingolipids. Specifically, the human mind has problems handling numerical thresholds: a slight increase in a metabolite may lead to a correspondingly slight change in some output measure or to no response at all, whereas a somewhat stronger increase has a distinctly different effect, such as the triggering of apoptosis. If several thresholds are in play, we simply can no longer make reliable predictions on responses. As a pertinent example, it is known that ceramide is a signaling compound associated with apoptosis, while sphingosine-1-phosphate (S1P) is associated with anti-apoptosis.[4,5,10] However, ceramide can easily be converted into S1P and vice versa. Thus, will slight changes in enzyme activities somewhere in the system ultimately lead to apoptosis or anti-apoptosis?

The sphingolipid pathway is usually shown as a more or less linear material flow system, or as a forward pathway with a few branches, beginning with the condensation of palmityl-CoA and serine and leading toward complex sphingolipids (CS), such as MIPC (for example see refs. 2,4,5,11,12). While these representations indeed capture the main flow, closer inspection reveals that there are numerous metabolic or regulatory feedback loops. For instance, dihydrosphingosine (DHS) and phytosphingosine (PHS) are key compounds at the center of the pathway. At the same time, they inhibit the production of phoshphatidylserine (PS), in a reaction that competes with serine palmitoyltransferase (SPT) for the same key substrate serine and of diacylglycerol (DAG), which is involved with the kinetics of complex sphingolipids. Palmitoyl-CoA is not only one of the inputs to biosynthesis, it is also produced in the lyase reaction with DHS-P and PHS-P. Thus, the uni-directionality of the sphingolipid pathway no longer holds as soon as these "loops" are taken into account. It may also happen that metabolites exert competing effects on some other part of the system, for instance, by serving simultaneously as input substrate and as a modulator of one of the catalytic steps in the system (e.g., see pathway diagrams in refs. 13, 14). These complexities render mathematical modeling a valuable tool with unique facilities that are difficult—if not impossible—to match with wet experiments.

A noteworthy feature of complex systems precluding reliable predictions is the fact that a pathway may exhibit new responses when it is put into its regulatory context. As an example, let us look at the simplest of pathways, a linear chain of reactions, which is here considered with one input and three intermediates (Fig. 1A). It does not require much imagination to predict what will happen if the input is raised to a higher level: the intermediates X, Y and Z will correspondingly change in concentration. Similarly, if the input is decreased, X, Y and Z will follow. We may not know how fast the adjustments will happen and what the final values of X, Y and Z might be, but we have a firm grasp of the qualitative response. Indeed, it is very easy to set up a mathematical model that captures the situation. While there are many options for such a model, the typical approach is a set of ordinary differential equations (ODEs), whose right-hand sides may be formulated as mass action kinetics, with Michaelis-Menten rate laws, as power-law functions, or with any number of other representations. For the present discussion, the particular mathematical format is essentially immaterial and we perform the illustration with a power-law model that is designed according to the guidelines of Biochemical Systems Theory (BST[15-19]). The result is shown in Eq. (1).

$$\begin{aligned} \dot{X} &= Input - k_1\, E\, X^{0.5} \\ \dot{Y} &= k_1\, E\, X^{0.5} - k_2\, Y^{0.5} \\ \dot{Z} &= k_2\, Y^{0.5} - k_3\, Z^{0.5} \end{aligned} \qquad (1)$$

In this formulation, X, Y and Z are generic representations of the metabolites. Their powers of 0.5 are typical in BST.[18] The parameters $k_1$, $k_2$ and $k_3$ are rate constants. E represents (the activity of) the enzyme that catalyzes the conversion of X into Y. We include it explicitly in these equations, because it will be the bridge to the extension following below. We could similarly include enzymes for the conversion of Y into Z and for the degradation of Z, but because their activities are assumed to be constant and because we do not explicitly use them in the following, we merge them with the rate constants $k_2$ and $k_3$, respectively. The left-hand sides of Eq. (1) are "dotted" variables that represent their change with respect to time; thus, for instance, $\dot{X} = dX/dt$.

To execute computational analyses, we need to specify parameter values. Because we only intend to illustrate the complications in predicting system responses, the parameters are chosen almost arbitrarily, but so that they are typical and that all variables have a nominal steady-state value of 1 if the Input has a magnitude of 1. Specifically, we set the rate constants $k_1$, $k_2$ and $k_3$ equal to 1.

For a baseline simulation, we start the system at the steady state (1, 1, 1). As expected, the system rests at this point and X, Y and Z do not change in value. Beginning at time $t = 10$, we reduce the input to 0.75 and, not surprisingly, the levels of X, Y and Z decrease. With the given settings, they approach the same value of 0.5625 (Fig. 2A). Doubling the input has the opposite effect: It raises the levels of X, Y and Z to 4 (results not shown). If we ignore the input in our discussion and just study X, Y and Z, we correctly conclude that changes in X cause corresponding changes in Y and Z.

Now suppose that Z is a sphingolipid with signaling function. Specifically, let us assume that it activates a transcription factor TF, which is responsible for the up-regulation of gene G, which codes for the enzyme catalyzing the conversion of X into Y (Fig. 1B). The former pathway is now embedded in a logic loop. This loop is easy to grasp in its organization, but its specific effects and the responses of the metabolic pathway are no longer easy to predict. Intuition may suggest that the positive feedback might magnify any changes in input, but it seems difficult to make specific predictions. For instance, how will changes in input affect the regulatory loop and the steady-state values of X, Y and Z? The answer may be surprising: with the given information alone, particular responses are not predictable with any reliability. It is again straightforward to set up BST equations capturing the situation. A possible implementation is shown in Eq. (2), which is an augmentation of the system in Eq. (1). The new parameters are again chosen so that all variables have "normal" values of 1 at the steady state. For simplicity of discussion, we will keep all parameters constant throughout the next set of simulations, except for the activation of TF. In other words, our "control parameter" is the strength of activation of TF by Z; it is coded here as $p$. The control parameter $p$ is always positive or 0, because a negative value would represent inhibition rather than activation. For the baseline case $p = 0$, Z has no influence on TF. Gene regulation is in effect decoupled from the metabolic pathway and the reaction between X and Y runs with the former baseline enzyme activity (E = 1); the situation is exactly the same as shown before in Figure 2A.

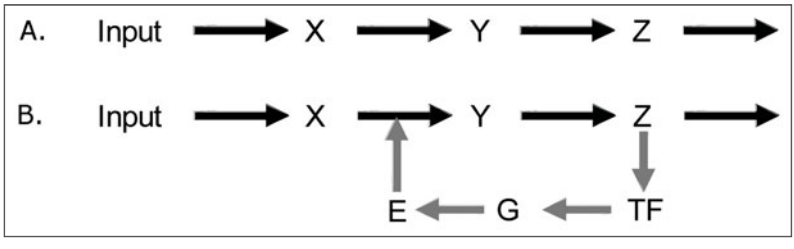


Figure 1. A) Simple linear pathway, in which an Input substrate is sequentially converted into metabolites X, Y and Z. Z is the substrate for another process or transported out of the system. B) Linear pathway from Figure 1A embedded in a "logic loop" consisting of a transcription factor TF, a gene G and an enzyme E. Grey arrows indicate activating effects. See Text for details.

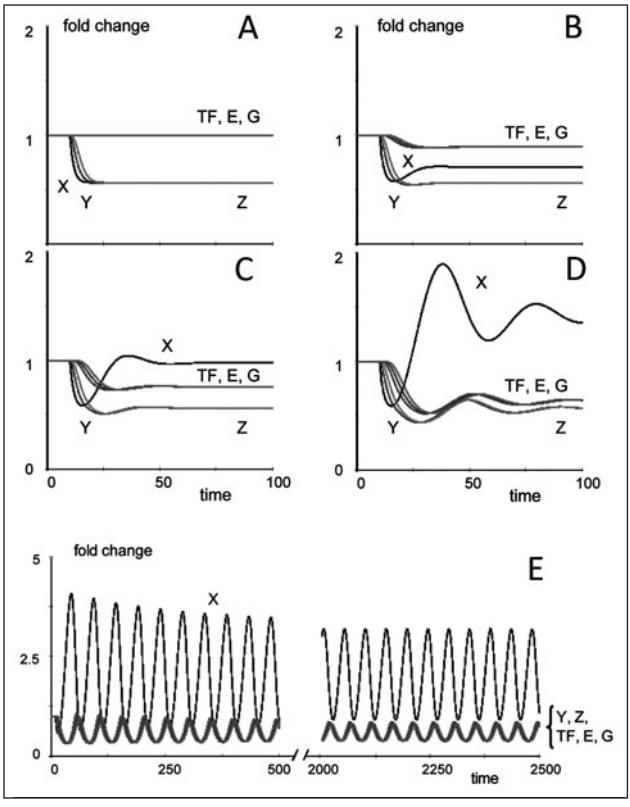


Figure 2. Simulation results for the pathways in Figures 1A,B and Equations (1) and (2). A) Response of the pathway in Figure 1A to a reduction of input to 75%. The situation is equivalent with Figure 1B if the control parameter is set as $p = 0$. B) Response of the pathway if $p = 0.1$. C) Response for $p = 0.24$. D) Response for $p = 0.4$. E) Limit cycle response for $p = 0.51$.

$$\begin{aligned} \dot{TF} &= Z^{P} - TF^{0.5} \\ \dot{G} &= TF^{0.5} - G^{0.5} \\ \dot{E} &= G^{0.5} - E^{0.5} \end{aligned} \tag{2}$$

If $p$ is greater than 0, Z activates TF, which subsequently leads to a change in the expression of gene G and a concordant alteration in enzyme activity E. One should expect the altered enzyme activity to change the balance between X, Y and Z. If so, Z would change in response to changes in X and Y and the change in Z would subsequently affect TF, G, E and, thus, indirectly the balance between X, Y and Z, leading to a cycle of events. Will the system spiral out of control? All the sudden, predictions become doubtful, thus demonstrating the necessity of a mathematical model.

In order to explore the effect of TF activation per simulation, let us set $p = 0.1$. The value is small in magnitude, which implies that the activation is not particularly strong. At the beginning of the experiment, the system is still in the steady state where each variable has a value of 1. As before we reduce the input to 75% at $t = 10$. Metabolite X again decreases initially but, in contrast to the unregulated system, "recovers" to some degree (Fig. 2B). If $p$ is set to a slightly increased value of 0.24, X actually returns close to its original value of 1. Y and Z assume the same values as before (Fig. 2C). Exploration of other values of $p$ show that small magnitudes in $p$ result in values for X that

are close to 0.5625, while larger values lead to higher values. Meanwhile, Y and Z always reach the same value of 0.5625, no matter what the value of $p$; or what the value of X, for that matter. These observations lead to an intriguing conclusion: If we did not know the structure of the pathway, we would surely conclude that Y and Z had nothing to do with X, because they always have the same values, independent of the value of X. Yet, X is their only precursor substrate!

Something quite different happens if $p$ is set higher: The metabolites begin to oscillate, before reaching their new steady state (Fig. 2D). For even larger values, the system "dies." For instance, if $p = 0.8$, the oscillations become so strong that one of the variables vanishes (result not shown). In between the former, reasonable values and these large values lies a small range of values for $p$ ($p \approx 0.51$) where the system exhibits yet another behavior: it oscillates in a stable fashion so that after some while the amplitude and frequency remain constant (Fig. 2E). The so-called limit cycle oscillations in this small range are very interesting mathematically, because they are able to tolerate perturbations, from which they recover. Sustained oscillations have been observed in the expression of actual genomes and in metabolic systems, such as glycolysis.[20-23]

The simulations with the simple pathway demonstrate that it is not necessarily possible to grasp intuitively the full functionality of a system if it is taken out of its context. Considering the complexity of sphingolipid metabolism and its regulation, one must therefore wonder to what degree intuition is sufficient when global responses are to be predicted.

## Sphingolipid Models and Their Potential Uses

Over a span of several years, we have been developing a series of increasingly more sophisticated models of sphingolipid metabolism.[13,14,24,25] The models were formulated as systems of nonlinear ordinary differential equations in the format of power-law functions, as suggested in BST.[18] Choosing this framework, it was straightforward to set up symbolic equations that reflect the known or assumed connectivity and regulatory signals of the pathway system. While this part of the model design phase was manageable with reasonable effort, the estimation of suitable parameter values was very challenging. Indeed, our case study confirmed common experience that parameter estimation is the bottleneck of biological modeling. In our case, the estimation was based on literature information, de novo experiments and some default assumptions based on experience with BST.[14] The resulting model was subsequently tested in the typical fashion, namely with stability, sensitivity and robustness analyses, through comparisons with experimental data that had not been used in the estimation phase and through qualitative reality checks based on biological experience. After many iterations and revisions, the model appeared reasonable and was semi-quantitatively validated with additional wet experiments.[13]

It would be counterproductive to use the limited space of this chapter to review the steps of a typical modeling process in general or even within the context of sphingolipid metabolism, because both have been described in recent years and at various levels of sophistication and detail (for example see refs. 10, 14, 18, 24). Instead, it seems more beneficial to ask what we may do with such models, once they are validated. Again, many typical uses of models have been described in the literature and we will simply mention some of them. However, other uses are less typical and will receive more attention in the following.

The first and foremost role of a mathematical model is the integration of diverse data and other pieces of information, such as kinetic characteristics of enzymes, expression profiles of genes, protein abundances and maybe even semi-qualitative clinical observations. This integration often shows very clearly whether we have a good grasp of the functionality of the pathway, because most initial efforts of merging all information into one mathematical construct fail. Typical failures become apparent in lacking stability or robustness of the model that is accompanied by unduly high sensitivities. In the former case, small variations in input or in some variable may cause the model to "crash" in a sense that one or more variables vanish. In the latter case, the system "overreacts" to small changes in parameters. For instance, a 5% increase in some enzyme activity could lead to a 220% increase in some metabolite, which is unreasonable in most cases. Relatively straightforward diagnostic tools weed out such systems (for example, see ref. 18).

If a model appears reasonable, we can further test our intuitive grasp of the system through simulation studies that represent *What-If* scenarios, as we employed them before. For example, the reduction in an enzyme activity is easily implemented in the model and simulation results can possibly be validated with wet experiments. An integrated model of sphingolipid metabolism also allows us to follow the fate of metabolites of interest, many of which are recycled or involved in several reactions. A good model, with slight adaptations, even permits the tracking of labeled substrates, from input to their ultimate fates.[10,14] As a variation on this theme, a reliable model may be used to study the relative contributions of different pathways to a common goal, such as the formation of rafts that become structural elements of membranes.

In a study of a slightly different nature, we asked the question whether a yeast cell mounts a response to stress by up-regulating a small number of enzymes (genes) a lot or whether it changes the activities of many enzymes a little bit. Intuitively one could easily find rationale for either strategy. On one hand, up- or down-regulating only one or two genes or enzymes appears to be the simpler strategy. On the other hand, drastic changes in some part of the pathway could lead to concomitant and undesired side effects. In our test case of the diauxic shift, the modeling analysis suggested that many enzymes are involved in the response.[24]

Sphingolipid metabolism has been analyzed experimentally in different organisms, some of which are phylogenetically close. This similarity permits the cautious extrapolation and use of the model in an untested organism. This type of model transfer was demonstrated by using the original *S. cerevisiae* model with some adaptations to study sphingolipid metabolism in *Cryptococcus neoformans* (*Cn*), an airborne fungal pathogen that may cause life-threatening infections.[25] The main challenge this organism faces is the distinct difference in pH between alkaline or neutral extracellular environments, such as alveolar spaces or the bloodstream and the acidic environment of the intracellular phagolysosome of the host's phagocytic cells, in which the organism lives and grows during a crucial phase of its virulence cycle.[26] Earlier work in Del Poeta's laboratory had suggested the involvement of sphingolipids in growth under acidic conditions, but it had not been possible to characterize the specifics of this process.[27] The model results together with subsequent validation studies led to the very specific proposition that inositol phosphoryl ceramide synthase (Ipc1) and inositol sphingolipid phospholipase C (Isc1) affect the function of the plasma membrane $H^+$-ATPase pump (Pma1) through modulation of the level of phytoceramides and complex sphingolipids.[25]

The successful use of the yeast model in the investigation of a fungal pathogen suggests that it might even be possible to study the evolution of design principles governing sphingolipid function, based on comparative model analyses of sphingolipid metabolism in closely related and more distant species. Along the same lines, a major future project should convert and test the yeast model for analyses of the mammalian analogues. This "extrapolation" is much more complicated, because mammalian sphingolipids are often bound to carbohydrates, which leads to a multiplication in the number of potentially relevant compounds, as discussed before.[28] This explosion in number may appear overwhelming for a model analysis. However, trying to understand the mammalian systems without computational approaches seems to be incomparably more complicated. It is clear that a mammalian model could be very useful for the exploration of pathways leading from health to diseases such as cancer, where we have strong indication that sphingolipids are involved (for example see ref. 29).

As a first step toward comparative studies, the sphingolipid system of the same organism may be studied at different grades of granularity. For instance, our present yeast model accounts only partially for compartmentalization. As a next step, it might be fruitful to distinguish sphingolipids and precursors with different fatty acid chain lengths. Accounting for this detail will require a substantial increase in model size and require additional biological information about the relevant compounds of different sizes and their respective roles.

As a more detailed example of an atypical model investigation, consider the immediate sphingolipid response in yeast to heat stress. Genome studies have strongly supported the involvement of several genes, such as *MSN2/4* and *YAP1*.[30] However, preliminary concentration

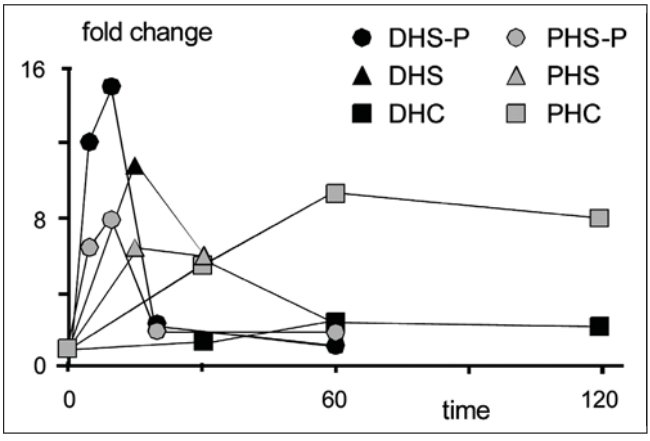


Figure 3. Fold changes in sphingolipids following heat stress at time $t = 0$. Abbreviations: DHS: dihydrosphingosine; PHS: phytoshpingosine; DHC: dihydro-ceramide; PHC: phyroceramide; –P: –phosphate. Data adapted from Jenkins et al[31] and Hannun, Y.A.: *pers. comm*.

measurements of key sphingolipids indicate that the heat stress response is much faster than any gene-regulatory mechanism could accomplish, exhibiting metabolic changes within a few minutes (Fig. 3[31]; and Y.A. Hannun, *pers. comm.*; see also ref. 32). Within about 8 minutes, dihydrosphingosine-phosphate (DHS-P) and phytosphingosine-phosphate (PHS-P) increase to 8- and 15-fold levels, respectively, before resuming almost normal values after about 20 minutes. DSH and PSH respond more slowly, peaking after about 15 minutes at 6- and 11-fold levels. Phytoceramide accumulates gradually over a period of about an hour, peaking at a level that is about 8 times higher than baseline. Dihydroceramide shows the same pattern, but with a peak accumulation of only about two-fold.

This fast change in metabolic profile is intriguing and not explainable with gene regulatory actions. We have seen a similarly quick response to heat stress in the trehalose cycle in yeast, which, according to careful in vivo NMR measurements, begins producing trehalose within two minutes.[30] Again, a gene regulatory response is too slow for such a response. As it turned out in the trehalose case, three key enzymes of the trehalose pathway are heat sensitive. The two enzymes controlling trehalose production are more active at higher temperatures, while trehalase is less active.[33] A preliminary mathematical model analysis suggests that the relatively slight changes in activity are sufficient to mount the fast, observed response.[30]

Given the similarity of the heat response task in the case of trehalose and sphingolipids it is reasonable to ask whether there are heat-sensitive enzymes within the sphingolipid pathway as well. If so, would a single enzyme be sufficient to mount the observed sphingolipid response? Would combinations of two or more enzymes be sufficient?

A mathematical model might help us identify such enzymes. First, it would of course be possible to launch a major simulation study, changing one or a few enzymes at a time and then testing whether metabolic concentration patterns like the one observed can be generated. However, some prudence might help us prescreen some possibilities and favor or disfavor particular hypothetical scenarios. To permit an objective, yet lucid exploration, we reduced the sphingolipid system to a minimum and converted it into a much simplified mathematical model. Thus, consider the reduced core of sphingolipid metabolism that contains only those components that appear most important (Fig. 4).

Looking at once at Figures 3 and 4, we can formulate the following as a framework for preliminary hypotheses. The concentration of phytoceramide at one to two hours exhibits a sustained level at eight times its baseline. This increased level requires that: (1) more material is produced per

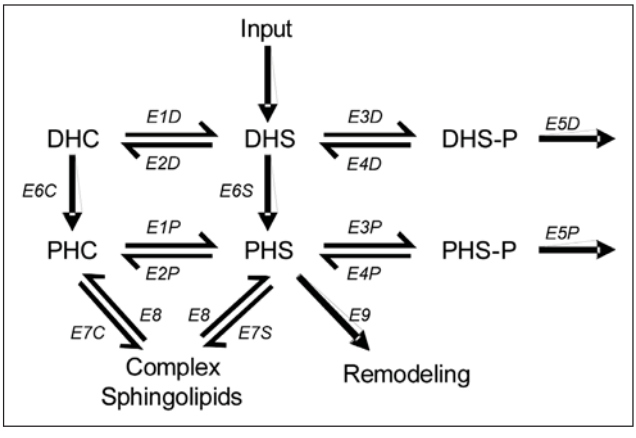


Figure 4. Reduced and simplified diagram of parts of the sphingolipid pathway involved in heat stress response. Abbreviations of metabolites are presented in Figure 3. Names of enzymes are not of relevance, but can be found in.[14] E7C, E7S and E8 are collective representations for the interactions between the simple and complex sphingolipids.

biosynthesis or recycling of complex sphingolipids (*cf.*); (2) material is simply reorganized within the pathway system; (3) the loss of sphingolipids is reduced; or (4) several of the previous options are combined. The second option may be discarded off-hand: A simple reorganization or shift of fluxes in the neighborhood of DSH and PSH would require decreased levels of the substrates of these reactions, but decreases in concentrations are not observed. Although some of the biosynthetic genes are affected by heat, the first option alone also seems questionable, because DSH-P and PSH-P respond most quickly, whereas one would expect a response through increased biosynthesis to cause increases in DHS and PHS first. Besides, if PHC is the target and biosynthesis was the mechanism, why should DSH-P and PSH-P be increased at all? Similar arguments seem to hold for the recycling of complex sphingolipids. One could surmise that a direct change in E7C and/or E8 could be a good strategy toward an increased level of PHC. However, this is apparently not a strategy pursued by the cell. Pursuing the third (or fourth) option, the observed metabolite profile could possibly be achieved through a reduction in the lyase reaction (*E5D*, *E5P*), which would lead to an accumulation of the phosphorylated forms. This change would have to be followed by increased sphingoid base PPase (*E4D*, *E4P*) and hydroxylase (*E4D*, *E4P*) activity, which would gradually shift the increasing amounts of DSH-P and PSH-P toward the dephosphorylated forms and from there to ceramide (*E6C, E6S*). It is interesting to note that the dihydro- and the phyto-forms of sphingolipids essentially form parallel pathways, but that the target profile after heat stress is distinctly different between the two pathways. This observation suggests that the involved enzymes might have different affinities for the dihydro- and the phyto-forms, at least under heat stress conditions.

Complex systems have a way of tricking our intuition and our hand waving arguments could simply turn out to be wrong. Nevertheless, it is possible to test these scenarios with a model. In order to keep our exploration as simple as possible, we created a reduced model reflecting the simplified pathway shown in Figure 4; it is shown in Eq. (3). Because the only purpose of this model is a proof of concept showing whether or not changes in enzyme activities could lead to something like the observed metabolite profile, we set the model up in a minimalistic fashion, again using (almost arbitrary) default parameter values and enzyme activities set to a nominal value of 1, so that the steady state consists of unity values. These intentional simplifications may show us the consequences of introduced changes more clearly than a model that is parameterized from actual data, but of course we cannot expect to obtain numerically valid simulation results.

$$
\begin{aligned}
\dot{DHC} &= 2\ E2D\ DHS^{0.5} - E1D\ DHC^{0.5} - E6C\ DHC^{0.5} \\
\dot{DHS} &= Input + E1D\ DHC^{0.5} - 2\ E2D\ DHS^{0.5} - 2\ E3D\ DHS^{0.5} \\
&\quad + E4D\ DHSP^{0.5} - 5\ E6S\ DHS^{0.5} \\
\dot{DHSP} &= 2\ E3D\ DHS^{0.5} - E4D\ DHSP^{0.5} - E5D\ DHSP^{0.5} \\
\dot{PHC} &= E6C\ DHC^{0.5} + 2\ E2P\ PHS^{0.5} - E1P\ PHC^{0.5} - 3\ E7C\ PHC^{0.5} + E8\ CS^{0.5} \\
\dot{PHS} &= 5\ E6S\ DHS^{0.5} + E1P\ PHC^{0.5} - 2\ E2P\ PHS^{0.5} - 2\ E3P\ PHS^{0.5} \\
&\quad + E4P\ PHSP^{0.5} - 3\ E7S\ PHS^{0.5} + E8\ CS^{0.5} - E9\ PHS^{0.5} \\
\dot{PHSP} &= 2\ E3P\ PHS^{0.5} - E4P\ PHSP^{0.5} - E5P\ PHSP^{0.5} \\
Input &= 7 \\
CS &= 1
\end{aligned}
\tag{3}
$$

As a first simulation, we start the model at the steady state (1, ..., 1). Because all values are 1, all later results automatically represent "fold" changes. At time $t = 2$, we reduce the lyase activity (*E5D*, *E5P*) (Fig. 5A). Even with different magnitudes and different changes for the lyases with respect to DHS-P and PHS-P, the resulting sphingolipid profiles are not even close to the observations in Figure 3; instead, they primarily lead to accumulation of DHS-P and PHS-P (Fig. 5B).

For a second exploration, we double the (biosynthetic) input starting at $t = 2$. The result is increased mass in the system, but the resultant profile is similar to that in Figure 5B (results not shown). As a variation on the same theme, we increase the input more gradually and assume that after a while the substrates for biosynthesis become limiting, thereby reducing the input flux (Fig. 5C). The model now yields a peaking profile, but all metabolites respond in parallel (Fig. 5D).

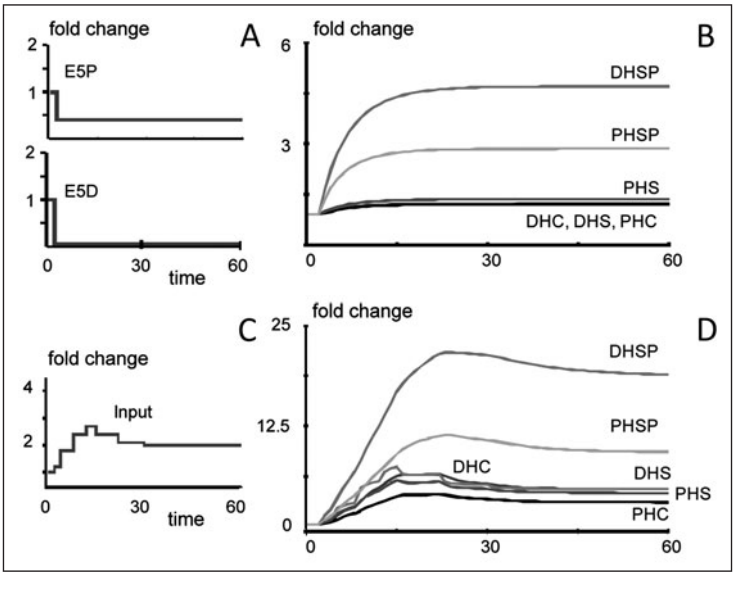


Figure 5. Two simulation scenarios with Equation (3) exploring the feasibility of simple changes in enzyme activities. A) The two lyases are reduced two minutes after initiation of heat stress. As a consequence (B), the phosphorylated sphingolipids accumulate. If in addition the input to the system changes in direct response to heat, the same metabolites increase more. Assuming that increased biosynthesis leads to substrate deprivation, the sphingolipid profiles begin to decrease.

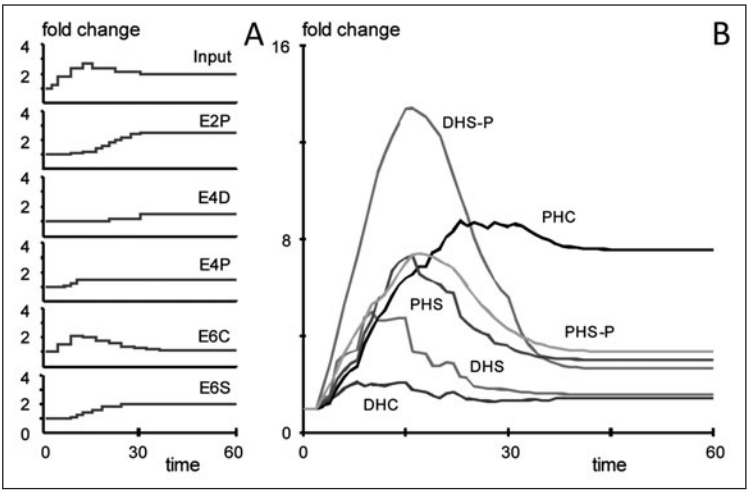


Figure 6. Simulation scenario with Equation (3) in which several enzymes have temperature dependent activities. Profiles of changes in enzymes and input are given in (A); see Figure 3 for abbreviations. The corresponding sphingolipid profiles show a qualitatively similar pattern as it was observed (Fig. 4). The "jagged" appearance of the simulated profiles is due to discrete changes in enzyme activities.

Uncounted simulations of this type may be executed and in the end it would be wise to formulate an optimization program that would guide the progression of simulations. Through manual exploration, we developed a more complex heat stress response pattern in enzyme activities (Fig. 6A) that actually produces a metabolic profile that qualitatively resembles the observations (Fig. 6B). This profile is clearly not unique and not refined or optimized, because it is based on the simplified model with "invented" parameter values, rather than a fully parameterized model. Nonetheless, the simulation constitutes proof of concept that temperature dependent enzymes could be the drivers of the very fast sphingolipid response to heat, as it is the case in the trehalose cycle. Interestingly, "successful" profiles like the ones in Figure 6A seem to require different affinities of the enzymes to the dihydro- and phyto-forms of the metabolites.

In addition to simulation studies, the model format of an S-system within BST could be used to exhaust all possible means of achieving a desired profile at steady state. We have shown in a completely different context how such an analysis could be pursued[10] (see also ref.19). In a nutshell, the mathematical features of the S-system model allow an elegant algebraic analysis of the entire space of steady-state solutions in terms of enzyme activities that are consistent with a desired metabolic concentration profile. Among these consistent solutions, optimal transient solutions could be determined through dynamic analyses or nonlinear optimization studies.

## Conclusion

The pathways of sphingolipid biosynthesis, utilization and recycling form an intriguingly complex system whose dynamics is difficult to predict with intuition alone. Mathematical modeling provides an aid in this regard, because it permits the integration of many pieces of information into computational structures that are very easy to diagnose, interrogate and analyze through *What-If* simulations. The bottleneck of setting up such models is the determination of parameter values, which may be based on literature information characterizing genes, enzymes and metabolites in a steady-state situation, or on dynamic time trends, which can be measured with modern methods of mass spectrometry or nuclear magnetic resonance. Parameter estimation is the bridge between wet experimentation and modeling and the need for improved parameter values, which are valid in

vivo and under relevant physiological conditions, gives clear indication that mathematical modeling is crucially dependent on solid experimental work. At the same time, once reliable models are established, they become incomparably rich tools for analyses that are often unattainable with wet experimentation. For instance, it is at least theoretically feasible to determine all possible combinations of enzyme activities that lead to an observed metabolite profile at steady state. As we indicated here with an intentionally simplified analysis, it is also possible in principle to determine mathematically which enzymes would have to altered and in what manner, to obtain dynamic metabolite profiles as they are observed in responses to perturbations such as heat stress.

Ultimately, reliable mathematical models will be used as valuable tools for exhaustive prescreening studies for all kinds of scenarios and for creating novel and optimally discerning hypotheses that are then to be tested in the laboratory. If the history of physics is an indication of the future of biology, we might one day execute experiments only once the theory describing the underlying system is sufficiently worked out and understood. Even if this procedure will become the norm in the future, one must expect that it will take many years and dedicated effort, both experimentally and methodologically, to establish models of sufficient scope and reliability. Thus, the rise of mathematical modeling as a biological technique should not be seen as threatening to experimentation, but simply as an additional tool that is able to elucidate different aspects of a system. Modeling has improved tremendously over the past decades and successful collaborations between biological and computational scientists in the recent past have begun to show that their team efforts will be rewarding to both sides and reveal insights that neither side could have obtained without the other.

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