Systems Biology in an Imperfect World: Modeling Biological Systems with Incomplete Information

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ABSTRACT

One of the primary goals of systems biology is to understand the complex underlying network of biochemical interactions which allow an organism to respond to environmental stimuli. Models of these biological interactions serve as a tool to both codify current understanding of these interactions as well as a starting point for scientific discovery. Due to the massive amount of information which is required for this modeling process, systems biology studies must often attempt to construct models which reflect the whole of the system while having access to only partial information. In some cases, the missing information will not have a confounding effect on the accuracy of the model. In other cases, there is the danger that this missing information will make the model useless.

The focus of this thesis is to study the effect which missing information has on systems level studies within several different contexts. Specifically, we study two contexts : when the missing information takes the role of incomplete molecular interaction network knowledge and when it takes the role of unknown kinetic rate laws. These studies yield interesting results. We show that when metabolism is isolated from gene expression, the effects are not limited to those reactions under strong control by gene expression. Thus, incomplete understanding of molecular interaction networks may have unexpected effects on the resulting analysis. We also reveal that under the conditions of the current study, mass action was shown to be the superior substitute when the true rate equations for a biological system are unknown.

In addition to studying the effect of missing information in the aforementioned contexts, we propose a method for limiting the parameter search space of biochemical systems. Even in ideal scenarios where both the molecular interaction network and the relevant kinetic rate equations are known, obtaining appropriate estimates for the unknown system parameters can be challenging. By employing a method which limits the parameter search space, we are able to acquire estimates for parameter values which are much closer to the true values than those which could be obtained otherwise.

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Thank you!

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Chapter 1: Introduction

Background

Introduction

Systems biology is a manifestation of general systems theory. General systems theory itself grew out of the recognition that in many cases a mechanistic viewpoint was insufficient to describe the complex actions of systems (Bertalanffy 1973). In biology, the discrepancies between the behavior of isolated components and their actions within a cellular context can be especially stark (Kaneko 2006). In order to address these discrepancies, a method of studying biology which took the interactions of these components into account was necessary. Systems biology studies aim to integrate knowledge of how, and the contexts in which, these components dynamically interact in order to gain a better understanding of biological processes as a whole. Ultimately, these studies aim to create models which accurately reflect the system being studied. These models must therefore take into account the underlying complex series of dynamic molecular interactions which allow the organism to respond to a variety of stimuli in its characteristic manner (Kitano 2002). In order to study biology in this way, much more information must be obtained than in reductionist approaches. Systems biology studies require that not only the components be identified and characterized (the business of molecular biology), but also how these components interact. Although there has been much success in generating massive amounts of 'omic data via high-throughput transcriptomics and metabolomics studies, there is still difficulty obtaining the information necessary to build accurate biological models. This difficulty in obtaining the necessary levels of information is due to a combination of factors including technical, experimental as well as other limitations. In turn, this lack of complete information can result in an inability to thoroughly determine the model parameters for the system (Kotte & Heinemann 2009 ; S. Sahle et al. 2008). Because the ability to create biologically relevant models depends on the quality and quantity of systems level data available, it is important to assess the effect that incomplete knowledge may have on a systems level study.

The focus of this thesis is on determining the effect that different types of incomplete or missing

information can have on a systems biology study. In some cases, the form of information which is missing may not have drastic consequences. In other instances, it may be necessary to perform additional experiments, when possible, to improve the quality of the analysis. In the worst case scenario, it will be difficult to impossible to determine that the information is missing and this lack will significantly affect the outcome of the given study.

In this chapter, the importance of modeling to gain an understanding of biology is discussed. In addition, the concept of using artificial biological networks for benchmarking the effects of missing information on the formation of these models will be advanced. Finally an introduction to the remaining chapters is given.

Modeling in Systems Biology

Modeling is the essence of science(Rosen 1991). Constructing models forms a pivotal role in the process of biological inference. According to Fisher, the general inference follows three core steps: 1. the specification of a model 2. parameter estimation and 3. estimation of precision (Fisher 1992). The process of biological discovery traditionally follows an idealized process where a hypothesis concerning a given system is constructed, a series of experiments are performed to test this hypothesis, and a conclusion is made. With the advent of high-throughput experiments, this process has become less linear. Information on multiple 'omic states of a cell may now be gathered in an unbiased manner, making it possible to formulate hypotheses after the experimental stage. These resultant hypotheses do not suffer from the initial selection bias that other hypotheses may have. In systems biology, models form part of an interactive process where experimental data can be used to create new hypothetical models or to refine aspects of a predefined model (Kell & Oliver 2004). In this manner, systems biology models may take on the roles of both hypothesis and experiment, depending on how they are used. Within a useful model, information is codified into a more simplified construct that allows the basic principles governing the much more complex studied system to be identified(Wolkenhauer & Ullah 2007). These constructs can then be used to make predictions on how the target system would behave in a given scenario. Ideally, a model will strike a balance between simplicity and correctness. The model should be much more simplistic than the studied system, yet retain the essential features

(Mendes 2001). While it is often possible to create a model that encompasses multiple aspects of the system, including all possible minutiae will likely make the model less general as well as less tractable. The purpose of a model is not to include all possible interactions of all possible components, but rather to be a partial representation which can offer an explanation of which features of a system are essential to understand it (Noble 2002).

Models of biochemical systems are usually created by following one of two distinct methodologies or by following a hybrid of the two. In the more traditional method, referred to as the bottom-up approach, the global model is seen as being composed of a series of distinct modular parts. These modules represent the individual biochemical reactions of the system. In the bottom-up approach, the selected components of the final system are first studied *in vitro*, where information concerning a given enzyme's rate law is determined in isolation from all other aspects of its native system. These parameter values are then combined with those of the other constitutive biochemical moieties to form an initial version of the system model. The initial *in vitro* measurements thereby serve as an initial estimate of the true system parameters. The model is then refined by calibrating it against *in vivo* measurements of the system (for example of metabolite concentrations). The bottom-up derived model thereby assumes a certain independence of parts and the global model is seen as arising from a combination of these. The quality of the resultant model will depend on a number of factors, including 1) the initial selection of variables to be included and their relative independence, 2) the accuracy of the rate laws used, 3) how widely the incorporated kinetic values of the constituent enzymes vary from those measured *in vitro*, 4) the order in which variables are added to the model, and 5) the quantity and quality of the experiments used to calibrate the model. Due to the biased nature of a bottom-up derived model, it is critical for the model's usefulness that the components accurately reflect the underlying biochemical network. Any interactions which are unknown will be missed by the bottom up approach. For example, feedback loops, if not already known, will be impossible to ascertain from the data if using a bottom-up approach.

A more recent modeling approach has been made possible by high-throughput methods in the 'omics disciplines. The top-down approach to modeling biochemical systems relies heavily on the availability of large systems level experimental datasets. In top down models, as opposed to bottom-up models, the initial model is formed in an unbiased manner based upon information gleaned from a set of 'omics

datasets. Unlike in the bottom up approach, top down models use only *in vivo* datasets . In a top-down model, constituents are related to each other via mathematical equations. A conceptual framework for performing top-down modeling was proposed by Mendes (Mendes 2001) . According to this framework, the initial model represents an initial guess as to how these elements relate to each other. The mathematical functions which approximate reaction rates in terms of the constituent elements are then identified. Finally the model parameters are estimated based on the available data. The conceptual model is then iteratively refined as new, relevant experimental data is added (Mendes 2001). Top down modeling is essentially a problem of network inference. One concern about using top down modeling is that formulating higher level cellular interactions does not necessarily mean that the correct lower order reaction mechanisms will be identified (Noble 2002). In general there are multiple disparate models which may explain the same datasets. Thus, determining which methods of network inference best uncover the true underlying biological network has become a problem of great interest.

Parameter Estimation

Independent of the method used to construct a given model of a biological system, parameter estimation is a crucial step in reconciling the model to the experimental data available. Parameter estimation is important both at the level of the individual parameters and for the model as a whole. In bottom up construction, parameter estimation is needed to properly assign values to the parameters of the rate laws determined from *in vitro* kinetic data and to optimize the fully constructed model using the *in vivo* data of the system. In particular, certain parameters that depend on the state of the system cannot be determined separately for the model to make sense and therefore must be estimated within the context of the full network (*i.e.* living organism). Models determined via top down methods rely on parameter estimation for initial stages of their formation as well as for refinement.

Artificial Biological Networks

One of the concerns surrounding top down modeling methodologies is that it is impossible to be sure if the resultant model formed from the available data is the correct one. There are several reasons why it is difficult to ascertain the veracity of a given model. One of the reasons is the nature of network

inference itself. Network inference is a type of inductive inference, meaning that it is an attempt to make generalizations about all possible data for a given system using only a subset of the data. The generalization in this case is the model being formed from a given dataset that is intended to encompass the system's behavior. While it is possible to make them mathematically rigorous, inductive inferences are uncertain in nature (Fisher 1935). The subset which is sampled may not accurately reflect the whole space of behaviors. The uncertainty implicit in inductive inference propagates into uncertainty within inferred biological system models. An aspect of this uncertainty is that for any given set of systems data, there are multiple models that will be able to explain it. Therefore, model selection is a critical step in the top down modeling process (Burnham & Anderson 1998) . In the case of complex systems, there will not be a global model which will accurately describe the system. While a combination of models may help to circumvent this issue in a complex system, even this approach is not guaranteed to work for all such systems (Rosen 1985).

Uncertainty in network inference is also related to its dependence on systems level data. Top down modeling is data driven and therefore the quality of the resultant models is dependent to a large degree upon the quantity and quality of data available for the given system. The issues of quantity and quality of data also cause concerns for bottom-up modeling. Biological systems datasets are not ideal in this respect. Results from biological experiments are not always reliable, as the techniques employed can have inherent errors (Wimsatt 2007). For example, experimental methods to determine protein interactions will often result in considerable numbers of both false positives and false negatives (Deane et al. 2002) . All other techniques produce some level of noise, therefore biological data sets always contain implicit noise which makes top down modeling challenging. The implicit noise is also due to the stochastic nature of biological systems (Kaern et al. 2005). Unlike deterministic systems, biological systems may not always respond identically to the same perturbation each time. Instead, biological systems react probabilistically and the same perturbation may result in different outcomes in different instances. This combined noise from various sources makes it difficult to extract the true biological signal which is needed to construct accurate models.

In addition to the difficulties associated with forming models from unreliable data , there is a lack of sufficient experimental data available to fully determine a model. In most cases, systems biology datasets are high dimensional with low sample number. While 'omics methodologies make it possible

to gather information on multiple variables in each sample, gathering a sufficiently large number of samples is problematic due to cost and logistics. These high dimensional, low sample number experimental datasets cannot be analyzed by traditional statistical methods, because it is impossible to 'sphere the data'. Data sphering is accomplished by multiplying the data matrix by the root inverse of the covariance matrix. If the data is normally distributed, a plot of these transformed variables would resemble a sphere. However, in high-dimensional, low sample number datasets this process is impossible because the required root inverse of the covariance matrix does not exist. This inverse does not exist because the covariance matrix is not of full rank for high dimensional, low sample number datasets. This is known as the large *p*, small *n* problem(Hall et al. 2005) . Most systems biology models are therefore under-determined, meaning that there are many models will fit the available evidence (Kotte & Heinemann 2009). As a result of this, many parameters within the model can take multiple values while still agreeing with the available experimental data.

This uncertainty in top down modeling leads to a predicament. Even if a fit of the model to the data seems good, it is still possible that the model is partially or entirely incorrect. While it is possible to use well-studied biological systems to determine the quality of a network inference method, they are not exempt from this problem because it is unknown if the current paradigms are true(P. Mendes et al. 2003; Camacho et al. 2007) . If an inference method conceived a result which differed from the current paradigm, it could not necessarily be ruled out. This difference could be due to errors or insufficiencies in the current model or models used to explain the given system.

One method to assess the uncertainty associated with top down modeling is to begin with a system where everything is known. While this is not feasible for true biological systems, it is for artificial biological systems. These artificial biological systems generally resemble true biological systems. In order to be useful, they must contain features similar to their nature counterparts. If a given network inference method performs well on data derived from an unrealistically simplistic artificial network, there is no guarantee that it would function as accurately when confronted with the more complex biological data. Therefore, proper design of artificial biological networks is critical for their effectiveness. An aspect of this design is that the artificial network should mirror the type of 'omics system that the top down modeling approach intends to model. Artificial biological systems have been successfully utilized to benchmark a number of network inference algorithms optimized for specific

'omics data, in particular metabolomics and transcriptomics data. In addition to making it possible to determine if the true model was determined, artificial biological networks can also examine the effect that noise has on network inference algorithms. As mentioned earlier in this section, data from biological samples is comprised of both signal and noise. It is therefore useful to determine under what levels of noise the method can be expected to remain functional.

Introduction to Later Chapters

Incomplete Knowledge and Systems Biology

Missing and incomplete information affects all branches of science, but its influence is pervasive in systems biology studies. Much of the influence this lack of information has over systems biology is due to systems biology's overall goal of understanding the whole of biology. As the complexity of the biological interactions increases, so does the amount of information which is required to identify and characterize these interactions.

The specific aims of this thesis are 1) to address several forms of missing information in systems biology studies, 2) to assess the effect that this form of missing or incomplete information may have on the overall conclusions of the given study, and 3) to propose methods to minimize the effect of this missing information in certain cases.

The Effect of Incomplete Network Information

In chapter 2, the effect that partial network information has on the ability to recover system properties is addressed. The focus here is on assessing how studying one level of a biological system, to the exclusion of others, may affect the interpretation of results. It is not uncommon for systems level studies to focus on the transcriptome or the metabolome without integrating any of the other levels of cellular organization. In chapter 2, the metabolic reactions of the Claytor artificial biological network are studied in isolation from the full network. The Claytor artificial biological network is ideal for these purposes because it contains many of the complex characteristics of natural biological networks, while

having the beneficial qualities of being fully known and much more tractable to analysis. The metabolic reactions are studied in a manner which is parallel to metabolomics time course experiments. However, unlike true metabolomics studies, all of the members of the metabolome are identified and there is no noise in the measurements. These ideal measurements are used to provide estimates for the parameters of the metabolic rate equations. In this scenario, the true structure of the rate equations is assumed to be known. Thus, this study aims to detect neither the effects of noise nor the effect of unknown rate equations. Instead, the study's sole intent is to determine what effect ignoring transcriptional and translation regulation will have on the ability to correctly ascertain the true kinetic parameters of the system. It is hypothesized that the rate equation parameters which will be most detrimentally affected by an exclusive analysis of the metabolome will be those which are primarily under transcriptional control. In order to test this hypothesis, a method is proposed to assess the level and type of control each reaction is most associated with.

The Effect of Unidentified Rate Laws

In chapter 3, the effect which unidentified rate laws have on creating useful systems biology models is discussed. Due to the prevalence of unavailable rate laws for metabolic reactions, many general kinetics equations have been proposed. In chapter 3, three methods of generalizing kinetic rate laws are compared using the Claytor artificial biological network mentioned above. Specifically, these methods are: generalized mass action, linlog, and convenience kinetics. In each case, the methods are assessed on their ability to properly fit metabolite concentration time-course data. The methods are compared both on their ability to fit the time-course data when integrated into the full network, where the transcriptome, metabolome, and proteome are all included, and in their ability to fit data when only the metabolic portion of the network is assumed. In this manner, it can be determined if any of the aforementioned methods is sensitive to partial network knowledge.

Reducing the Parameter Search Space in Biochemical Networks

One of the main steps in a systems biology study is to codify available information into a useful model. The goal, in this case, is to integrate data and models (Krohs & Callebaut 2007). In order to accomplish this goal, the parameters for the model must be estimated using the available data. A confounding issue

at this stage is the large "volume" of parameter space which must be searched through. In addition to increasing the amount of time necessary for the optimization methods to work, increased parameter search space can increase the likelihood of obtaining improper parameter estimates. Many systems biology models are under-determined (Ashyraliyev et al. 2009) . This means that for the limited amount of information available there are several models which would explain it. In chapter 3, a data-driven method for limiting the parameter search space for biochemical networks is proposed. The proposed method assumes that the ordinary differential equations ODEs based upon the kinetic rate laws for the given system are known and that the only issue is properly parametrizing this model (finding the values of its constants). The underlying premise of the model is that these ODEs may be separated by initially substituting functions which only depend on time for the concentrations of dependent chemical species in each equation. This method is demonstrated on an artificial metabolic network obtained from the curated portion of the BioModels database (Le Novere et al. 2006).

Conclusions and Future Directions

In the final chapter of this thesis, the outcome of the studies in previous chapters and their implications will be discussed. In addition, future work related to these implications will also be proposed.

Chapter 2: The Effect of Studying Metabolism Isolated from Gene Expression

Background

Introduction

In many systems biology studies, the underlying network of molecular interactions that constitute gene expression and signaling is implicit within the model. Although this information may not be explicitly formulated in the model, assumptions of how the composite biological moieties interact imply a given network. However, this underlying network is generally not known in its entirety. This problem is exacerbated in models of living systems, because it may not be possible to ever know whether or not *all* of the components of the complete underlying biological network have been fully identified, much less characterized. Therefore it is almost guaranteed that any biological model ignores a number of molecular interactions. Depending on the type of model, how it was formulated, and which framework is used, these unknown interactions may be implicit in the model or otherwise entirely absent. While it may not be necessary for all purposes that the underlying biochemical network be entirely known, it is important to determine to what extent the aspects of the network which are not included affect the outcome of a modeling study. Understanding the influence of biological interactions which are not the focus of a study is important both in minimizing any deleterious effect they may have and in efforts to simplify complex networks.

In this chapter, the focus in on how missing information about the network influences the ability to estimate appropriate parameter values . In particular, it examines the case where the full metabolic network is known, but influences from the transcriptome and proteome are not taken into account. By using an artificial biological network, it is possible to assess the effect that studying an organism's metabolism in isolation will have on predictions of system level properties. In order to achieve this a computational study was carried out using a dynamic model (gold standard) that includes the metabolome, proteome and transcriptome. This network model is used as if it was the original biological system, and used to simulate experiments. The resulting data from these *in silico* experiments is is then used to calibrate a smaller model which only includes the metabolic reactions

and ignores the proteome and transcriptome (reduced model). Unlike natural biological systems, the true values of all parameters as well as all interactions of the gold standard model are known. Thus it is possible to compare not only how well the reduced model fits the experimental data, but also how close the estimated parameter values of the reduced model are to the true ones in the gold standard.

These *in silico* experiments are carried out in an ideal scenario where the true form of the kinetic rate equations is known and there is no noise in the data. It is hypothesized that those moieties which will have the most difficulty being fit in this scenario are those which are the most influenced by transcriptional elements. As an extension to this reasoning, it is thought that those reactions which contain the highest number of transcriptionally influenced members will also obtain estimates for their parameters which are the most distinct from their true values.

Materials and Methods

Overview

In order to determine the effect that studying one aspect of a biological system in isolation could have on the resulting analysis, the metabolic portion of an artificial biological network was extracted and studied separately. The artificial biological network used, the Claytor network, is described in detail later in this section. After determining the subset of reactions which constituted metabolism, their respective kinetic parameters were estimated via least-squares fitting to the data generated in the *in silico* experiments. The data fitting was carried out with distinct training and validation datasets to prevent over-fitting. The training dataset is the only one used to adjust parameter values, while the validation dataset is used to assess how well the model predicts the system's response to a perturbation that was not used to fit the model. The validation set is also used as a stopping criterion, such that if at some point in the fitting iterations, the distance between the model prediction and the validation data starts increasing, the fitting procedure will stop. It is considered that in such a case, further refinement of the parameter values would result in a model that fits only a particular set of data. The use of this stopping criterion with a validation dataset thus ensures that the model extrapolates as well as possible.

After fitting the reduced model to the data from the *in silico* experiments (obtained with the Claytor

network), the resultant parameter values of the isolated metabolism were then compared to their true counterparts in the intact Claytor network (the gold standard). It is hypothesized that those reactions which are under primarily metabolic control would achieve more accurate parameters than those which are under primarily transcriptional control. In order to test this hypothesis, the primary control type is predicted for each reaction under multiple experimental conditions.

Artificial Biological Networks

Claytor Artificial Biological Network

In order to ascertain the effect that incomplete or missing information had on the systems level analysis, the Claytor artificial biochemical network was used. The Claytor network is shown in figure 2.1 . As was mentioned previously in this chapter, artificial biological networks are important modeling resources. Without a predefined artificial network, it would be impossible to make a meaningful comparison between studies where all information is known and those where knowledge is limited. Initially introduced as dream1n3 to the DREAM challenge (Stolovitzky et al. 2007; Stolovitzky et al. 2009) as a means of benchmarking network inference, or top down modeling methods, the Claytor network represents a small biochemical network that includes metabolism, signal transduction, and gene regulation aspects. The Claytor artificial biological network was chosen for this study because its complexity mirrors that of true biochemical systems, while remaining much more tractable (e.g. it has only 20 genes).

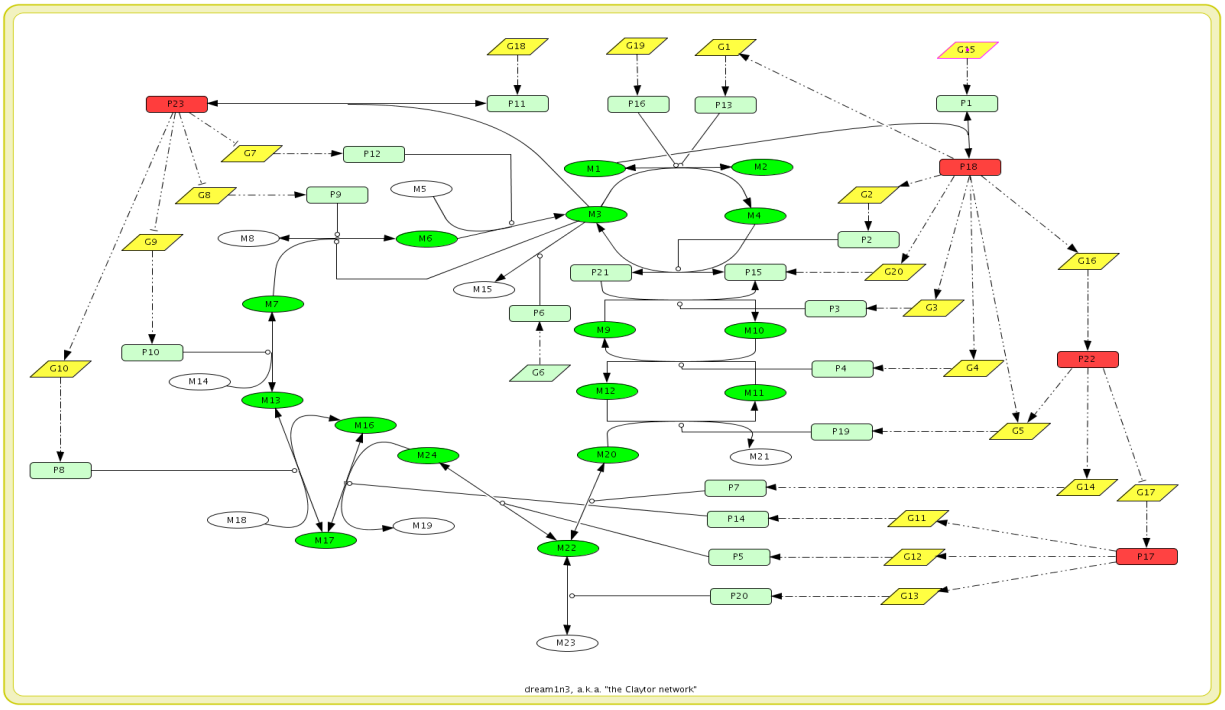


Figure 2.1 The Claytor artificial biological network. Rectangle represent proteins. Red rectangles act as transcription factors, while green ones act as enzymes or directly participate as reactants or products in reactions. Clear ellipses are external metabolites, while green ellipses represent internal metabolites. Yellow parallelograms represent genes as mRNA concentrations. Used with permission from P. Mendes.

Properties of the Full Claytor Network

The complete Claytor network contains 59 internal state variables, of which 16 are metabolites, 23 are protein forms, and 20 are genes. The Claytor network also encompasses a simplified, artificial environment. This environment encompasses 8 external metabolites which may be used to perturb the full network in a manner reminiscent of exo-metabolite perturbations in metabolomics studies. The metabolic and transcriptomic portions of the Claytor network follow realistic kinetics which depend on the nature of their interactions. As a result, the kinetics of the various metabolic reactions and those involved in transcription, translation, and degradation of proteins and mRNA encompass a wide range of dynamic scales and steady state levels. In addition to realistic kinetics, the components of the Claytor network are subject to both both hierarchical (gene regulation) and metabolic control.

The metabolic portion of the Claytor network contains subnets which are involved in the processes of catabolism, biosynthesis of a co-factor, and redox chain reactions to remove a toxic external compound, M1. In addition, the metabolic portion of the full Claytor network includes other features which appear in biochemical networks but are not usually included in artificial ones. There is competition for the main energy source, M12, between the subnets functioning in catabolism and generation of redox potential. The redox chain, which is crucial for detoxification, contains a mixture of small molecules and proteins that can exist in both an oxidized and reduced state.

The full Claytor network also contains signaling pathways which can sense presence of the toxic M1 or the detoxified M3. The signaling pathways are reminiscent of those found in biochemical systems, where receptor proteins bind to their target and form complexes, which can then act as transcription factors.

Transcriptional control is also present in the Claytor network, beyond its role in signaling: two additional transcription factors encode genes that are subject to regulation by other transcription factors. Each gene codes for a specific mRNA that can produce a protein. Thus, knocking out a given gene will have an effect on its target protein concentration. However, similar to biological systems, several genes have redundant functions so that knocking out a given gene in the network will not necessarily eliminate the function since there may be another protein carrying out that function (though with different properties).

Claytor Metabolism Model

The Claytor metabolism model consists of the metabolic portion in isolation from the full network. Unlike the complete version of the network, the Claytor metabolism model does not represent transcriptional control. Thus, the Claytor metabolism model mirrors studies where the focus is the metabolic portion of a system and the rest of the network is excluded (Broeckling et al. 2005; Draeger et al. 2009; Guy et al. 2008; Moco et al. 2009). Because the true systems level properties of the entire network are known, it is possible to quantify the effect of using only a portion of the network in an analysis. The subset of reactions which constituted the metabolism portion were identified by including reactions which contained metabolites. The only proteins included explicitly are those that

react with metabolites (in the redox cycles), the proteins that catalyze the metabolic reactions are represented only implicitly in the kinetic parameters of the reactions, and therefore became constants of the model. The metabolism reactions used in this study were R1, R2, R3, R4, R5, R6, R7, R8, R9, R10, R11, R12, R13, R14, and R19. It was unclear whether or not the signaling reactions for M1 and M3 should be included as part of the metabolism set of reactions. Under transcriptional control, these reactions act as a signaling mechanism to detect M1 and M3 levels. However, when transcriptional regulation is removed from the model, the proteins in the reaction should act as receptors and sequester the relevant metabolite. A comparison was made between metabolism models which contained the M1 and M3 signaling reactions and one which did not. The Akaike's information criterion (Akaike 1974), which is described later, was used as a basis for this decision. As a result of this comparison, the model without M1 and M3 signaling was selected. This model is depicted in figure 2.2. These reactions were the targets for the kinetic estimation procedures in addition. They were replaced by the relevant generalized rate laws in both the studies where the rest of the Claytor network remained intact as well as those where only the metabolic reactions were included. Details of this comparison are in the results and discussion section.

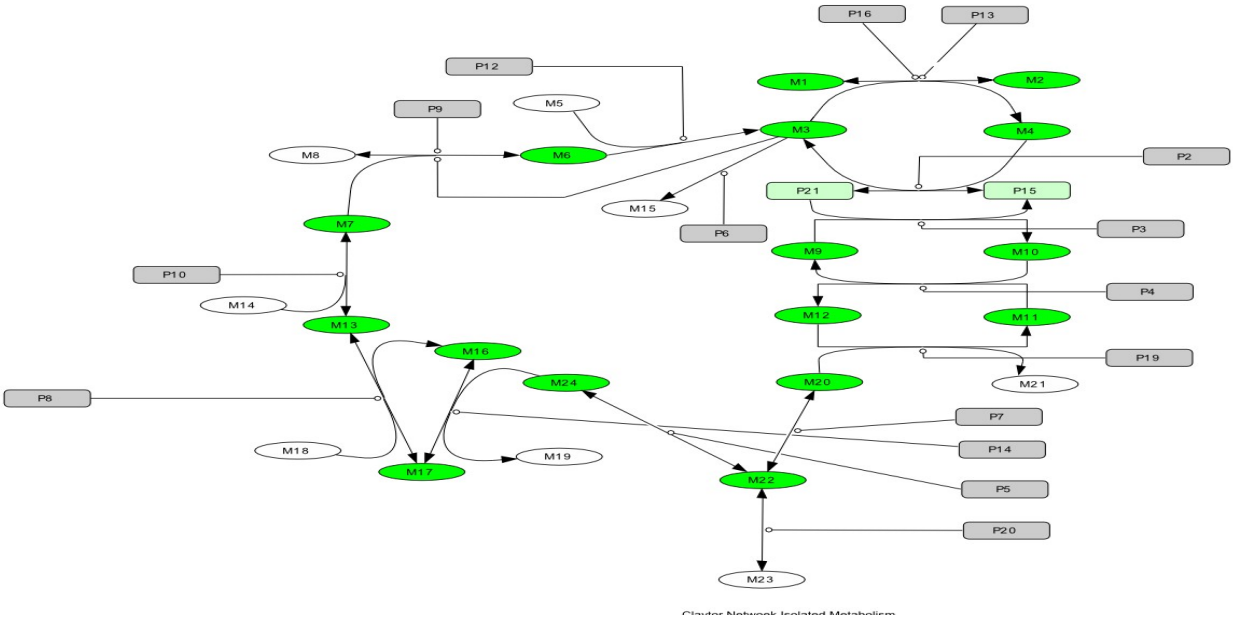


Figure 2.2 The Claytor isolated metabolism model is shown. This model consists of a subset of reactions from the Claytor network. Gray rectangles are enzymes. Unlike the full Claytor network, these are initial enzyme concentrations and do not change. The green rectangles are proteins which directly participate in reactions as products or reactants. Their concentrations are therefore allowed to change. The metabolite representations are identical to those in the complete Claytor network. Clear ellipses represent external metabolites, while green ellipses represent internal metabolites.

Datasets

Two classes of *in silico* "experimental" datasets were created for the purposes the current study. One set is used as a training set for estimating kinetic parameters and in certain models estimating the initial protein concentrations. The second set was used as a test set so that validation could be performed, thus preventing over fitting. Validation is employed both during the process of parameter estimation, and again to test the final model's performance on a novel dataset. In order to capture the inherent dynamics of the system, and to parallel the types of data necessary to study biological systems, time-series datasets were generated for both training and testing of models. These training and testing time-series datasets were composed of both metabolic and genetic perturbations. Both the training and test datasets were created by using the simulation package COPASI (S. Hoops et al. 2006) .

The training dataset consists of perturbations in the concentration of the metabolite M1. Because M1 is toxic to the network, many reactions must take place in order to remove it from the system. Both the redox chain and energy production elements of the metabolic portion of the Claytor network must be activated in order to remove M1. In order to form the training datasets, initial concentration of M1 was systematically perturbed starting at an initial concentration of 0.05 mmol/mL and ending with a final initial concentration of 0.25 mmol/mL. This resulted in 5 experiments each with a different initial concentration of M1. Figure 2.3 demonstrates how the metabolites within the full Claytor network model react to an initial M1 concentration of 0.05 mmol/mL . It is important to note that all metabolite concentrations change, but the differences in scale make some of these changes more apparent in the figure than others. As in biological systems, the response is a dynamic process. The Claytor network initially meets the increased M1 concentration with increases in the activity of both the redox chain and in energy production in order to detoxify its internal environment as quickly as possible. Eventually, the levels of M1 are successfully reduced.

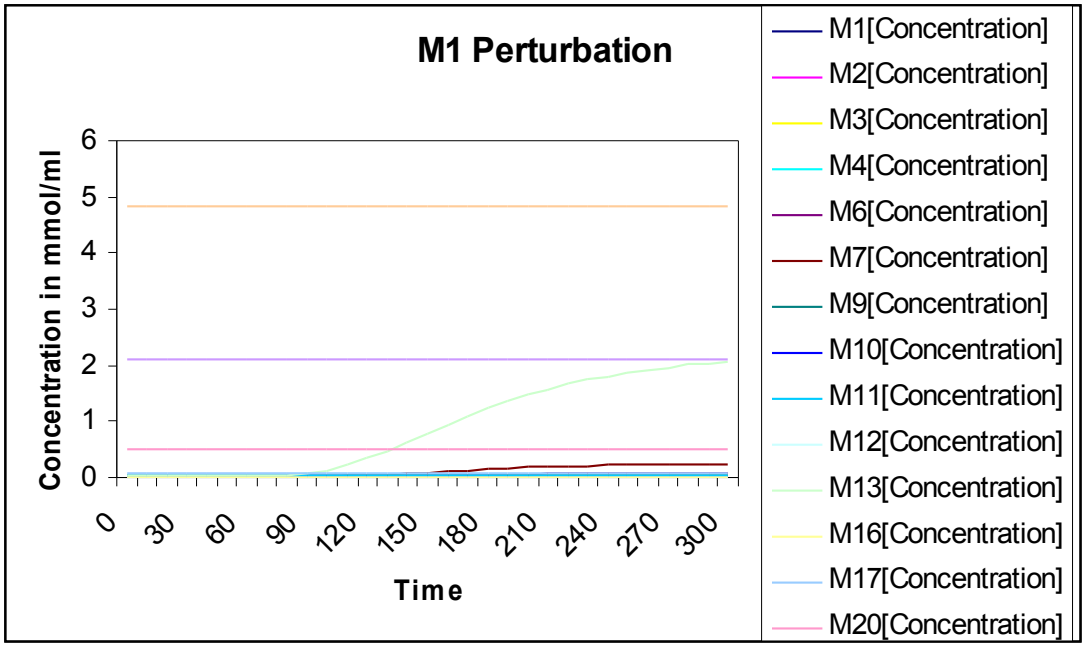


Figure 2.3. The response of the claytor network model metabolite concentrations to an increase of the initial M1 concentration from 0 to 0.05 mmol/mL.

In order to provide an internal validation dataset that was distinct from the training set, an artificial

gene knockout was created by setting the rate constant V of mRNA synthesis of gene G16 to 0. By setting the parameter V to 0, no mRNA synthesis occurs for the selected gene. In figure 2.4, the effect of knocking out G16 is shown. By knocking out G16, a disturbance is created in the network that is soon compensated for by other members. Thus demonstrating the redundancy inherent in the system. This is comparable to the effect that non-lethal gene knockouts can have in biological systems.

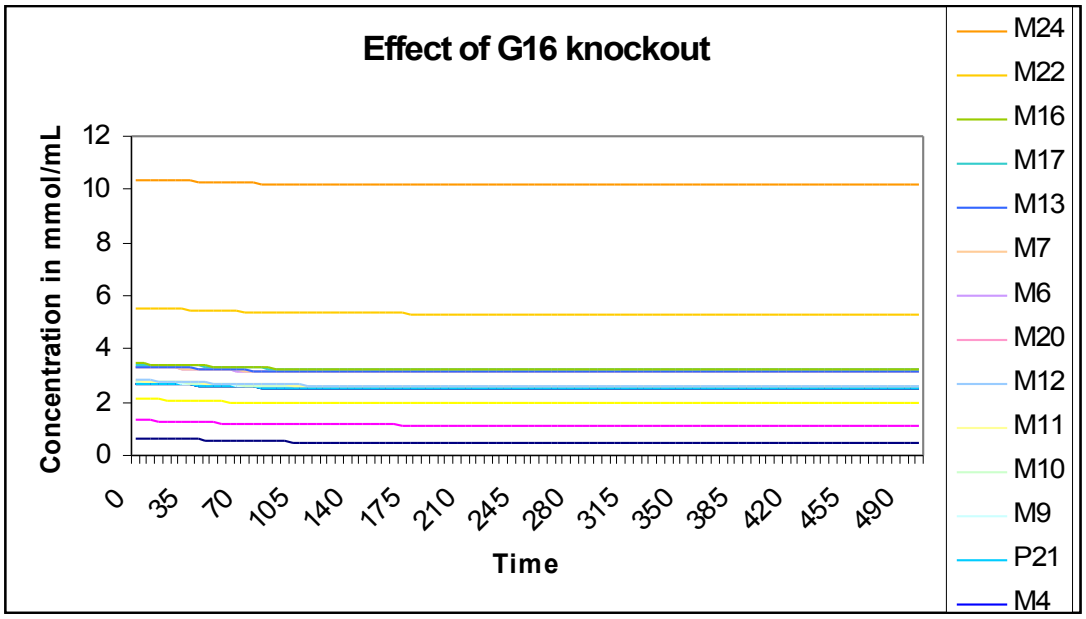


Figure 2.4. The effect of knocking out G16 on the dependent variable in the Claytor metabolic network is shown. In some instances, the G16 knock out (KO) had no effect on a variable's concentration. These non-affected variables are not shown. In most of the species, the G16 KO caused a perturbation in the earlier time points which subsided by the end of the simulation.

The final validation sets were composed of both metabolic and genetic perturbations. In the metabolic perturbation, the initial concentration of metabolite M23, which serves as an energy source for the Claytor network, is increased incrementally from 0.05 to 2.0 mmol/mL. As can be seen in figure 2.5, even an initial M23 concentration of 0.05 mmol/mL, which is much less than the full model initial concentration of 1 mmol/mL, results in a lot of initial activity which soon dissipates as the supply of M23 is exhausted. The genetic perturbation consisted in a knock out of G19. This G18 knock out (KO) was achieved in the same manner as the G16 KO which was used in the earlier model validation. The G18 KO is more subtle as it effects metabolites which operate at different scales. Because the scales of the effected metabolites are often much smaller than the non-affected ones, the changes can be more

difficult to identify. In figure 2.6, a plot of the effect of the G18 KO for all dependent variables in the Claytor network metabolic model is shown. In addition, figure 2.6 B shows the small effect that the G18 KO has on M4, which is not seen due to scale issues, when all dependent variables are co-plotted. While M4 is not the only metabolite which responds to this genetic perturbation, it serves as an example.

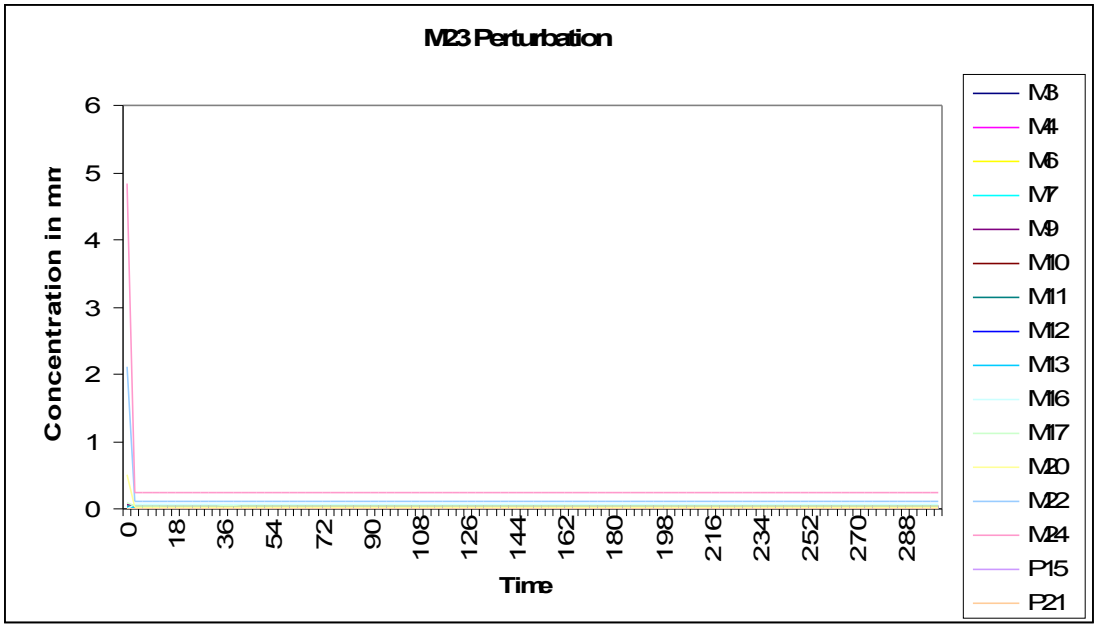
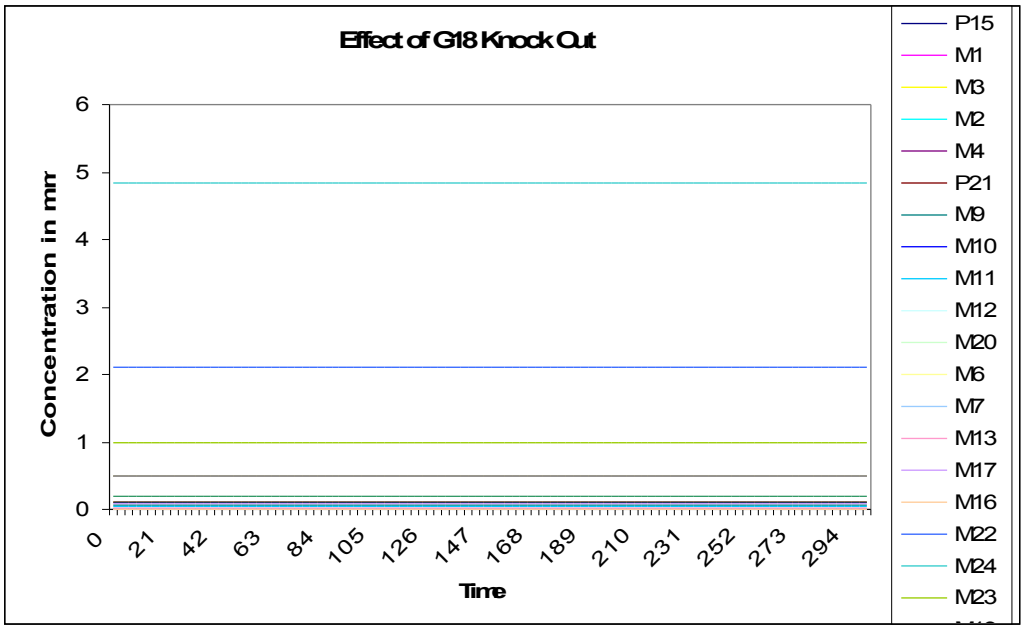


Figure 2.5. The effect on the dependent variables of the Claytor metabolism model of an initial M23 Concentration of 0.05 mmol/mL. As can be seen from the figure, most of the network response occurs in the early time points. Metabolites which were unaffected are not shown.

In addition to the aforementioned perturbations, additional genetic perturbations were applied to the Claytor network. However, several of these, such as a knock out of G9, resulted in lethality. This is reminiscent of the knock outs of critical genes in natural biological networks. However, such perturbations do not provide the kinds of information necessary for this study. Therefore, these perturbations were not used in the current study.

A.



B.

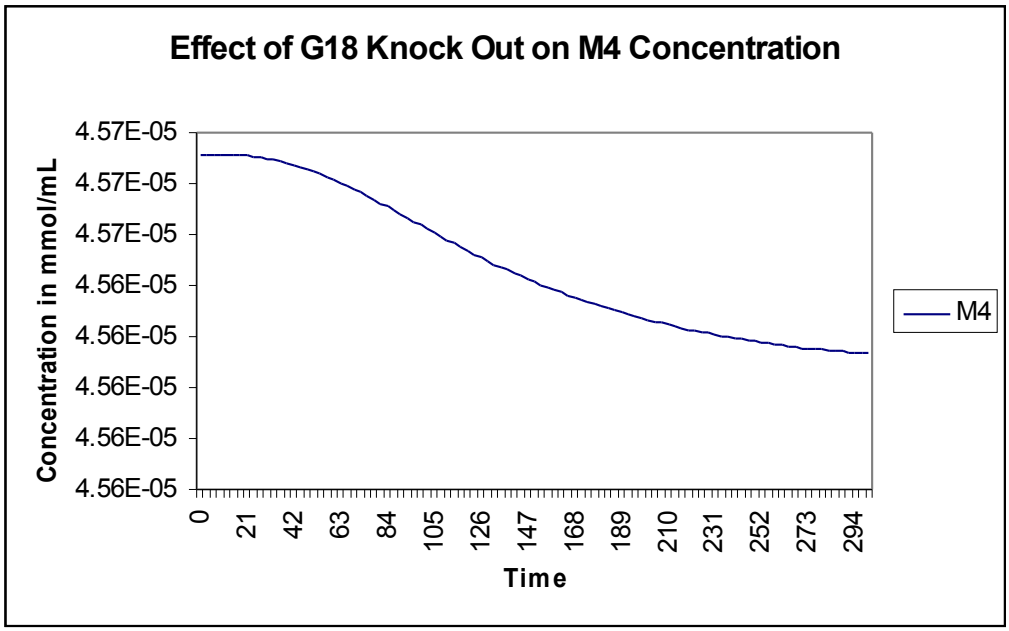


Figure 2.6. The effect of knocking out gene G18 on the Claytor network metabolism model. **A.** The effect show on all dependent variables in the model. **B.** The effect of knocking out G18 demonstrated for M4.

Simulation and Parameter Estimation

Parameter estimation as well as all other simulations were performed using the package COPASI (S. Hoops et al. 2006). COPASI is a biochemical network simulation tool which can perform internal validation in addition to employing a number of methods for parameter estimation. For this study, two parameter estimation methods were used: particle swarm (Kennedy & Eberhart 1995) and genetic algorithms with stochastic ranking (SR)(Runarsson & Yao 2000) . These algorithms were chosen because of their ability to explore a large portion of the parameter space, and thus have a chance of avoiding local minima. In the particle swarm optimization particles represent candidate solutions that exist in a multidimensional space of the parameters; multiple particles are initialized with differing velocities and starting positions. Then, in a manner which mimics swarm species searching for food, the particles move across the parameter space. Each particle remembers its last best position and objective value. This information is then reciprocally shared with its neighboring swarm particles. Thus, there is an individual best result, a local neighborhood best result, and a global best result. These results are used to guide the subsequent movements of the swarm (Kennedy & Eberhart 1995). Usually, the individual results converge for the entire population at the end of the simulation. These results are often better than non-swarm approaches (Clerc & Kennedy 2002).

While particle swarm optimization attempts to mimic social intelligence, genetic algorithms (GAs) attempt to invoke the concept of natural selection (Goldberg 1989). In genetic algorithms, populations are also used, but rather than moving around in space to search for a solution, the individuals are now reproducing and generating new ones with some errors (mutations) or recombination. Each individual in a GA is composed of chromosomes. These chromosomes are generally bit strings which encode a solution to the current problem. In COPASI, these solutions are encoded using floating point notation. The fitness of each individual chromosome is dependent upon its overall fitness, as determined by its objective value. The fittest individuals are selected at each iteration and allowed to reproduce. The offspring are composed of the best solutions from the previous generation with crossover and mutation effects added in. After a given number of generations, the GA will stop searching for a more optimal solution. Although GAs have been shown to reach the global optimum, there is no guarantee of when this optimum will be achieved and if the current solution is that optimum (Schmitt & Droste 2006) . Due to the stochastic nature of these algorithms, multiple simulations were performed with

combinations of both algorithms being employed on the same dataset. The parameters which were used in running these optimization methods is shown in table 2.1.

Table 2.1 : Parameters used with each optimization method

Optimization Method	Parameters
Particle Swarm	Number of Generations: 2000 Population Size: 35
Genetic Algorithms SR	Number of Generations: 1500 Population Size: 55

These parameter estimation tasks were initially performed using a subset of the training and test datasets. In the training set, the initial subset was composed of the M1 perturbation time-series monitored at every 50 seconds as opposed to every 10 seconds. For validation, the initial test dataset was comprised of a gene knockout of G16. After a satisfactory preliminary estimate of the true model was reached, the remainder of the available data was then added and used to obtain a final model estimate.

Due to the large parameter search space, parameter estimation was performed by iteratively searching larger portions of the solution space. Parameters in the model were initially confined to a range which was near the true values for each reaction. These search space restrictions were imposed in order to improve the chances for the metabolic model of Claytor network to find an optimal solution. After each parameter estimation run was completed, the location of each parameter estimate with respect to its range was investigated. In cases where the estimate was near the boundary of that range, the range was increased for the next iteration of parameter estimation. This process was continued until the sum of squares for each model showed a minimum of improvement on each new run. Each set of model parameter estimations was done for several different random starting values in addition to the use of the true parameter values.

Determining Significance of Pairwise Comparisons of Parameter Values Obtained in Intact and in Isolated Metabolism Studies of the Claytor Network

One of the main benefits to using an artificial biological network is that the true values of parameters are known. This makes it possible to compare the parameter values obtained for the Claytor metabolism model to those in the intact network. By comparing these parameters, it is possible to assess the effect that studying the metabolome in isolation has on the analysis of the metabolic reactions overall as well as the effect on individual reactions. In order to determine when differences between parameter values were significant, the Wilcoxon signed rank test was employed. The Wilcoxon signed rank test was preferred over the t-test, as it is nonparametric and does not make the assumption of normality (Whitley & Ball 2002).

The Wilcoxon signed rank test was applied using the internal R stats package (R Development Core Team 2009) . The two sided pairwise Wilcoxon test was used to obtain the significance of differences between parameter values on both the overall set of reactions as well as on individual reactions. By making these comparisons at different resolutions, it is possible to determine whether individual reactions are more affected by studying the metabolome in isolation than others as well as to assess the parameter differences overall.

Predicting Control Type of Differing Experiments

In biological systems, reactions are often under both transcriptional, or hierarchical, and metabolic control (Heinrich & Schuster 1996; Kacser & JA Burns 1995; A de la Fuente et al. 2002). Depending upon environmental conditions and the overall network structure, a given reaction may be under one form of control more than another at a given point in time. If a given metabolic reaction is under stronger hierarchical control during a perturbation, then it is reasonable to hypothesize that an analysis which takes transcription into account would give more accurate results for this reaction. Similarly, an isolated study of metabolism might be sufficient to understand reactions which are only weakly affected by transcription. Therefore, the degree to which an isolated metabolic study may be successful for a series of reactions should correlate to the distribution and type of control its components are under.

In this study, the type and amount of control a metabolic reaction is under is predicted in the following

manner. For each dependent variable in the metabolic network, a series of p-values are calculated based upon its Kendall's correlation (Kendall 1938) to the concentrations of predetermined transcriptional regulatory species as well as to other metabolites. In this study, transcriptional elements are considered to be the transcription factor and mRNA concentrations for a given experimental perturbation of the Claytor network. The metabolic elements include all of the metabolite concentrations as well as those of the proteins which were directly involved as substrates or products in the metabolic reactions, namely P15 and P21. Enzymes were classified as contributing to transcriptional control of a dependent variable of metabolism when they catalyzed a reaction in which it participates. In all other cases, correlations with proteins are classified as a form of metabolic control. This is the result of reasoning that a strong correlation between an enzyme concentration and that of the dependent variable is due either to direct interaction or to indirect action. When the correlation is due to direct interaction, the enzyme catalyzes a reaction which either creates or depletes the variable concentration. In cases where there is an indirect interaction, the enzyme could be responsible for either limiting or producing a needed precursor for the synthesis of the dependent variable, or affecting the rate at which it is depleted. When there is direct interaction between the enzyme and dependent variable, the control is hierarchical as it depends upon the concentration of the enzyme which is itself controlled via transcription. In the case of indirect interaction, the control is exerted via the structure of the metabolic network, and is thus considered metabolic in nature.

In this study, Kendall correlations are used to assess association between the dependent variables in metabolism and elements affiliated with a given control type. The Kendall correlation coefficient, $\boldsymbol{T}$, is a nonparametric rank statistic which measures the strength of associations via cross tabulations (Kendall 1938) . These $\boldsymbol{T}$ values can be subsequently converted into p-values (Kendall 1975; Valz & Thompson 1994). In this study, the Kendall correlation coefficients were obtained using the R correlation package (R Development Core Team 2009). These T values were then separated according to whether they suggested correlation, via positive values, or anti-correlation, via negative values, or no correlation as when T was 0. The probability values of each correlation, or anti-correlation coefficient were obtained using the SuppDists R package (Wheeler 2009). All Kendall correlations between the dependent variables of metabolism and elements associated with a control type in the Claytor network which achieved p-values significant at the $\boldsymbol{\alpha} = 0.05$ level were considered for further analysis. These p-values were then converted to Z-score using the inverse normal cumulative distribution, $\theta^{(-1)}$. For

each p-value, p_i, the Z-score is calculated by:

$$Z = \theta^{(-1)}(1 - p_i)$$

Aggregate Z-scores for correlations to transcriptional regulatory elements and to metabolic elements are then calculated for each dependent variable of Claytor metabolism. For each dependent variable ***n***, the aggregate Z-score for a given regulation was calculated as:

$$Z_{var_{aggregate-regulation}} = \frac{\sum_{n=1}^{K} Z_{ni}}{\sqrt{K}}$$

where Z_{ni} represents the Z-score for a given correlation probability of a dependent variable to a regulatory element ***i***. K is either the number of transcriptional regulatory elements or metabolic elements which had a significant correlation to the given dependent variable. Finally, these individual aggregate Z-scores for dependent variables, ***n***, were combined according to reaction to obtain an aggregate Z-score of reaction:

$$Z_{reaction_{aggregate-regulation}} = \sum_{i=1}^{R} Z_{var_{(aggregate-regulation-i)}} \quad ,$$

where R, represents the number of metabolic species in a given reaction. In this way, the primary control type of a given reaction could be determined along with the individual control types of each of its members. Each reaction was considered to be under hierarchical control if its aggregate reaction Z-score for transcriptional regulation was higher than that for metabolic regulation. Similarly, when the aggregate reaction Z-score for metabolic regulation was higher, the reaction was considered under metabolic control. If the aggregate Z-scores for the two regulation types were not significantly different the reaction was considered under shared metabolic and hierarchical control.

This analysis was carried out to predict the primary control type of reactions in the following experimental conditions: a perturbation of the initial M1 concentration from 0 to 0.05 mmol/ml, a decrease in the initial concentration of M23 from 1 to 0.05 mmol/mL, and a knock out of gene G18. In

each case, a time course ranging from 0 to 500 model time units was simulated and measurements were sampled at every 50^{th} interval.

In some cases, a given experiment can provide no information concerning the control of a given dependent variable. For example, this can occur in perturbations where there is no effect upon a variable's concentration. When the concentration of a variable does not change, calculations of its Kendall's correlation with other variables is impossible. In these cases, the variable is excluded.

It is important to note that this procedure is significantly different than metabolic control analysis (Kacser & JA Burns 1995; Kacser & J. A. Burns 1979; Heinrich & Schuster 1996) or hierarchical control analysis (HCA) (H.V. Westerhoff & Kahn 1993; ter Kuile & H. V. Westerhoff 2001; A de la Fuente et al. 2002) and gives a different type of result. The prediction made here is based upon how correlated the dependent variables of metabolism (those which are substrates and products of metabolic reactions) are with moieties that are associated with a given form of control. These correlations can differ depending upon the experimental condition. The goal of this analysis is to determine if high levels of correlation with these purported regulatory elements makes it possible to predict how likely it is to get close to the true system parameters under certain experimental conditions by only examining metabolism.

Determining Model Performance

The ability of a given model to accurately describe the system level properties of the Claytor network was determined in two ways. As a first measure, the sum of squares between a given model and the data was used. In particular, the sum of squares of the overall fit of the model to the data as well as that of the individual fits to the pertinent dependent variables were taken into account.

As a second measure, a visual assessment of the fit of the dependent variables of interest to the data was taken. The dependent values of interest in this case were the metabolites and the proteins which played a direct role, as opposed to a modifying role, in the metabolic reactions. The quality fit of each dependent variable was visually graded using a binary scheme. Each fit was assigned a bit string based on an assessment of certain features. These features and the criteria for each one to be defined as either

one or zero are displayed in table 2.2. Employing both the sum of squares and a descriptive method of comparing the model fits has two main benefits. For one, by using the sum of squares it becomes possible to make quantitative claims about the fit quality. Secondly, it is possible to determine which fit features contributed to the sum of squares value.

Table 2.2 Description of the quality bit score

Bit Position	Description	Conditions to Be Defined as '1'
1	Overall fit	Overall visual quality of fit must be good
2	Shape	Shape of prediction curve is close to that of data
3	Scale	Predictions can be viewed simultaneously with data without either losing important shape features
4	Direction	Overall direction, increasing or decreasing, of predictions match those of data
5	Alignment	Prediction fit curve must align near data curve

Model Selection

In the case where a version of the Claytor metabolism model had to be selected, Akaike's information criterion was used. Akaike's information criterion (AIC) is a measure of how well a model fits the given data. It is based on the concept of information entropy. The AIC can be described in terms of the likelihood as:

$$AIC = -\ln(L) + 2k$$

where L represents the maximized likelihood of the data given the model and where k represents the number of parameters in the given model. The AIC can also be expressed in terms of the sum of squares, as it was used in this study. In this case, the AIC is written as:

$$AIC = N(\ln(SSQ/N)) + 2k \quad ,$$

where k again represents the number of parameters, N is the number of observed data points, and SSQ is the residual sum of squares. The goal of AIC is to identify the model which best fits the data using the smallest number of parameters. Thus, increased numbers of parameters are penalized. This ensures

that a model will not be selected simply because it fits the dataset perfectly as there is some preference for simpler models. In cases where the AIC values of differing models were compared, the one with the smallest AIC was selected (Akaike 1974; Burnham & Anderson 1998).

Results and Discussion

Determining the Subset of Reactions to be Included in the Metabolism Model

It is a common practice in many systems biology studies for the metabolome, the genome, and the transcriptome to be studied separately. In order to determine the effect of analyzing the metabolome in isolation, the metabolism portion of the Claytor network was extracted from the full network and parametrized as described in the experimental design section . As mentioned in the experimental design section on forming the Claytor metabolism model using the original kinetics, a decision had to be made whether to include the signaling reactions for M1 and M3. While all of the other metabolic reactions are primarily composed of metabolites, proteins play a primary role in the M1 and M3 signaling reactions. In these reactions, the signaling metabolite, M1 or M3, interacts with a receptor protein to form a transcription factor. This transcription factor subsequently activates a cascade of transcriptional-mediated events which compensate for the presence of the signaling metabolites. Because the metabolites act as part of a signaling cascade in these reactions, it was unclear if they should be included in the metabolism models. In order to solve this dilemma, two Claytor metabolism models were constructed and their respective parameters estimated. In one model, the M1 and M3 signaling reactions were included, while in the other they were not. The two models were compared using the Akaike's information criterion (AIC). The results of this comparison are shown in table 2.3. The model which excluded the M1 and M3 signaling reactions obtained the lowest AIC value. Therefore, this model was chosen to represent the metabolic portion of the Claytor network both here and in the later metabolism models where the true rate equations are replaced with estimated versions.

Table 2.3 : A comparison of two different versions of the metabolism model considered

Metabolism Model	Number of Parameters	Number of Data Points	Sum of Squares	AIC
Including M1 and M3	33	7280	1.10E-04	3.49E+05

Signaling Reactions				
Excluding M1 and M3 Signaling Reactions	29	6240	6.81E-03	2.76E+05

Determining the Primary Control Type for Metabolic Reactions

The control types for reactions under various experimental conditions were predicted as described in the Materials and Methods section. In tables 2.4, 2.5, and 2.6, the aggregate hierarchical and metabolic control based Z-scores of each dependent variable in Claytor metabolism is given for the M1 and M23 perturbations as well as for the G18 knock out. The $\log_2$ ratio of the metabolic aggregate Z-score to the hierarchical aggregate Z-score is also shown as a way of comparing the relative preference of one control over the other.

Table 2.4 : The aggregate hierarchical and metabolic control based Z-scores of each dependent variable in Claytor metabolism for an initial M1 concentration of 0.05 mmol/mL

Metabolic Dependent Variable	Hierarchical Aggregate Z-score	Metabolic Aggregate Z-score	Log_2(MAZ/HAZ)
M1	5.888	12.814	1.122
M3	11.311	15.895	0.491
M2	5.888	11.800	1.003
M4	10.179	15.895	0.643
M9	6.216	10.355	0.736
M10	6.216	9.057	0.543
M11	6.216	7.608	0.292
M12	6.216	5.952	-0.063
M20	5.512	9.306	0.756
M6	12.154	13.016	0.099
M7	12.154	11.902	-0.030
M13	12.154	10.681	-0.186
M17	5.136	9.108	0.826
M16	5.136	7.595	0.564
M22	5.502	6.384	0.215
M24	4.952	4.539	-0.126
P15	11.824	8.084	-0.549
P21	8.649	8.547	-0.017

Table 2.5 : The aggregate hierarchical and metabolic control based Z-scores of each dependent variable in Claytor metabolism for a M23 initial concentration of 0.05 mmol/mL

Metabolic Dependent Variable	Hierarchical Aggregate Z-score	Metabolic Aggregate Z-score	Log_2(MAZ/HAZ)
M1	No Information	No Information	No Information
M3	2.950	13.522	2.197
M2	No Information	No Information	No Information
M4	3.909	13.095	1.744
M9	4.194	13.041	1.637
M10	4.194	12.075	1.526
M11	4.194	11.054	1.398
M12	3.648	9.549	1.388
M20	2.328	9.720	2.062
M6	4.328	11.689	1.433
M7	5.012	11.043	1.140
M13	6.104	5.626	-0.118
M17	2.328	10.975	2.237
M16	2.328	12.123	2.381
M22	2.328	8.324	1.838
M24	2.328	6.737	1.533
P15	5.326	8.122	0.609
P21	5.326	6.418	0.269

Table 2.6 : The aggregate hierarchical and metabolic control based Z-scores of each dependent variable in Claytor metabolism for a G18 knock out

Metabolic Dependent Variable	Hierarchical Aggregate Z-score	Metabolic Aggregate Z-score	Log_2(MAZ/HAZ)
M1	No Information	No Information	No Information
M3	6.551	12.704	0.955
M2	No Information	No Information	No Information
M4	3.870	10.024	1.373
M9	No Information	No Information	No Information
M10	No Information	No Information	No Information
M11	No Information	No Information	No Information
M12	2.467	3.901	0.661
M20	No Information	No Information	No Information
M6	7.770	10.685	0.460
M7	8.081	9.046	0.163
M13	8.647	6.884	-0.329
M17	4.739	6.900	0.542
M16	7.810	5.548	-0.493
M22	No Information	No Information	No Information

M24	No Information	No Information	No Information
P15	No Information	No Information	No Information
P21	No Information	No Information	No Information

As can be seen in table, many of the aggregate Z-scores for the dependent variables show a preference for metabolic control in all of the experimental conditions under study. In the M1 perturbation experiment, 12 of 18 variables showing at least a weak preference. In the perturbation of the concentration of M23, all but one variable, M13, has a higher metabolic aggregate Z-score value than a hierarchical one. The metabolic aggregate Z-scores for this experiment are much higher than in the M1 perturbation.

No control preference could be assigned to M1 or M2 in this case, as neither dependent variable's concentration was affected by the M23 perturbation. This result is to be expected, as M1 is a toxic element and M2 is involved with its removal. Unless M1 is directly perturbed, these concentrations are unlikely to change. The knock out of G18 affected the least number of metabolic dependent variables, resulting in information for only 8 of the 18 total variables. This phenomenon is paralleled in other biological studies. Certain experimental perturbations will yield more information than others and care must be taken to account for this. In this case, the more information rich dataset, the M1 perturbation, is used to estimate the model parameters. If one of the less information rich datasets had been used, it is unlikely that the resultant model would be able to account for changes associated with an M1 perturbation.

Of the three experiments, the M1 perturbation contains the highest number of dependent variables with stronger hierarchical associated control aggregate Z-scores. However, many of the log2 ratios of these are close to 0, and therefore cannot be confidently classified as under transcriptional control. In all three experiments, metabolite M13 was unique in that it consistently had a higher aggregate Z-score for hierarchical control than for metabolic control.

For each reaction, the substrate and reactant aggregate Z-scores are summed for each control type, as mentioned in the materials and methods section. These reaction sums of the aggregate Z-scores for each control type and experimental condition are shown below in tables 2.7, 2.8, and 2.9 along with

the $\log_2$ ratio of the two sums. In the perturbation of M1 concentration and in the G18 knock out, reaction R9 has a slightly higher hierarchical aggregate Z-score reaction sum. In the G18 knock out, reaction R11 also has a slightly higher hierarchical aggregate Z-score reaction sum .The metabolic reactions in the perturbation of M23 concentration, by contrast, consistently have higher metabolic aggregate Z-score sums.

Table 2.7 : The aggregate hierarchical and metabolic aggregate Z-scores for each reaction as calculated for an initial M1 concentration of 0.05 mmol/ml

Reaction	Hierarchical Aggregate Z-score Sum	Metabolic Aggregate Z-score Sum	Log_2(MAZS/HAZS)
R1	23.088	40.509	0.811
R2	30.651	32.526	0.086
R3	26.689	26.985	0.016
R4	18.648	27.019	0.535
R5	17.944	22.865	0.350
R6	11.311	15.895	0.491
R7	23.465	28.912	0.301
R8	24.308	24.919	0.036
R9	24.308	22.583	-0.106
R10	17.290	19.789	0.195
R11	15.224	21.243	0.481
R12	10.454	10.923	0.063
R13	5.502	6.384	0.215
R14	11.014	15.690	0.511
R19	23.088	40.509	0.811

Table 2.8 : The aggregate hierarchical and metabolic aggregate Z-scores for each reaction as calculated for an initial M23 concentration of 0.05 mmol/ml

Reaction	Hierarchical Aggregate Z-score Sum	Metabolic Aggregate Z-score Sum	Log_2(MAZS/HAZS)
R1	2.950	13.522	2.197
R2	14.562	27.634	0.924
R3	14.846	27.581	0.894
R4	12.581	36.171	1.524
R5	10.170	30.323	1.576
R6	2.950	13.522	2.197

R7	7.277	25.211	1.793
R8	9.340	22.732	1.283
R9	11.116	16.669	0.585
R10	8.433	16.602	0.977
R11	6.985	29.835	2.095
R12	4.657	15.061	1.693
R13	2.328	8.324	1.838
R14	4.657	18.044	1.954
R19	2.950	13.522	2.197

Table 2.9 : The aggregate hierarchical and metabolic aggregate Z-scores for each reaction as calculated for a knockout of G18

Reaction	Hierarchical Aggregate Z-score Sum	Metabolic Aggregate Z-score Sum	Log_2(MAZS/HAZS)
R1	6.551	12.704	0.955
R2	3.870	10.024	1.373
R3	No Information	No Information	No Information
R4	No Information	No Information	No Information
R5	2.467	3.901	0.661
R6	6.551	12.704	0.955
R7	14.321	23.390	0.708
R8	15.851	19.731	0.316
R9	16.728	15.929	-0.071
R10	13.386	13.784	0.042
R11	12.549	12.449	-0.012
R12	No Information	No Information	No Information
R13	No Information	No Information	No Information
R14	No Information	No Information	No Information
R19	6.551	12.704	0.955

Based upon these results, it is predicted that using an isolated model of metabolism to study these reactions would be appropriate and that regulation by transcription/translation and signaling were minor effects in the data. Because most of the dependent variables of metabolism under study as well as their reactions are more associated with metabolic control elements, excluding transcriptional control should not significantly negatively impact the study. In cases where a given dependent variable has difficulty being fit in a certain perturbation, it is expected that it will have a higher hierarchical control based aggregate Z-score or only a slightly higher metabolic one. It is further predicted that those reactions which have the lowest metabolic control based aggregate Z-score sums will achieve

parameter values most different from their true counterparts.

Claytor Network Metabolism Model Fitting Results

Once the reactions which comprised the metabolic portion of the claytor network were established, its ability to estimate the true kinetics of the system could be determined. The sum of squares values for all experiments can be seen in table 2.10. In table 2.11, the sum of squares values for fits to the dependent variables as well as the bit string scores for experiments1 and 5 are given. Experiments 1 and 5 were chosen because they represent the lowest and highest M1 initial concentrations respectively, as well as the lowest and highest experimental sum of squares values. As mentioned in the material and methods section, experiment 1 has an initial M1 concentration of 0.05 mmol/ml, while experiment 5 has a higher M1 starting concentration of 0.25 mmol/ml. Fixed variables are not shown in this or any of the following tables as they are not fit in any of the simulations (i.e. their values are the same throughout). These fixed variables include: M5, M8, M14, M15, M18, M19, and M23.

Table 2.10 : Residual sum of squares for fits of Claytor metabolism model to each experiment

Experiment	Initial M1 concentration in mmol/ml	Sum of Squares
1	0.05	5.78E-004
2	0.10	9.28E-004
3	0.15	1.35E-003
4	0.20	1.72E-003
5	0.25	1.87E-003

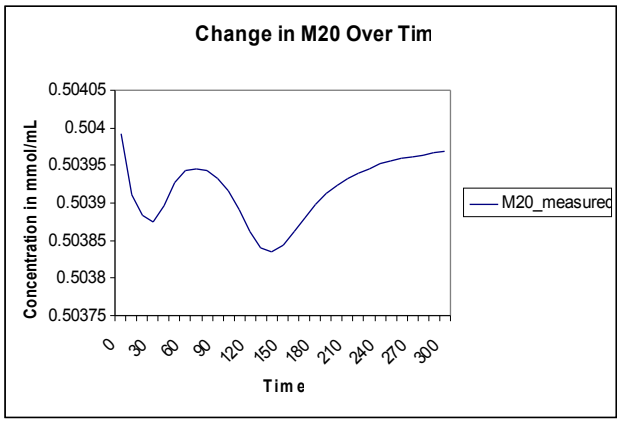
Table 2.11 : Residual sum of squares for fits of each dependent variable of metabolism and their quality bit strings in experiment 1 and 5

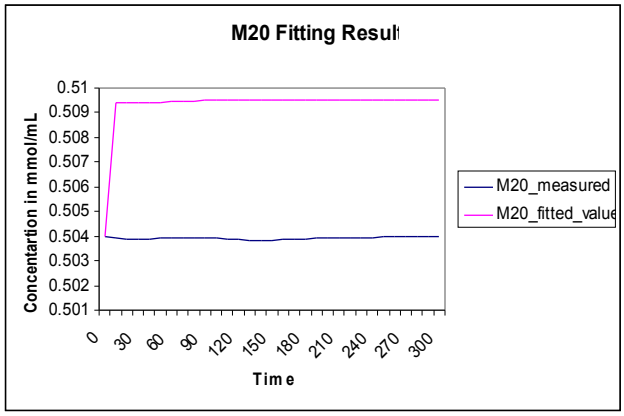
Dependent Value	Sum of Squares	Experiment 1 Fit Quality Bit String	Experiment 5 Fit Quality Bit String
M1	6.20E-005	11111	11111
M3	7.61E-005	00110	00110
M2	2.58E-005	11111	11111
M4	1.32E-004	00100	00100
M9	1.39E-005	11110	01110
M10	8.67E-005	01110	01110
M11	3.02E-005	11110	01110

M12	9.61E-005	11110	01110
M20	2.32E-006	00000	00000
M6	1.82E-004	10111	10111
M7	6.18E-004	10111	10111
M13	4.62E-003	00110	00110
M17	3.36E-005	00100	00100
M16	3.04E-004	00100	00100
M22	8.28E-005	00000	00000
M24	6.49E-005	00000	00000
P15	2.65E-006	00100	00100
P21	7.29E-06	01110	00110

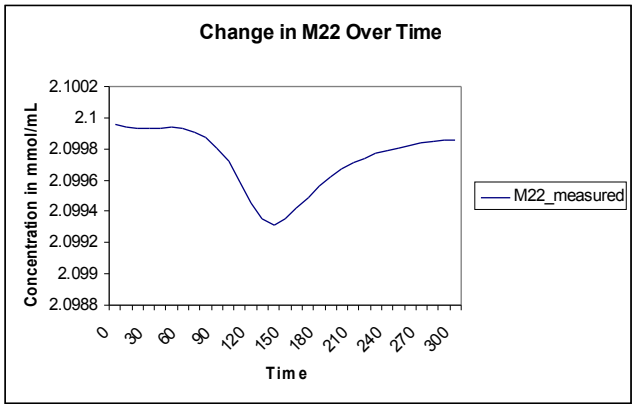
As can be seen from tables 2.10 and 2.11, the metabolism only model of Claytor network, fits data from the full network fairly well. Although the fits to the dependent variables are not perfect, most of them meet the criteria to achieve a '1' in at least one position of the bit score. Only three metabolites M20, M22 and M24, have a bit score of 00000 in both experiments. The fits to these metabolites achieved in experiment 1 are shown in figure 2.7. As can be seen in figure 2.7, the difficulty in fitting these metabolites is most likely due to the presence of valleys. Of these three metabolites, only M24 was predicted to have difficulty being fit based upon its association with transcriptional control elements, as shown in table 2.6. Interestingly, these metabolite fits do not have the highest sum of squares values for the set. In fact, the fit to M13 received the highest sum of squares value, but has a bit score of 00110 in both experiments. The fit to M13 for experiment 1 is also shown in figure 2.7. Metabolite M13 was predicted to have difficulty being fit based upon its association with hierarchical control elements as shown in table 2.6. The difference between the sum of squares value and quality bit score highlights the advantage of both. Although a fit may have a low sum of squares value, it may not contain certain characteristic features that are needed to correctly portray the data. However, the quality bit score is a qualitative measure that is necessarily subjective. By employing both approaches, a better sense of the overall fit is achieved.

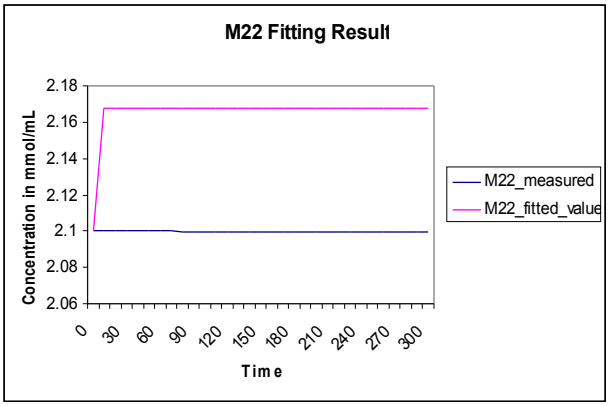
A.


Change in M20 Over Tim
Concentration in mmol/mL
0.50405
0.504
0.50395
0.5039
0.50385
0.5038
0.50375
0 30 60 90 120 150 180 210 240 270 300
Time
M20_measured

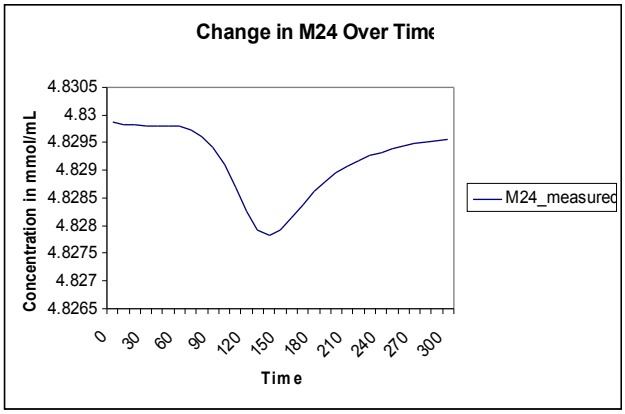

M20 Fitting Resul
Concentartion in mmol/mL
0.51
0.509
0.508
0.507
0.506
0.505
0.504
0.503
0.502
0.501
0 30 60 90 120 150 180 210 240 270 300
Time
M20_measured
M20_fitted_value

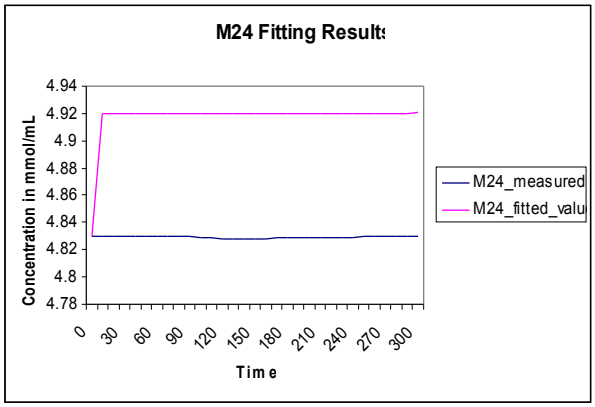
B.


Change in M22 Over Time
Concentration in mmol/mL
2.1002
2.1
2.0998
2.0996
2.0994
2.0992
2.099
2.0988
0 30 60 90 120 150 180 210 240 270 300
Time
M22_measured


M22 Fitting Result
Concentration in mmol/mL
2.18
2.16
2.14
2.12
2.1
2.08
2.06
0 30 60 90 120 150 180 210 240 270 300
Time
M22_measured
M22_fitted_value

C.


Change in M24 Over Time
Concentration in mmol/mL
4.8305
4.83
4.8295
4.829
4.8285
4.828
4.8275
4.827
4.8265
0 30 60 90 120 150 180 210 240 270 300
Time
M24_measured


M24 Fitting Results
Concentration in mmol/mL
4.94
4.92
4.9
4.88
4.86
4.84
4.82
4.8
4.78
0 30 60 90 120 150 180 210 240 270 300
Time
M24_measured
M24_fitted_valu

D.

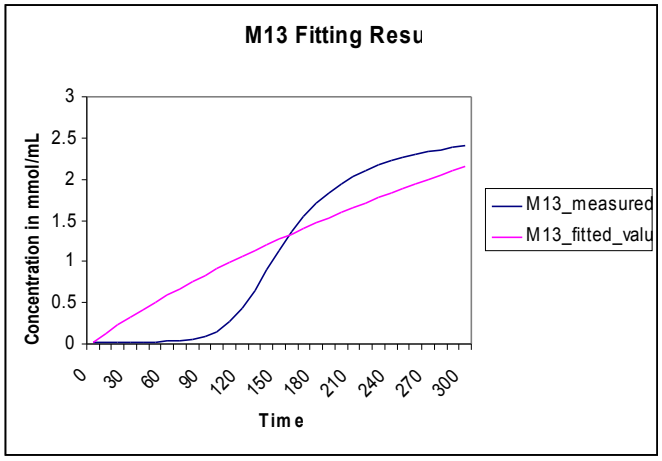
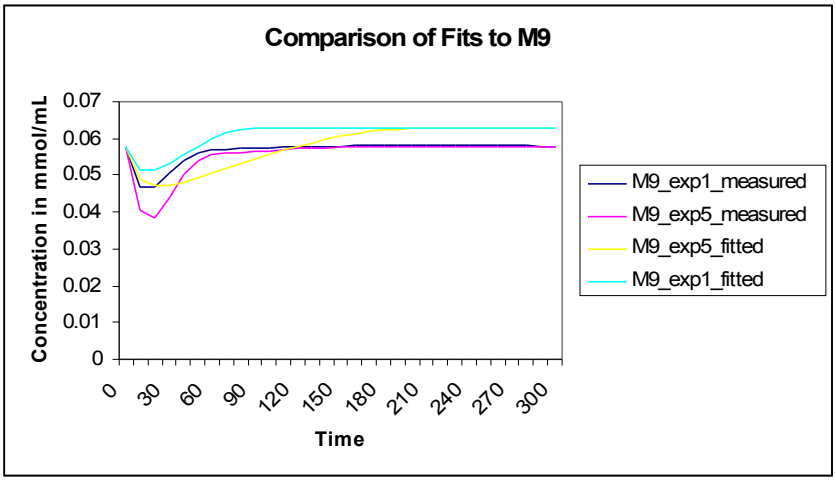


Figure 2.7. The dependent variables which received the lowest quality bit score are shown along with the metabolite which received the largest sum of squares value. Displayed are the results from experiment 1. **A.** A plot of the concentration of metabolite M20 is shown in isolation and with its fitted value. **B.** A plot of the concentration of metabolite M22 is shown in isolation and with its fitted value. **C.** A plot of the concentration of metabolite M24 is shown in isolation and with its fitted value. **D.** A plot of the fit to the concentration of M13 .

While both experiments resulted in identical quality bit scores for many of the dependent variables, there were some instances where they differed. In the dependent variables M9, M10, M11, M17, and P21, the fits received different quality scores in each experiment. In all of these cases, the fit to experiment 1 has a better quality bit score than in experiment 5. Most of the differences between the quality bit scores was in the category of "Overall Fit". This difference occurred in fits to the metabolic concentrations of M9, M10, and M11. In figure 2.9, a plot of the measured M9 concentrations in both experiments and their respective fits in these two experiments is given. The quality bit scores also differed between experiments in the category of "Shape" for P21. Plots of the fits to these metabolites and protein for each experiment are also given in figure 2.9.

A.



B.

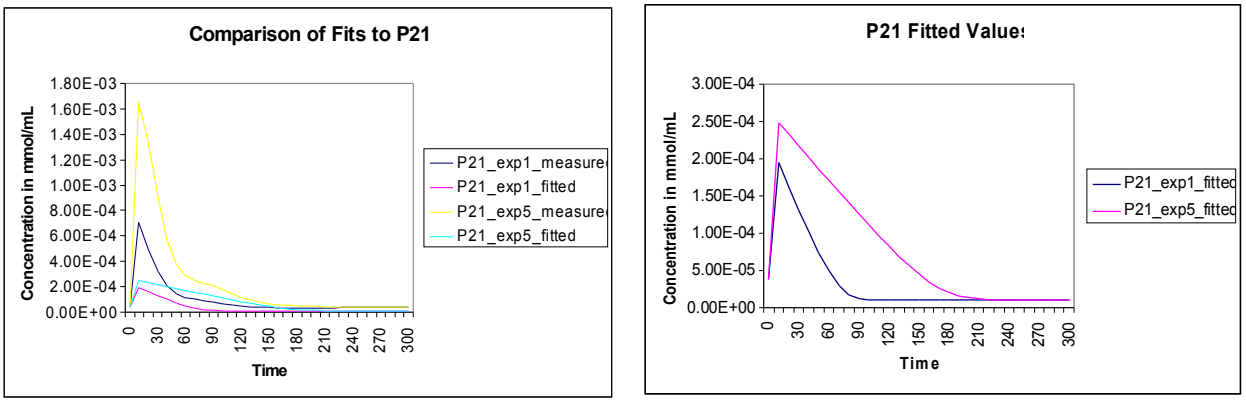


Figure 2.8. A comparison between the fits in experiments 1 and 5 for M9 and P21. **A.** The difference in overall quality of fit is depicted for M9, with the best fit being achieved for experiment 1. **B.** A simultaneous plot of the fitted and measured values of P21 in experiments 1 and 5 is shown on the left . On the right, the fitted values for P21 are shown in isolation from the measured values in order to demonstrate that the fit to P21 in experiment 1 has the same overall shape as its measured value. Although the fit to P21 in experiment 5 has a similar shape, it was not adequate to receive a quality bit score of 1 in this category.

The top three dependent variables of metabolism which were predicted to be most likely to have difficulty being fit were P15, M13, and M24. Metabolite M13 received the highest sum of squares value, but not the lowest quality bit score. Metabolite M24, by contrast, had a low sum of squares value by comparison, but had one of the lowest quality bit scores. Protein P15 had a low quality bit score but also a low sum of squares value. Of the remaining dependent variables which were predicted to have

fitting difficulty, M6 , M7 , and M12 received some of the highest sum of squares values, yet had acceptable quality bit scores. Protein P21 had a low sum of squares value and an acceptable quality bit score. Certain metabolites which were not fit well, M22 , M20 and M16 for example, were not predicted as having difficulty via the control association method. Therefore, while a metabolite's correlation with control elements seems to have some effect on it's ability to be fit, it is not the only factor.

Claytor Metabolism Model Applied to New Perturbations

After the Claytor metabolic model was parametrized by being fit to the M1 perturbation and G16 knock out datasets as described above, its ability to accurately describe new experimental conditions was tested. In tables 2.12 and 2.12, the sum of squares values for results of the fits of the model to perturbations in the concentration of M23 along with their respective quality bit scores is given. Metabolite M13 was predicted by the control association method to have difficulty being fit in the current experiment. This prediction is verified in this case, as M13 has one of the lowest quality bit scores and the highest sum of squares values. However, other badly fit dependent variables present were not predicted via the control association method. In particular, M4 which received the lowest quality bit score, was not targeted by the method. Its fitting difficulty is most likely perpetuated from the low quality fit that was achieved to the M1 perturbation dataset which was used to determine the parameter values for the model.

Table 2.12 : Results of the dependent variable fits to different perturbations of the concentration of M23

Experiment	Initial M23 concentration in mmol/ml	Sum of Squares
1	0.05	7.72E-05
2	0.44	1.65E-04
3	0.83	1.65E-04
4	1.22	1.64E-04
5	1.61	1.64E-04
6	2.00	1.63E-04

Table 2.13 : Residual sum of squares values and quality bit scores assigned to each dependent variable in experiments 1 and 3

Dependent Value	Sum of Squares	Experiment 1 Fit Quality Bit String	Experiment 3 Fit Quality Bit String
M1	5.20E-49	11111	11111
M3	1.38E-05	00010	00010
M2	5.20E-49	11111	11111
M4	6.53E-08	00000	00000
M9	3.05E-08	01110	01110
M10	4.80E-08	01110	01110
M11	3.34E-08	01110	01110
M12	4.10E-08	01110	01110
M20	5.48E-10	11111	11111
M6	2.76E-05	00010	00010
M7	1.10E-04	00010	00010
M13	7.46E-04	00010	00010
M17	9.29E-08	01110	01110
M16	9.88E-08	01110	01110
M22	1.99E-08	11111	11111
M24	1.55E-08	11111	11111
P15	4.42E-11	01010	01010
P21	5.51E-10	01010	01010

The fit results of the Claytor metabolism model fits to the G18 knock out experiment are shown in tables 2.14 and 2.15. In this experiment, metabolites M13 and M16 were predicted as being hard to fit . The results in table 2.15 agree with these predictions. However, many other dependent variables also had low quality bit scores in this experiment, although their sum of squares values are low. Similar to the M23 perturbation, some of the depend variables have difficulty achieving high quality bit scores because they did not do so in the M1 dataset. Although the Claytor metabolism model did not perform well using the qualitative measure of fit represented as the quality bit score, the sum of squares values for fits to the variables is very good. This seems to indicate that although the Claytor metabolism model may miss certain qualitative features of the perturbation, in most cases it predicts a value which is very close to the true one.

Table 2.14 : Residual sum of squares for the fit of the Claytor metabolism model to the G18 knock out experiment

Experiment	Sum of Squares
G18 Knock Out	0.000164982

Table 2.15 : Residual sum of squares for the fits of each dependent variable of metabolism and their associated fit quality bit string in a G18 knock out experiment

Dependent Value	Sum of Squares	G18 KO Experiment Fit Quality Bit String
M1	0.00E+00	11111
M3	2.53E-06	00000
M2	0.00E+00	11111
M4	3.01E-09	00000
M9	6.30E-10	00100
M10	5.80E-09	00100
M11	1.55E-09	00100
M12	6.67E-09	00100
M20	8.94E-11	00100
M6	5.07E-06	00000
M7	2.03E-05	00000
M13	1.37E-04	00000
M17	8.12E-10	00000
M16	9.16E-09	00000
M22	3.25E-09	00100
M24	2.52E-09	00100
P15	3.95E-12	00100
P21	2.91E-11	00100

Comparison of Parameter Values in Isolated Metabolism and Intact Claytor Network

As mentioned in earlier sections, one of the great benefits of using an artificial biological network is that the true values of all parameters are known. It is therefore possible to determine whether or not studying the metabolome in isolation has a significant effect on the parameters which are estimated. In other words, whether a model that has only the metabolic part is a sufficient to explain the observations of those metabolic components. In table 2.16, a comparison is made between parameters estimated using the metabolic portion of the Claytor network and the true system parameters for these reactions. In order to determine whether or not isolating the metabolome had a significant effect on these system level properties, the nonparametric Wilcoxon signed rank test using a two tailed distribution was applied (Whitley & Ball 2002). The null hypothesis used was that the difference in parameter values between the two cases was not significant. The Wilcox signed rank test value for the pairwise

comparison between all parameters in the metabolic portion of the Claytor network was $W = 2119$, with a p-value of $P = 0.007997$. These results indicate that there is a significant overall difference between the parameter values of the isolated and the intact Claytor metabolic networks. By contrast, when comparisons are made between the parameters in individual reactions, these differences are no longer significant at the $\alpha = 0.05$ level, as shown in table 2.16. This is to be expected as the number of samples being tested is much smaller. When each reaction is tested individually, multiple testing must be taken into account. After the Bonferroni correction (Weir 1996) is applied, the new significance level required is $\beta = .0033$. The reaction which is closest to being significantly different between the isolated metabolic and full Claytor networks is R11, which has a Wilcoxon p-value of P=0.0625. However, even this reaction is far from the acceptable significance level employed. The least significant p-values were obtained for reactions R1 and R2. While the difference in the quality of fits achieved by members in each reaction could in part explain this difference, it does not explain it fully. While reactions R1 and R2 contain some of the dependent variables which were fit the best, according to their quality bit score, R19 also contains these well fit metabolites. Similarly, R11 contains the fixed metabolite M19 and several metabolites which did not receive very high quality bit scores. However, except for M19, these metabolites are present in other reactions. While M19 is not shared, other fixed metabolites are present in reactions which received much higher p-values. For example, R10 which has a Wilcoxon p-value of $P = 0.8438$, contains M16 and M17. Metabolite M24 is also shared with reaction R12, which has a Wilcoxon p-value of $P = 0.625$. Therefore, it is unlikely that any one metabolite fit, or misfit, is responsible for the lower R11 p-value.

According to the reaction sums of the control associated aggregate Z-scores for the M1 perturbation experiment , reactions R3, R8, and R9 were predicted as having the parameters which were the most different from the true values. Reaction R9 is predicted as having the largest difference between its predicted parameters and the true ones. These predictions are not verified by the results in table 2.16 . The G18 knock out experiment also predicted difficulty in obtaining good parameter estimates for reaction R9 as well as for R10 and R11. Although reaction R11 has the smallest Wilcoxon p-value, it as well as the p-values for reactions R9 and R10 are far from being significant.

Table 2.16 : Comparison of the true kinetic parameters of the Claytor network with those estimated using only the metabolic reactions

Reaction	Equation	Parameter	True Value	Estimated Value	Wilcoxon W	Wilcoxon P-value
R1	M1 + M3 = M2 + M4; P13	Ka	5.91E-02	1.68E-01	11	1
		Kb	1.42E-06	2.27E-01		
		Kcatf	4.64E+05	3.22E+03		
		Keq	1.76E-07	5.07E-01		
		Kp	7.63E-07	1.44E+00		
		Kq	2.33E+01	2.21E-02		
R2	M4 + P15 = P21 + M3; P2	Ka	4.61E-03	5.15E-02	11	1
		Kb	7.48E+02	3.64E-03		
		Kcatf	4.34E-03	1.02E+04		
		Keq	1.54E+04	2.20E+00		
		Kp	1.63E-02	2.47E-03		
		Kq	3.56E-02	5.81E-01		
R3	M9 + P21 = P15 + M10; P3	Ka	4.82E+02	2.36E-01	16	0.3125
		Kb	1.79E+04	7.01E-04		
		Kcatf	4.96E-02	5.66E+03		
		Keq	1.20E+05	6.25E-01		
		Kp	3.45E+03	5.80E-03		
		Kq	8.51E-06	3.69E-04		
R4	M10 + M11 = M9 + M12; P4	Ka	2.77E+01	1.28E-01	12	0.8438
		Kb	2.00E-01	3.61E-03		
		Kcatf	3.51E-06	7.56E+03		
		Keq	1.01E+02	2.21E+00		
		Kp	3.96E-02	1.81E-01		
		Kq	2.15E-03	2.69E-02		
R5	M12 + M20 = M11 + M21; P19	Ka	1.49E-05	5.01E-06	9	0.8438
		Kb	9.82E+02	1.22E-01		
		Kcatf	1.80E-07	5.17E+03		
		Keq	6.35E+00	8.61E+00		
		Kp	9.43E+00	4.71E-03		
		Kq	6.90E-03	1.59E+01		
R6	M3 -> M15; P6	Km	1.94E+00	3.11E-03	3	0.5
		Kcat	1.77E+02	1.21E-01		
R7	M5 + M6 = M3; P12	Ka	6.88E-06	2.32E+00	9	0.8125
		Kb	2.57E+01	7.66E-01		
		Kcatf	2.50E+04	7.90E+01		

		Keq	8.63E-05	8.88E-01		
		Kp	7.56E-06	4.80E-02		
R8	M8 + M7 = M6; P9 M3	Ka	1.78E+02	5.36E-01	17	0.6875
		Kb	5.89E+03	8.71E+00		
		Kcatf	1.38E+02	4.75E+02		
		Keq	1.11E-03	5.71E-01		
		Ki	3.23E+04	1.38E+00		
		Kp	6.36E-05	6.87E-01		
		ni	2.59E-01	2.00E+01		
R9	M13 + M14 = M7; P10	Ka	3.14E-04	4.61E+00	9	0.8125
		Kb	1.98E+00	6.31E+00		
		Kcatf	8.56E+02	1.56E+02		
		Keq	1.01E+04	1.33E+00		
		Kp	2.21E-07	2.90E-01		
R10	M17 + M18 = M13 + M16; P8	Ka	3.62E-04	1.90E-01	12	0.8438
		Kb	6.70E-02	2.96E-02		
		Kcatf	4.76E+04	2.90E+03		
		Keq	3.77E+02	1.74E+00		
		Kp	2.14E+00	3.37E+00		
		Kq	1.78E-03	7.21E-02		
R11	M16 + M24 = M17 + M19; P14	Ka	3.50E+03	1.02E-02	20	0.0625
		Kb	9.32E+00	4.87E+00		
		Kcatf	5.93E+04	8.24E+03		
		Keq	8.54E+04	1.03E+01		
		Kp	6.78E+00	1.02E-01		
		Kq	1.05E+00	3.88E+00		
R12	M22 = M24; P5	Ka	1.17E+03	3.12E+00	7	0.625
		Kcat	1.12E-02	1.16E+03		
		Keq	3.13E+05	2.27E+00		
		Kp	2.24E-05	1.67E+01		
R13	M23 = M22; P20	Ka	2.96E+03	2.38E+00	7	0.625
		Kcat	4.50E-04	3.21E+03		
		Keq	1.28E+04	2.17E+00		
		Kp	2.80E+01	1.22E+00		
R14	M22 = M20; P7	Ka	4.41E-06	8.63E-02	4	0.875
		Kcat	2.54E-07	2.93E+02		
		Keq	1.30E-02	2.35E-01		
		Kp	6.09E+02	6.57E-02		
R19	M1 + M3 = M2 + M4;	Ka	1.57E+01	2.59E+00	17	0.2188

	P16					
		Kb	2.50E+01	1.44E-01		
		Kcatf	2.78E+01	9.25E+00		
		Keq	6.31E+00	1.27E+01		
		Kp	7.65E+00	1.87E+00		
		Kq	2.90E-06	1.07E-02		

Conclusions

It is common practice to make systems level predictions using only one aspect of the full biological network, commonly the transcriptome or metabolome. This is due to both the technical difficulty associated with obtaining data at more than one level as well as the difficulty in integrating the results when multi-level experiments are done. Even under the most ideal circumstances, the true underlying biological interaction network and the true system parameters are not known.

The goal of this study was to determine if there was an effect on the ability to assess system level properties when the metabolic portion of the system is studied in isolation. By utilizing the Claytor artificial biological network it was possible to characterize the effect that both incomplete network knowledge as well as studying only the metabolic level had on this ability. It was hypothesized that the elements which would be most negatively impacted would be those which were were most influenced by transcription. The comparative influence of metabolic and transcriptional control was predicted by using aggregate Z-scores for control association. These aggregate Z-scores were based upon the correlation of the metabolically dependent variable in question and predetermined transcriptional and metabolic control affiliated elements. It is important to note that correlation in metabolic systems can be the result of factors which are not associated with control (Camacho et al. 2005). Therefore, it is possible that the given method for predicting association with a control type may be an overestimation. It is also likely that the influence of a given control type over a reaction is not purely the additive contributions of its members. Therefore, there may be other more reliable indicators of transcriptional influence.

The results of this study demonstrate that studying metabolism in isolation does affect the ability to correctly estimate system parameters. Although association, as determined via aggregate Z-scores,

with hierarchical control did seem to influence this ability it is unlikely to be the sole factor. In particular, it is likely that translational regulation, which was not assessed, also influences this ability.

Chapter 3: A Comparison of Generalized Kinetic Rate Laws

Background

Introduction

Enzyme kinetics play a critical role in forming dynamic models of biological systems . In order to be able to simulate dynamics, it is not enough to merely know that an interaction or reaction occurs, it is also necessary to know its rate of occurrence and how it depends on the concentration of the various chemical species of the model – its kinetics, expressed through a mathematical function known as rate law, rate function or kinetic function. Certain thermodynamic factors may make a given series of reactions unfavorable or even impossible under cellular conditions. While rate laws for many enzymes have been established by *in vitro* methods, there are still many enzymes for which appropriate kinetics remain unknown. Although *in vitro* determined kinetics are useful for identifying the boundaries for reactions, they may not always be the same as in the *in vivo* conditions (Teusink et al. 2000) . Enzyme kinetic parameters are usually dependent on factors such as temperature and *pH,* and those are often different *in vitro* from *in vivo* conditions. Unfortunately, no reliable method of determining *in vivo* enzyme kinetic parameter values currently exists, making it difficult to determine whether most rate functions determined in vitro have a correspondence to those occurring in the cell . However, even the existence of such a method would not completely solve the problem. Enzymes, like other biochemical moieties, behave differently depending on their tissue location and physiological condition (Steuer et al. 2006), partly because of macromolecular crowding (Minton 2006) and other enzyme-enzyme interactions (P. Mendes et al. 1992) . This lack of global, quantitative kinetic data combined with observed inconsistencies in the in vivo data, has hindered the creation of network-scale kinetic models of biochemical systems (Famili et al. 2005).

In order to compensate for this lack of available *in vivo* kinetic data, several methods for generalizing rate laws have been proposed. The purpose of this study is to compare several of these methods using the Claytor artificial biological network. These methods include: convenience (Liebermeister & Klipp 2006a; Liebermeister & Klipp 2006b), linlog (Visser & J. J. Heijnen 2003a) , and generalized mass

action kinetics (Guldberg & Waage 1879). In order to properly benchmark these differing methods, an artificial biological network is used . The Claytor network , which was described in detail in chapter 2, is ideal for these purposes as it contains many of the complex characteristics found in natural biological systems while being much more tractable. In each case, the true values for the metabolic rate equation are replaced with one of the generalized versions. In order to determine if some methods were more sensitive to missing network information, two models were created to test each method. In one model, the non-metabolic portion of Claytor network is left intact and in the other it is removed.

The Role of Kinetic functions in Forming Realistic Biological Models

It is important when modeling biological systems to take into account that the various chemical species interact in a dynamic manner. The dynamics, or kinetics, of a given set of interactions affects both the probability that the interaction will occur and what the impact of this interaction will be on the overall system. Two methods which have been developed to account for the dynamic character of biological systems are chemical kinetics and enzyme kinetics. In chemical kinetics, only the relationships between products and reactants are considered . Chemical kinetics are portrayed using mass action kinetics which was established by Guldberg and Waage (Guldberg & Waage 1879). Enzyme kinetics is based on chemical kinetics and considers the effect of enzymes and also of modifiers (inhibitors, activators, etc.). Mass action rate laws describe the kinetics of elementary reactions, whereas enzyme kinetic equations employ a more macroscopic view, summarizing the behavior of all of the elementary reactions that compose a full enzyme catalytic cycle. Both forms of kinetics can be useful for describing the dynamics of biological systems depending on the assumptions that one is prepared to accept. The following sections will focus on the properties of mass action and enzyme kinetics and briefly discuss their use in biological studies.

Mass Action Kinetics

The law of mass action forms the basis of chemical kinetics (Klipp et al. 2005a). It emphasizes the relationship between the rate of a reaction and the concentration of its components. Under mass action kinetics, the rate of an elementary reaction is proportional to the probability of collisions of its

reactants . The probability of chemical collisions is itself proportional to the concentrations of the reactants raised to the power of their molecularity (Guldberg & Waage 1879). Molecularity is numerically the same as the stoichiometric coefficient for elementary reactions, however they are disparate conceptually. The stochiometric coefficient relates to the quantitative relationships between reactants and products within a balanced chemical reaction. Molecularity, by contrast, is a theoretical concept which is defined as the number of colliding molecular entities involved in a single reaction step. For a simple, reversible reaction

$$S_1 + S_2 \Leftrightarrow 2\mathrm{P} \tag{3.1}$$

where S_1 and S_2 are the substrates and P is the product of the reaction the mass action reaction rate would be :

$$v = k_f S_1 S_2 - k_r P^2 \quad . \tag{3.2}$$

The net rate of the reaction , $\boldsymbol{v}$, is the difference of the forward reaction and the reverse reaction rates. The parameters $\boldsymbol{k_f}$ and $\boldsymbol{k_r}$ are the rate constants for the forward and reverse reactions respectively. The law of mass action can be generalized for substrates S_i and products P_j with respective molecularites m_i and m_j (Heinrich & Schuster 1996)with the equation :

$$v = k_f \prod_i S_i^{m_i} - k_r \prod_j P_j^{m_j} \quad . \tag{3.3}$$

The use of the law of mass action to represent the kinetics in systems biology models is pervasive. Mass action kinetics have been used in models of systems such as apoptosis (Albeck et al. 2008), bistability of protein interaction networks (Sabouri-Ghomi et al. 2007), the ErbB signaling network (Chen et al. 2009), and the *Drosphila melanogaster* circadian network (Bagheri et al. 2008) in addition to many others. The primary difficulty in its use is in identifying the values of the elementary rate law parameters. Mass action kinetics are defined in terms of elementary reactions, which are reactions where individual chemical entities directly react in a single step to form the product. Therefore, in order to employ mass action kinetics, the biochemical reactions within the model of interest must be known at the level of resolution of this single reaction step. However, even in the rare cases where ***in vivo*** kinetics are known they are often known only in their aggregate form. These aggregate rate laws combine multiple elementary steps of a specific mechanism into a single reaction step. These aggregate rate laws may not be based on an underlying mechanism, and in some cases may be phenomenological formulas constructed to fit available data. It can be challenging to extract the elementary rate law

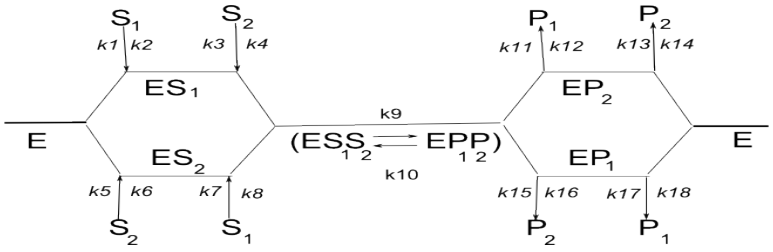
parameters from these aggregate ones for several reasons: the experimental data may not be reliable; there may be multiple elementary reactions and multiple elementary rate law parameter estimates that can explain a single aggregate rate law; finally, the fitting algorithms may have difficulties solving the nonlinear equations for a given system, or may become trapped in local minima (Zhao et al. 2008).

Enzyme Kinetics

Enzyme kinetics rate laws model the dynamics of cellular interactions by describing the overall enzyme catalyzed reaction as if it was a single step, however reflecting the underlying mechanisms. These kinetic mechanisms consist of the order of addition of reactants and release of products from an enzyme's active site, as well as any internal transformations (formation and destruction of chemical bonds). These mechanisms are grouped into three main classes, according to the order of binding of the substrates and products : random, sequential and ping-pong. These mechanisms can be further classified according to the number of kinetically important reactants in both reaction directions, where Uni, Bi, and Tri represent one, two, and three effectors respectively (substrate and/or products). In sequential mechanisms, all reactants must bind to the enzyme's active site before the reaction can proceed. This binding can be either ordered or random. An example of a random Bi-Bi sequential mechanism for the conversion of S1 and S2 into P1 and P2 is shown in figure 3.1 A using Cleland's shorthand notation (W W Cleland 1963). In ping-pong mechanisms, by contrast, products are released between the addition of two reactants in a manner reminiscent of a ping-pong game. Here, the first reactant will bind to the enzyme's active site, undergo transformation, and be released as a product while leaving a fragment of itself behind. This fragment is then used by the second binding reactant to assist in undergoing the chemical transformation that results in it being released as the second product(Cook & W.W. Cleland 2007). An example of a Bi-Bi ping pong mechanism for the previously mentioned conversion of S1 and S2 into P1 and P2 is shown in figure 3.1B. using Cleland's shorthand notation (W W Cleland 1963).

The enzymes involved in sequential mechanisms have one stable form, while those involved in ping-pong mechanisms have two, or more, stable forms. Enzymes with random mechanisms may have one or several stable forms. The main characteristic of these mechanisms is that the binding of substrates (or products in the reverse reaction) is devoid of a fixed sequence.

A.



B.

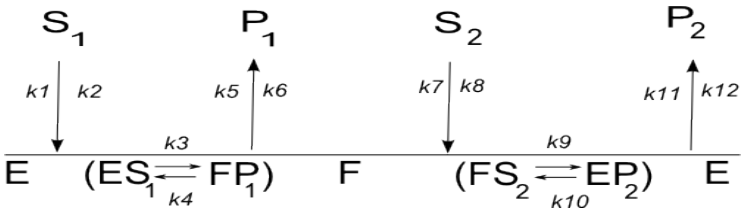


Figure 3.1 Cleland shorthand notation for substrates S1 and S2 being converted into products P1 and P2. In the diagrams E represents the enzyme. F is an alternate stable form of that enzyme. The various k values represent the microscopic rate constants. **A.** The reaction is demonstrated as following a random Bi Bi sequential mechanism. Binding of the substrates must happen sequentially, but the order of binding does not matter. The products are released sequentially as well without regard to order. **B.** The reaction is shown following a Bi Bi Ping Pong mechanism. The first substrate must bind and be converted into the first product before the next substrate can bind and be converted.

Unlike mass action kinetics, enzyme kinetics rate laws, once established, do not require that the elementary reactions be known. However, establishing the kinetic mechanisms for a given set of reactions is often challenging. The numerator in the rate law of an enzyme catalyzed reaction represents its thermodynamic character, as well as the overall speed attainable. It gives information on which direction the reaction is most likely to proceed, either toward or away from product creation, under certain conditions. The denominator represents all of the various forms of the enzyme during the catalytic cycle of the reaction. It also reflects the regulation that is conferred by the enzyme action including the effect of inhibitors or other modifiers. In order for the denominator to be correct, the reaction mechanism must be known. Unfortunately, determining reaction mechanisms is challenging as

it requires knowing the exact binding order of substrates and subsequent release of product. Even in cases where there are relatively few substrates and products believed to be involved, the number of possible binding sequences which must be accounted for in the experiment can become large. Within each step of the binding study, the microscopic kinetic constants must be properly measured. Once both the reaction mechanism and the microscopic rate constants have been determined, the rate equation can be calculated using a variety of methods (King & Altman 1956; Cornish-Bowden 1977; Cook & W.W. Cleland 2007; I. Segel 1975). However the true difficulty lies at the experimental stage, particularly in establishing *in vitro* experiments that reveal the mechanism of reaction. This usually requires spectroscopic methods and very rapid sampling (sub-second). Although the reaction mechanisms for a few reactions have been determined, the mechanisms, and therefore the true rate equations for most remain unknown.

Generalizing Enzyme Rate Laws

Assigning appropriate rate laws to reactions is a critical step in forming useful mathematical models of dynamic biological systems. However, the reaction mechanisms for many metabolic networks, such as those stored in the databases KEGG (Kanehisa et al. 2006; Kanehisa & Goto 2000) and METACYC (Caspi et al. 2006) are often unknown. Due to the lack of available kinetic data, combined with their necessity for creating relevant systems biology models, several methods of approximating unknown rate laws have been proposed. The resulting approximate rate laws are either continuous or discrete and either deterministic or stochastic (Albert 2007).Within these confines, these rate laws tend to be probabilistic (Gillespie 2000), phenomenological (Smallbone et al. 2007), semi-mechanistic(Liebermeister & Klipp 2006a), or a hybrid of these approaches.

Convenience Kinetics

Convenience kinetics have been proposed by Liebermester *et al*, as a method of compensating for the scarcity of *in vivo* kinetic models (Liebermeister & Klipp 2006a; Liebermeister & Klipp 2006b). Convenience kinetics is a general rate law based upon a simple random order mechanism. It is an extension to the reversible form of the general kinetic rate law proposed by Michaelis and Menten

(Michaelis & Menten 1913) which itself was based upon the relationship between enzyme and substrate concentration originally identified by Henri (Henri 1903). Both the reversible form of Michaelis-Menten and convenience kinetics describe saturable kinetics, making them more biologically credible approaches than those general rate laws which do not. The reversible form of the Michaelis-Menten equation for a given reaction $S \Leftrightarrow P$ with a substrate concentration of S and product concentration of P is:

$$v(S,P) = E\frac{(k_{catf}S - k_{catr}P)}{(1 + S/k_s + P/k_p)} \quad , \tag{3.4}$$

where E represents the enzyme concentration, $\boldsymbol{k_{catf}}$ and $\boldsymbol{k_{catr}}$ are the forward and reverse conversion rate constants, and $\boldsymbol{K_s}$ and $\boldsymbol{K_p}$ are the Michaelis-Menten rate constants. The convenience rate law equation is based upon this equation. However, it is expanded to include multiple substrates and products, multiple inhibitors and effectors . In spite of these additions, it can be specified with a relatively small number of parameters. Given a reaction $S_1 + S_2 + \ldots S_i \Leftrightarrow P_1 + P_2 + \ldots P_j$ catalyzed by an enzyme with reaction concentration E without activators or inhibitors, the convenience kinetics would be defined as:

$$v(S,P) = E\frac{(k_{catf}\prod_i S_i/k_{si} - k_{catr}\prod_j P_j/k_{pi})}{\prod_i (1 + S_i/k_{si}) + \prod_j (1 + P_j/k_{pi}) - 1} \quad , \tag{3.5}$$

where $\boldsymbol{K_i}$ and $\boldsymbol{K_j}$ represent the MichaelisMenten constants for each substrate S_i and product P_i. The convenience equation can also be extended for more general reaction stoichiometries, as in the reaction $\alpha_1 S_1 + \alpha_2 S_2 + \ldots \alpha_i S_i \Leftrightarrow \beta_i P_1 + \beta_i P_2 + \ldots \beta_j P_j$ and to include the presence of known activators or inhibitors with the equation:

$$v(S,P) = E\frac{k_{catf}\prod_i (S_i/k_{si})^{\alpha_i} - k_{catr}\prod_j (P_j/k_{pi})^{\beta_j}}{\prod_i (\sum_{m=0}^{\alpha_i} (S_i/k_{si})^m) + \prod_j (\sum_{m=0}^{\beta_j} (P_j/k_{pi})^m) - 1} \cdot \prod_m h_A(A, K_m^A)^{\gamma_m} h_I(I, K_m^I)^{\delta_m} \quad . \tag{3.6}$$

The effect of the activator with a concentration of $\boldsymbol{A}$,activation constant $\boldsymbol{K^A}$, and reaction stoichiometry $\boldsymbol{\gamma}$ is given by:

$$h_A(A, K^A)^\gamma = \left(\frac{A}{K^A + A}\right)^\gamma \quad . \qquad 3.7$$

The effect of an inhibitor with concentration ***I***, inhibition constant $\boldsymbol{K^I}$, and reaction stoichiometry $\boldsymbol{\delta}$ is similarly given by:

$$h_I(I, K^I)^\delta = \left(\frac{I}{K^I + I}\right)^\delta \quad . \qquad 3.8$$

Convenience kinetics' versatility combined with its relatively small number of parameters has made it a promising source of rate laws for models where the true kinetics are unknown. It and other generalized rate laws can be automatically added to systems biology models using programs like SBMLsqueezer (Drager et al. 2008), thus speeding up the generation of simulation ready models. In addition to being used only for the subset of reactions where the kinetics are unknown, convenience kinetics has also been successfully used to fully replace all rate laws in models of leucine and valine biosynthesis. This was done both in an evolutionary algorithms benchmarking study (Drager & M. Kronfeld 2007) and later was shown to give the best system level results in combination with Michaelis-Menten rate laws when compared to other methods of assigning approximative rate laws (Draeger et al. 2009) .

Linlog Kinetics

Linlog kinetics is based on concepts from Metabolic Control Analysis (MCA). In MCA, relationships between various biochemical moieties are described in a mathematical framework. In particular, it quantifies the dependence of control coefficients on local system properties, called elasticities. Control coefficients are network dependent properties which relate the effect that a change in a parameter, such as enzyme activity, has on the relative change in the steady state system variables such as flux. Elasticities describe the effect that changing the local environment, by altering substrate concentrations for example, has on the local reaction rate. By quantifying both the local and global effects on control, MCA demonstrated that control is a distributed property and thus refuted the concept of rate-limiting steps in biological systems (Kacser & JA Burns 1995; Heinrich & Schuster 1996). Because MCA quantifies the contributors to flux, it logically seems to be an excellent starting point for a method to generalize enzyme kinetics. Linlog kinetics is an attempt to do this.

With linlog kinetics, kinetic modeling is performed using approximate, non-mechanistic rate laws, which leads to a reduction in the number of parameters that must be estimated. This in turn reduces the number of experiments which must be conducted. The linlog rate equation for a given reaction $S_1 + S_2 \rightarrow P_1$ catalyzed by enzyme E is

$$v = E(a + b\ln(S_1) + c\ln(S_2) + d\ln(P_1)) \quad , \tag{3.9}$$

with the parameters b and c being positive and parameter d being negative. The linlog rate equation is based upon the equation for the thermodynamic reaction affinity. The equation for the thermodynamic reaction affinity for the above reaction would be

$$A = \Delta G_R^0 + RT\ln(S_1) + RT\ln(S_2) - RT\ln(P_1) \quad . \tag{3.10}$$

where R is the gas constant, T is the absolute temperature in Kelvin, and ΔG^O_R is the standard Gibbs free energy of reaction. Theoretically, the reaction affinity and rate of reaction are linearly related in reactions which are close to equilibrium (Onsager 1931). However, this linear relationship has also been observed for several cellular processes without the near equilibrium constraint (Rottenberg 1973; van der Meer et al. 1980) . There is, however, some debate about the linear relationship between reaction rate and its affinity observed in several experiments. For example, Pekař argued that in several cases this linear relationship which was observed was due to the experimental methods and arrangements used in the given study (Pekar 2007). Linlog utilizes this linear relationship but differs from it in several important ways. Linlog accounts for the effects of modifiers by including a logarithmic concentration terms for each allosteric effector. In addition, linlog allows all of the kinetic parameters to be chosen independently, rather than being restricted by reaction stoichiometry as is the case for the thermodynamic reaction affinity.

The linlog rate equation integrates MCA into this kinetic modeling structure via a reference state. A reference state can be one of many possible steady states for the given system. In the previous reaction, a reference state could be written as:

$$J^o = E^o(a + b\ln(S_1^o) + c\ln(S_2^o) + d\ln(P_1^o)) \quad , \tag{3.11}$$

where J^o is a given steady-state flux and E^o , S_1^o, S_2^o, P_1^o are the enzyme and metabolite concentrations at this steady state. Dividing the linlog rate equation by the reference state allows the coefficients to be written in terms of the elasticities of MCA, giving:

$$\frac{v}{J^0} = \frac{E}{E^0}\left(1 + \epsilon^0_{S_1}\ln\left(\frac{S_1}{S_1^0}\right) + \epsilon^0_{S_2}\ln\left(\frac{S_2}{S_2^0}\right) + \epsilon^0_{P_1}\ln\left(\frac{P_1}{P_1^0}\right)\right) \quad . \tag{3.12}$$

Thus, linlog kinetics can be expressed in terms of elasticities and thereby integrated with MCA by use of a reference steady state. As an extension of this relationship, it was shown that linlog kinetics could be successfully employed to determine the control coefficients of a system by applying it to multiple steady state experiments via linear regression (Wu et al. 2004). Note that for mechanistic enzyme kinetic rate laws, the elasticities are the partial derivatives of the rate law towards the substrate, product and effectors.

Linlog kinetics have been successfully used to substitute for the true rate equations in several instances. Linlog was first demonstrated by Visser *et al* on an artificial branched network (Visser & J. J. Heijnen 2003a) which was designed by Mendes *et al* (P. Mendes & Kell 1998). It was subsequently used to substitute for all of the kinetic equations in the primary metabolism of *Esherichia coli* (Visser et al. 2004). In addition to these and similar usages, linlog kinetics have been employed as part of a method to ameliorate constraint-based and kinetic modeling. The resulting method combined flux balance analysis with linlog kinetics to approximate a yeast model of glycolysis without the need for experimentally derived kinetic data (Smallbone et al. 2007).

However, in spite of the utility of linlog kinetics in these circumstances, it must be used with caveats. For one, linlog kinetics are by definition most accurate when near a given reference state (Visser & J. J. Heijnen 2003a). When concentrations are remote from this state, the linlog approximations may not be as useful. Indeed, without an appropriate reference state, the linlog equation becomes quite stiff, making parameter estimation difficult. Finally, it is important to note that the natural logarithm function will not appropriately mirror the true system kinetics. In biological systems, increasing the substrate concentrations will increase the reaction rate but not linearly, in fact the rate law saturates and in the limit of infinite substrate concentration it has a finite rate. This saturation is an effect of the fact that in a cell (or test tube) there are a finite number of active sites and once all are occupied with substrate, no other substrate molecules can react, thus leading to the saturation. When saturation occurs, the reaction rate will asymptotically approach a maximum (Cook & W.W. Cleland 2007). By contrast, the natural logarithm function applied to a given concentration will tend to infinity as the concentration of substrate gets very large. All chemical reactions, including those catalyzed by enzymes, have zero rate when the substrate concentration is zero, however the linlog rate law tends to a negative value when the substrate concentration approaches zero. Therefore, in order for the linlog rate equations to be

applicable, it is important that the relevant concentrations fall within a range where the linlog kinetics will mirror that of the true kinetics. In particular, kinetics involving extremely large or extremely small biological concentrations may not be correctly estimated. However, if these constraints are observed, linlog has been shown to give useful approximations of the true kinetics.

Hypothesis

It is hypothesized here that the convenience kinetics method will provide the overall best approximation, because it has the form most similar to that of commonly used enzyme rate equations. It is also thought that generalized mass action kinetics will be the least affected by reducing the available model information to only include the metabolism reactions. This is hypothesized because mass action kinetics are believed most affected by local interactions, rather than longer distance ones.

Materials and Methods

Overview

Two models based upon the Claytor network are created for each generalized rate equation method. In one model, the metabolic portion is separated from the transcriptional and translational portions of the network in the same manner as in chapter 2. In the second model, these metabolic reactions remain affiliated with the full Claytor network. In each case, the true rate equations for the metabolic reactions are replaced with those proposed by the given method. Because there are two forms of the linlog equation, one where a steady state is directly included and one where it is not, there were more models needed to test the efficacy of linlog than were needed for the other methods. Therefore, four models were created to test the linlog generalized rate equation. In one set of linlog models, the form of equations which included a steady state was applied to the metabolic reactions of both the fully intact Claytor network and the isolated metabolic portion of this network. In the other set of linlog models, the form of equations which did not include a steady state was applied to the full and isolated versions of Claytor network metabolism.

These models each have their parameter values estimated via fits to perturbations of M1 concentration, with a G16 knock out dataset being used for internal validation. All parameter estimation is performed using the package COPASI (S. Hoops et al. 2006) . The parameter results for each method using the intact and isolated Claytor metabolism are compared. Finally, the results of each method are compared using Akaike's Information Criterion (AIC) (Akaike 1974) .

Claytor Network with Altered Kinetics

As was mentioned earlier , it is often not possible to know the true reaction kinetics of a biological system. Yet, in order to build dynamic models of these systems, knowledge of these rate laws is essential. Because of the deficiency of kinetic information, several generalized rate laws have been proposed as a means of circumventing this issue. Of particular interest to this study were convenience kinetics, linlog kinetics and mass action kinetics. In order to monitor the effect of substituting the true kinetic rate laws by convenience, linlog, or mass action rate laws , three versions of the full Claytor network were created. The Clayor artificial biological network was described in detail in chapter 2. In one version of this altered Claytor network, the true rate laws of the metabolic portion of the Claytor network are replaced with convenience kinetics where appropriate. In the other versions, these rate laws are instead substituted with mass action or linlog kinetics. In each case, only the rate laws for the metabolic portion of the Claytor network were replaced. Tables 3.1 and 3.2 display these metabolic equations, their true rate law, and the form of the rate law used for each generalized kinetics method. Of special note is the fact that two forms of the linlog rate equation were used. In one, no knowledge of a reference state is assumed. In the other, a reference state based on a steady state analysis of the full Claytor network was used. All steady state and MCA analyzes were performed in COPASI (S. Hoops et al. 2006).

Table 3.1 : The true rate equations and the generalized kinetics equations which replaced them in each compared method.

Reaction	Equation	Rate Law	Convenience Rate Law	LinLog Rate Law	Mass Action Rate Law
R1	M1 + M3 = M2 + M4; P13	Bi Bi with Explicit Enzyme	Convenience A+B =P+Q, with Explicit Enzyme	Linlog A+B=P+Q with Explicit Enzyme : with and without reference state	Mass Action A+B = P+Q with Explicit Enzyme
R2	M4 + P15 = P21 + M3; P2	Bi Bi with Explicit Enzyme	Convenience A+B =P+Q, with Explicit Enzyme	Linlog A+B=P+Q with Explicit Enzyme : with and	Mass Action A+B = P+Q with Explicit Enzyme

				without reference state	
R3	M9 + P21 = P15 + M10; P3	Bi Bi with Explicit Enzyme	Convenience A+B =P+Q, with Explicit Enzyme	Linlog A+B=P+Q with Explicit Enzyme : with and without reference state	Mass Action A+B = P+Q with Explicit Enzyme
R4	M10 + M11 = M9 + M12; P4	Bi Bi with Explicit Enzyme	Convenience A+B =P+Q, with Explicit Enzyme	Linlog A+B=P+Q with Explicit Enzyme : with and without reference state	Mass Action A+B = P+Q with Explicit Enzyme
R5	M12 + M20 = M11 + M21; P19	Bi Bi with Explicit Enzyme	Convenience A+B =P+Q, with Explicit Enzyme	Linlog A+B=P+Q with Explicit Enzyme : with and without reference state	Mass Action A+B = P+Q with Explicit Enzyme
R6	M3 -> M15; P6	Michaelis-Menten with Explicit Enzyme	Convenience A->P with Explicit Enzyme	Linlog A->P with Explicit Enzyme ; with and without reference state	Mass Action A-> P with Explicit Enzyme
R7	M5 + M6 = M3; P12	Bi Uni with Explicit Enzyne	Convenience A+B=P with Explicit Enzyme	Linlog A+B=P with Explicit Enzyme; with and without reference state	Mass Action A+B = P with Explicit Enzyme
R8	M8 + M7 = M6; P9 M3	Bi Uni with Uncooperative Inhibition and Explicit Enzyme	Convenience A+B=P with Explicit Enzyme and Inhibition	Linlog A+B=P with Explicit Enzyme; with and without reference state	Mass Action A+B = P with Explicit Enzyme
R9	M13 + M14 = M7; P10	Bi Uni with Explicit Enzyne	Convenience A+B=P with Explicit Enzyme	Linlog A+B=P with Explicit Enzyme; with and without reference state	Mass Action A+B = P with Explicit Enzyme
R10	M17 + M18 = M13 + M16; P8	Bi Bi with Explicit Enzyme	Convenience A+B =P+Q, with Explicit Enzyme	Linlog A+B=P+Q with Explicit Enzyme : with and without reference state	Mass Action A+B = P+Q with Explicit Enzyme
R11	M16 + M24 = M17 + M19; P14	Bi Bi with Explicit Enzyme	Convenience A+B =P+Q, with Explicit Enzyme	Linlog A+B=P+Q with Explicit Enzyme : with and without reference state	Mass Action A+B = P+Q with Explicit Enzyme
R12	M22 = M24; P5	Michaelis-Menten with Explicit Enzyme	Convenience A=P with Explicit Enzyme	Linlog A=P with Explicit Enzyme; with and without reference state	Mass Action A = P with Explicit Enzyme
R13	M23 = M22; P20	Michaelis-Menten with Explicit Enzyme	Convenience A=P with Explicit Enzyme	Linlog A=P with Explicit Enzyme; with and without reference state	Mass Action A = P with Explicit Enzyme
R14	M22 = M20; P7	Michaelis-Menten with Explicit Enzyme	Convenience A=P with Explicit Enzyme	Linlog A=P with Explicit Enzyme; with and without reference state	Mass Action A = P with Explicit Enzyme
R19	M1 + M3 = M2 + M4; P16	Bi Bi with Explicit Enzyme	Convenience A+B =P+Q, with Explicit Enzyme	Linlog A+B=P+Q with Explicit Enzyme : with and without reference state	Mass Action A+B = P+Q with Explicit Enzyme

Table 3.2: The formulas for each rate equation mentioned in Table 3.1.

Rate Law	Equation
Bi Bi with Explicit Enzyme	$E\frac{Kcat_f*(AB-PQ/K_{eq})}{((1+A/K_a+P/K_p)(1+B/K_b+Q/K_q))}$
Bi Uni with Explicit Enzyne	$E\frac{Kcat_f*(AB-P/K_{eq})}{(1+A/K_a+B/K_b+P/K_p)}$
Bi Uni with Uncooperative Inhibition and Explicit Enzyme	$E\frac{Kcat_f*(AB-P/K_{eq})}{((1+A/K_a+B/K_b+P/K_p)*(1+(I/K_i)^n_I))}$
Michaelis-Menten with Explicit Enzyme	$E\frac{Kcat_f*(A-P/Keq)}{(1+A/K_a+P/K_p)}$

Convenience A+B =P+Q, with Explicit Enzyme	$E\frac{(Kcat_f(a/Km_a)(b/Km_b)-Kcat_r(p/Km_p)(q/Km_q))}{(1+(a/Km_a)+(b/Km_b)+((ab)/(Km_a Km_b)+(p/Km_p)+(q/Km_q)+((pq)/(Km_p Km_q))))}$
Convenience A->P with Explicit Enzyme	$E\frac{(Kcat_f*(a/Km_a))}{(1+(a/Km_a))}$
Convenience A+B=P with Explicit Enzyme and Inhibition	$E\frac{(Kcat_f(a/Km_a)(b/Km_b)-Kcat_r(p/Km_p))}{(1+a/Km_a+b/Km_b+p/Km_p+(a\,b/(Km_a Km_b)))}$
Convenience A=P with Explicit Enzyme	$E\frac{(Kcat_f(a/Km_a)-Kcat_r(p/Km_p))}{(1+a/Km_a+p/Km_p)}$
Linlog A+B=P+Q with Explicit Enzyme	$E(1+b\log(A)+c\log B+d\log P+e\log Q)$
Linlog A+B=P+Q with Explicit Enzyme with reference state	$E(1+b(\log(A)-\log(A_o))+c(\log(B)-\log(B_o))+d(\log(P)-\log(P_o))+e(\log(Q)-\log(Q_o)))$
Linlog A->P with Explicit Enzyme	$E(1+b\log(A)+c\log P)$
Linlog A->P with Explicit Enzyme with reference state	$E(1+b(\log(A)-\log(A_o))+c(\log(P)-\log(P_o)))$
Linlog A=P with Explicit Enzyme	$E(1+b\log(A)+c\log P)$
Linlog A=P with Explicit Enzyme with reference state	$E(1+b(\log(A)-\log(A_o))+c(\log(P)-\log(P_o)))$
Linlog A+B=P with Explicit Enzyme	$E(1+b\log(A)+c\log B+d\log P)$
Linlog A+B=P with Explicit Enzyme with reference state	$E(1+b(\log(A)-\log(A_o))+c(\log(B)-\log(B_o))+d(\log(P)-\log(P_o)))$
Mass Action A+B = P+Q with Explicit Enzyme	$E(k_1 AB-k_2 PQ)$
Mass Action A-> P with Explicit Enzyme	$E\,k_1 A$
Mass Action A+B = P with Explicit Enzyme	$E(k_1 AB-k_2 P)$
Mass Action A = P with Explicit Enzyme	$E(k_1 A-k_2 P)$

Claytor Metabolism Models with Altered Kinetics

Claytor metabolism models were also created for each of the generalized kinetics models mentioned earlier. This was done in order to determine if the methods differed in their ability to handle incomplete network information. Specifically, metabolism-only models were created using convenience, linlog, and mass action to substitute for the known kinetics. As before, two forms of the linlog equation were used.

Datasets

Two datasets were used in order to estimate the parameters for all models in the current study. One dataset , a metabolic perturbation, is used as a training set, while the other, a genetic perturbation, is used as an internal validation set. The training set consists of a series of perturbations of the concentration of metabolite M1. The validation dataset is formed from a time-series produced after knocking out gene G16. Both of these datasets are described in greater detail in chapter 2.

Parameter Estimation

The parameter values for each of the generalized kinetics models were obtained via minimizing the sum of squares of the residuals between the simulated data and the measured data. The numerical minimization methods used were the particle swarm (Clerc & Kennedy 2002; Kennedy & Eberhart 1995) and genetic algorithm with stochastic ranking (GASR) (Goldberg 1989). These stochastic methods were preferred as they have less of a tendency of becoming trapped in local minima. In order to prevent the possibility of overflow in the optimization of the mass action functions, parameter estimation was performed iteratively. The analysis was at first restricted to a narrow range near the starting point. If a boundary was approached during successive runs, the range for that parameter was increased. Parameter estimation was performed until there was no or minimal improvement in the overall sum of squares results for the model.

For the convenience kinetics models, the starting values and initial search range were based upon the true kinetics values when applicable. These values were approximated using the parameter values of the true rate equations. The linlog parameter range was based upon elasticities taken from a MCA analysis of the full Claytor network. When the form of the linlog equation which did not require a reference state was employed, this range was increased to account for this lack. The mass action kinetics models, by contrast, used a range of 0 to 2000 for all parameters. This parameter range is based upon the ranges observed in parameter values that were obtained experimentally and are stored in the the BRENDA enzyme database (Chang et al. 2009; I. Schomburg et al. 2002), and was also used as the range for the generalized kinetics comparisons made by Drager et al (Draeger et al. 2009) .

Comparison of Parameter Values Estimated in Intact and in Isolated Claytor Network Metabolism

In order to compare the parameter value estimates obtained using the intact and isolated Claytor metabolic network, the paired Wilcoxon signed rank test was used. The Wilcoxon signed rank test, with test statistic W, is a nonparametric test for the null hypothesis, H_0, that the difference between two values is equal to 0 (Whitley & Ball 2002) . The values for W as well as their associated p-values were obtained using the R statistics package (R Development Core Team 2009). The Wilcoxon signed rank test was used to compare the overall parameter value differences between the intact and isolated Claytor metabolism models for each method. In addition, the parameter values for individual reactions were also compared.

Determining Individual Model Performance

The quality of fit obtained by each generalized kinetics method was determined in the same way as discussed in chapter 2. In brief, the fits of each dependent variable of metabolism are assessed using both the individual sum of squares value for that variable as well as a quality bit score. The quality bit score is a binary bit string which assesses the qualitative fit of the model to the data. It assigns a 1 or 0 to the following fit properties: overall fit, shape, scale, direction, and alignment. Although the quality bit score is somewhat subjective, it is useful for ascertaining how well the given model reflects the data.

Comparison of Models Employing Differing Generalized Rate Laws

The final models obtained via each of the generalized kinetics methods were compared using Akaike's Information Criterion (AIC). The AIC compares model performance based upon both the sum of squares values and the number of model parameters, where preference is given to more parsimonious models. The form of the AIC equation used in this study was:

$$AIC = N(\ln(SSQ/N)) + 2k \quad , \tag{3.13}$$

where k represents the number of parameters, N is the number of observed data points, and SSQ is the residual sum of squares. Comparisons of each generalized kinetics method were made by applying AIC to all isolated and all intact Claytor metabolism models.

Results and Discussion

Linlog Kinetics Model

As mentioned in the materials and methods section, two different forms of the linlog rate equation were used. One required information from a reference state and the other did not. Both models were used in the attempt to estimate their parameter values. However, neither parameter estimation converged. This result was seen both in the models where the non-metabolic reactions remained intact as well as in the models where metabolism was studied in isolation.

Two hypotheses emerged as to why linlog was unable to correctly fit the Claytor network data. One possibility was that the range over which parameter estimation was performed was too large (and therefore convergence could happen if the methods were allowed to run for much longer time). The other possibility was that the Claytor lake model itself contained features which made it impossible to being fit by linlog kinetics. Both hypotheses were tested by inserting individual reactions containing linlog rate equations into the full Claytor network and then attempting to estimate their parameters. This means that at each time only one linlog rate law was being estimated since all other reactions were the original ones (i.e. the true kinetics). By inserting one reaction at a time into a network where all other kinetics were known, the search space should be reduced. Rather than fitting parameters from all of metabolism, only one reaction was parametrized in this exercise. Once this reaction was parametrized sufficiently, the values obtained could be used as the basis for the parameter range in a new fit. Insertion and parameter estimation of individual reactions into a fully described model also had the benefit of testing whether all reactions could be fit using linlog kinetics. The results of this can be seen in table 3.3. While not all reactions could be fit using this methodology, most were. In some cases, reactions could not be fit using the more general form of the linlog equation, but were able to be fit when a reference state was used. Therefore, it may be concluded that these reactions, specifically R8 and R10, require more information in order to be defined using linlog kinetics. By contrast, reactions R1 and R19 were not fit by substituting either form of the linlog equation into the full Claytor network. It is likely that certain aspects of these reactions make them impossible to model using linlog kinetics. In both cases, all of the component's elasticities within the context of the reaction at the reference state

were zero. Because linlog parameters are equivalent to the elasticities when a reference state is incorporated, when all of the relevant elasticities are zero linlog fits the reaction kinetics as a constant. In both reactions R1 and R19, this is inappropriate. Reactions R1 and R19 constitute an important feature of the Claytor network. They make up the reaction cycle which detoxifies M1. Although linlog kinetics has had much success in estimating rate equations in a variety of organisms, these were often branched systems and did not include loop structures. It has been shown that feedback loops require multiple steady state perturbations for their elasticities to be identifiable (Kacser & J. A. Burns 1979; Giersch 1994; Giersch & CornishBowden 1996). Therefore, it is likely that in order for linlog kinetics to correctly approximate feedback loops, a combination of reference states would be needed for each participating reaction. As feedback loops comprise an important part of biological systems (Alberts et al. 2007; Iyengar & Bhalla 1999; Reeves & Fraser 2009), it is unlikely that linlog kinetics will remain relevant without addressing this issue.

These results confirm that, in large part, the lack of convergence in the estimation of all linlog parameters simultaneously was due to the size of the search space, since it was possible to estimate parameters of most linlog reactions when done in isolation. However, for two reactions, the problem was also the unidentifiability of the parameters (i.e. due to characteristics of the data and the equation).

Table 3.3 : Effectiveness of modeling individual metabolic reactions with linlog kinetics

Reaction	Equation	Fit Using Linlog	Fit Using Linlog Containing Reference State
R1	M1 + M3 = M2 + M4; P13	no	no
R2	M4 + P15 = P21 + M3; P2	yes	yes
R3	M9 + P21 = P15 + M10; P3	yes	yes
R4	M10 + M11 = M9 + M12; P4	yes	yes
R5	M12 + M20 = M11 + M21; P19	yes	yes
R6	M3 -> M15; P6	yes	yes
R7	M5 + M6 = M3; P12	yes	yes
R8	M8 + M7 = M6; P9 M3	no	yes
R9	M13 + M14 = M7; P10	yes	yes
R10	M17 + M18 = M13 + M16; P8	no	yes
R11	M16 + M24 = M17 + M19; P14	yes	yes
R12	M22 = M24; P5	yes	yes

R13	M23 = M22; P20	yes	yes
R14	M22 = M20; P7	yes	yes
R19	M1 + M3 = M2 + M4; P16	no	no

Convenience Kinetics Applied to Intact Claytor Network Metabolism

Convenience kinetics were used as approximations for the rate equations of the metabolic portion of the Claytor network. The remainder of the network was left intact. The results of parameter estimation with these approximative rate laws can be seen in tables 3.4 and 3.5 below. As before, the experiment with the lowest sum of squares and the one with the highest were chosen for further analysis. The quality bit score for the dependent variables of each of these experiments is given along with the sum of squares values. A low sum of squares value, in this case, does not always have a corresponding high quality bit score as can be seen by M20.

Table 3.4 : Residual sum of squares of fits to experiments using convenience kinetics as applied to full Claytor network.

Experiment	Initial M1 concentration in mmol/ml	Sum of Squares
1	0.05	5.58E-03
2	0.10	6.15E-03
3	0.15	7.02E-03
4	0.20	7.69E-03
5	0.25	7.32E-03

Table 3.5 : Residual sum of squares of fits to each dependent variable of metabolism and the corresponding quality bit score obtained in experiments 1 and 4.

Dependent Value	Sum of Squares	Experiment 1 Fit Quality Bit String	Experiment 4 Fit Quality Bit String
M1	3.90E-03	00110	00110
M3	8.65E-04	00010	00010
M2	1.77E-02	01010	01010
M4	1.38E-04	00000	00000
M9	5.81E-05	00110	00110
M10	3.52E-04	00110	00110
M11	1.24E-04	00110	00110

M12	3.86E-04	00110	00110
M20	9.30E-06	00000	00000
M6	2.17E-03	00010	00010
M7	6.57E-03	00010	00010
M13	1.07E-03	11110	11110
M17	4.19E-05	00100	00100
M16	3.79E-04	00100	00100
M22	4.15E-07	00010	00010
M24	2.45E-05	00010	00010
P15	4.44E-06	01010	01010
P21	1.25E-05	01110	01110

The dependent variables which achieved the lowest quality bit scores were M20 and M4. Neither of these received the highest sum of squares estimate, which was achieved by M2. Overall, the sum of squares values for the dependent variables are good. However, the qualitative aspects of each fit as assessed by quality bit score are not particularly good.

Convenience Kinetics Applied to Isolated Claytor Network Metabolism

The quality of fit was also examined for convenience kinetics applied to the isolated metabolic portion of the Claytor network. The results of this fit can be seen below in tables 3.6 and 3.7.

Table 3.6 : Residual sum of squares for each fit to experiments using convenience kinetics applied to the isolated metabolic portion of the Claytor network

Experiment	Initial M1 concentration in mmol/ml	Sum of Squares
1	0.05.	1.41E-03
2	0.10	2.94E-03
3	0.15	4.36E-03
4	0.20	5.58E-03
5	0.25	5.93E-03

Table 3.7 : Residual sum of squares for fits to each dependent variable of metabolism and the corresponding quality bit scores obtained in experiments 1 and 5

Dependent Value	Sum of Squares	Experiment 1 Fit Quality Bit String	Experiment 5 Fit Quality Bit String
M1	9.01E-05	11111	11111
M3	8.69E-04	00000	00000
M2	3.65E-05	11111	11111
M4	1.38E-04	00000	00000
M9	5.81E-05	00110	00110
M10	3.52E-04	00110	00110
M11	1.24E-04	00110	00110
M12	3.86E-04	00110	00110
M20	9.93E-06	00000	00000
M6	2.18E-03	00000	00000
M7	6.63E-03	00010	00010
M13	8.85E-03	10110	10110
M17	4.20E-05	00100	00100
M16	3.80E-04	00100	00100
M22	1.09E-05	00000	00000
M24	1.71E-05	00000	00000
P15	3.86E-05	01110	01110
P21	1.25E-05	01110	01110

The overall fit quality of the convenience kinetics method applied to the metabolic portion of the Claytor network is similar to that obtained using the fully intact model. In the case of several metabolites, for example M1 and M2, the fit achieved is actually better than that obtained using the full network.

Comparison of Convenience Kinetics Parameters in Intact and in Isolated Metabolism of Claytor Network

In table 3.8, a comparison is given between the convenience kinetics parameters which were estimated using the full Claytor network and those which were determined using the metabolic portion in isolation. The signed pairwise Wilcoxon W for the overall comparison was W=1415, with a p-value of $\mathbf{P} = 0.5356$ using the two-tailed distribution. Therefore, the parameter values obtained using the intact and isolated Claytor network metabolism do not differ at the $\alpha = 0.05$ level of significance. None of the comparisons performed on individual reactions was significant at this level either. Reaction R4 was

closest to having significantly different parameter values with a Wilcoxon p-value of ***P*** = 0.063

Table 3.8 : Comparison between the convenience kinetics parameter values obtained using the intact and isolated Claytor metabolism network

Reaction	Equation	Parameter	Intact Claytor Metabolism Estimate	Isolated Claytor Metabolism Estimate	Wilcoxon W	Wilcoxon p-value
R1	M1 + M3 = M2 + M4; P13	Kcatf	23841	25465	12	0.844
		Kcatr	2758	2025.75		
		Kma	0.084582	0.014721		
		Kmb	0.052174	0.10131		
		Kmx	2.86788	2.85725		
		Kmy	0.015881	0.005314		
R2	M4 + P15 = P21 + M3; P2	Kcatf	409.024	1310.66	5	0.313
		Kcatr	734.076	1411.89		
		Kma	0.019326	0.030204		
		Kmb	0.002745	0.002458		
		Kmx	0.004228	0.00493		
		Kmy	0.44848	0.230401		
R3	M9 + P21 = P15 + M10; P3	Kcatf	13236.4	19112.8	11	1
		Kcatr	1792.26	1462.23		
		Kma	0.021653	0.031312		
		Kmb	0.001159	0.000926		
		Kmx	0.010878	0.004181		
		Kmy	0.000543	0.000421		
R4	M10 + M11 = M9 + M12; P4	Kcatf	2273.32	3225.44	1	0.063
		Kcatr	2641.2	2864.77		
		Kma	0.002334	0.002127		
		Kmb	0.011703	0.013106		
		Kmx	0.01704	0.046201		
		Kmy	0.016144	0.017126		
R5	M12 + M20 = M11 + M21; P19	Kcatf	121.164	101.392	18	0.156
		Kcatr	554.954	384.499		
		Kma	0.000138	0.000133		
		Kmb	0.280794	0.392881		
		Kmx	0.009671	0.007587		
		Kmy	8.84385	1.21907		
R6	M3 -> M15; P6	Kcatf	127.07	63.4065	2	1
		Kma	0.002179	0.003746		
R7	M5 + M6 = M3; P12	Kcatf	89.8539	239.268	5	0.625
		Kcatr	3057.78	2103.25		

		Kma	1.26237	1.59467		
		Kmb	0.401477	0.612815		
		Kmx	0.008409	0.023496		
R8	M8 + M7 = M6; P9 M3	Kcatf	390.686	147.226	10	1
		Kcatr	10016.6	10184.8		
		Ki	0.046773	0.164359		
		Kma	2.74891	2.78144		
		Kmb	4.14592	0.500002		
		Kmx	0.325107	1.69325		
R9	M13 + M14 = M7; P10	Kcatf	889.909	521.604	12	0.313
		Kcatr	5078.07	3530.29		
		Kma	1.75466	3.35269		
		Kmb	0.281954	0.270574		
		Kmx	2.33196	1.69188		
R10	M17 + M18 = M13 + M16; P8	Kcatf	2647.08	3211.12	5	0.313
		Kcatr	317.705	203.631		
		Kma	0.306491	0.310403		
		Kmb	0.009161	0.022568		
		Kmx	1.8125	2.42383		
		Kmy	0.02226	0.036408		
R11	M16 + M24 = M17 + M19; P14	Kcatf	3045.18	2295.64	9	0.844
		Kcatr	9048.95	10686.3		
		Kma	0.001901	0.001664		
		Kmb	0.995868	3.31469		
		Kmx	0.003446	0.004802		
		Kmy	6.30099	5.80225		
R12	M22 = M24; P5	Kcatf	349.569	183.64	7	0.625
		Kcatr	411.171	332.07		
		Kma	1.56959	1.64002		
		Kmb	4.20917	6.96908		
R13	M23 = M22; P20	Kcatf	67.1862	68.4227	4	0.875
		Kcatr	474.594	445.471		
		Kma	0.871056	1.02657		
		Kmb	12.9019	13.8698		
R14	M22 = M20; P7	Kcatf	4777.87	5803.06	0	0.125
		Kcatr	12020.8	15852.3		
		Kma	0.018101	0.01885		
		Kmb	0.010711	0.01279		
R19	M1 + M3 = M2 + M4; P16	Kcatf	10.8604	0.330572	18	0.156
		Kcatr	3.93603	1.07049		

		Kma	0.858299	0.935993		
		Kmb	0.097779	0.043981		
		Kmx	2.87793	2.68964		
		Kmy	0.004153	0.001		

Mass Action Kinetics Applied to Intact Claytor Network Metabolism

Mass action is a classic method for representing chemical kinetics. Because it does not take enzyme actions into account, it generally requires the determination of elementary reactions to be effective. In this study, a general form of mass action kinetics was used to model the rates of each metabolic reaction. Each reaction rate was multiplied by the relevant enzyme concentration (which is know from the original Claytor network, and in a real experiment would have to be determined). However, all reactions remained in their original form and no elementary reactions were considered. The quality of fitting results obtained using generalized mass action kinetics on the intact Claytor network are given in tables 3.9 and 3.10. Experiments 2 and 5 are used in comparisons as they show the widest discrepancies in the sum of squares values, although not in initial M1 concentration.

Table 3.9 : Residual sum of squares for each fit by experiment where generalized mass action kinetics is applied to full Claytor network

Experiment	Initial M1 concentration in mmol/ml	Sum of Squares
1	0.05	1.69E-03
2	0.10	7.63E-04
3	0.15	8.57E-04
4	0.20	1.19E-03
5	0.25	1.73E-03

Table 3.10 : Residual sum of squares for each dependent variable of metabolism and the quality bit score for experiments 2 and 5

Dependent Value	Sum of Squares	Experiment 2 Fit Quality Bit String	Experiment 5 Fit Quality Bit String
M1	3.50E-05	11111	11111
M3	1.35E-04	11110	11110
M2	1.07E-05	11111	11111
M4	1.56E-04	00100	00100

M9	1.31E-05	00110	00110
M10	8.95E-05	00110	00110
M11	2.02E-05	00110	00110
M12	6.92E-05	00110	00110
M20	1.31E-05	00000	00000
M6	1.94E-04	11110	11110
M7	5.68E-04	11110	11110
M13	4.61E-03	11110	11110
M17	3.59E-06	00110	00110
M16	3.27E-05	00110	00110
M22	2.70E-04	00000	00000
M24	1.20E-07	00100	00100
P15	1.84E-08	11111	11111
P21	1.25E-05	01010	01010

As can be seen in table 3.10, both the sum of squares values and the quality bit scores for most of the fitted dependent variables is fairly good. This is somewhat surprising as generalized mass action was one of the simpler methods used.

Mass Action Kinetics Applied to Isolated Claytor Network Metabolism

The fit quality results for applying generalized mass action to the isolated metabolic portion of the Claytor network are shown below in tables 3.11 and 3.12.

Table 3.11: Residual sum of squares values for the fits to each experiment using mass action kinetics applied to isolated Claytor metabolism model

Experiment	Initial M1 concentration in mmol/ml	Sum of Squares
1	0.05	4.00E-04
2	0.10	9.01E-04
3	0.15	1.23E-03
4	0.20	1.71E-03
5	0.25	2.57E-03

Table 3.12 : Residual sum of squares for fits to each dependent variable of metabolism where mass action applied to the isolated Claytor metabolism model and the associated fit quality bit score achieved in experiments 1 and 5

Dependent Value	Sum of Squares	Experiment 1 Fit Quality Bit String	Experiment 5 Fit Quality Bit String
M1	1.28E-04	11110	11110
M3	7.97E-05	10110	10110
M2	3.88E-05	11111	11110
M4	1.26E-04	00110	00110
M9	1.27E-05	00110	00110
M10	8.65E-05	00110	00110
M11	5.58E-05	00110	00110
M12	1.95E-04	00110	00110
M20	1.33E-06	00000	00000
M6	1.90E-04	10110	10110
M7	6.00E-04	10110	10110
M13	4.79E-03	10110	10110
M17	4.88E-05	00110	00110
M16	4.40E-04	00110	00110
M22	8.04E-07	00000	00000
M24	2.60E-06	00000	00000
P15	7.98E-07	00110	00110
P21	1.20E-05	01010	01010

The results obtained are very similar to those obtained by applying generalized mass action kinetics to the full Claytor network. However, in several cases the fits obtained using the intact Claytor network are better than those obtained here.

Comparison of Mass Action Kinetics Parameters in Intact and in Isolated Metabolism of Claytor Network

The parameter values obtained using the intact and isolated Claytor network metabolism were compared using the Wilcoxon signed rank test. The pairwise Wilcoxon W for the overall comparison between the isolated and intact Claytor metabolism parameters is $W = 193$, with a p-value of $P = 0.6089$ using the two-tailed distribution. Therefore, there is no difference between the two sets of parameters at the $\alpha = 0.05$ level of significance. The differences in the parameter values obtained using the intact and isolated metabolic portions of the Claytor network were also assessed for each reaction. These results are shown below in table 3.13. In no case were any of the Wilcoxon p-values for these differences close to the predetermined significance level.

Table 3.13 : Comparison between the generalized mass action kinetics parameter values obtained using the intact and isolated Claytor metabolism network

Reaction	Equation	Parameter	Intact Claytor Metabolism Estimate	Isolated Claytor Metabolism Estimate	Wilcoxon W	Wilcoxon p-value
R1	M1 + M3 = M2 + M4; P13	K1	511.625	17.1905	1	1
		K2	50.8187	1356.79		
R2	M4 + P15 = P21 + M3; P2	K1	766.447	1999.99	0	0.5
		K2	615.397	843.489		
R3	M9 + P21 = P15 + M10; P3	K1	1601.3	1331.13	1	1
		K2	410.809	780.591		
R4	M10 + M11 = M9 + M12; P4	K1	1876.45	1414.79	3	0.5
		K2	1454.43	437.275		
R5	M12 + M20 = M11 + M21; P19	K1	1659.93	1342.75	1	1
		K2	847.193	1709.66		
R6	M3 -> M15; P6	K1	976.05	0.008269	1	1
R7	M5 + M6 = M3; P12	K1	959.421	1704.14	0	0.5
		K2	1408.18	1949.3		
R8	M8 + M7 = M6; P9 M3	K1	1121	165.16	3	0.5
		K2	1907.98	275.087		
R9	M13 + M14 = M7; P10	K1	1173.45	1337.79	0	0.5
		K2	942.408	1066.14		
R10	M17 + M18 = M13 + M16; P8	K1	792.183	452.936	2	1
		K2	317.589	1996.87		
R11	M16 + M24 = M17 + M19; P14	K1	1703.04	1985.09	0	0.5
		K2	620.324	159.536		
R12	M22 = M24; P5	K1	1869.82	1794.08	3	0.5
		K2	772.203	779.669		
R13	M23 = M22; P20	K1	1391.84	1864.14	0	0.5
		K2	697.072	885.376		
R14	M22 = M20; P7	K1	369.717	339.94	1	1
		K2	1497.65	1430.42		
R19	M1 + M3 = M2 + M4; P16	K1	967.457	1884.15	1	1
		K2	1720.9	1943.64		

In table 3.14, the convenience and generalized mass action kinetics methods applied to the fully intact Claytor network metabolism are compared using their AIC values. The generalized mass action model is preferred in this instance as it achieves the lowest AIC value.

Table 3.14 : A comparison of the fits of convenience and generalized mass action models applied to the intact Claytor metabolic network

Generalized Kinetics Method	Sum of Squares	Number of Parameters	AIC
Convenience	3.38E-02	219	2.05E+06
Mass Action	6.23E-03	170	1.57E+06

Comparison Between Varying Kinetics Methods Applied to Isolated Metabolism Model of Claytor Network

The results of the comparison between the convenience and mass action kinetics models fitted to the isolated metabolic portion of the Claytor network is shown below in table 3.15. In addition, the results obtained using the true kinetic rate equations are shown for comparison. In this case, the generalized mass action model would be again selected, even over that of the model which used the true form of the rate equations. This preference is due to the smaller number of parameters employed by the mass action model as well as its low sum of squares value.

Table 3.15 : Results of comparing models formed using the true rate equations, convenience, and generalized mass action kinetics applied to isolated metabolic portion from the Claytor network

Kinetics Method	Sum of Squares	Number of Parameters	AIC
True	6.44E-03	79	6.94E+05
Convenience	2.02E-02	78	6.90E+05
Mass Action	6.81E-03	29	2.14E+05

Conclusions

The goal of this study was to compare three different methods for modeling unknown kinetic rate laws: convenience, linlog, and generalized mass action. As the modeling demand for rate equations increases while the number determined ***in vivo*** does not, there is an increasing need to evaluate the performance of generalized rate equations. An advantage of the current study is its use of the Claytor artificial biological network. The Claytor network contains many of the complex features found in natural biological networks and is thus a useful tool for testing these methods. These complex interactions made it possible to identify weaknesses in certain methods. In particular, it was shown than the parameter values for the linlog approximation could not converge when applied to the Claytor network data. This is likely due to its inability to handle feedback loops, which are a common feature in many biological systems. Thus, this study illustrates that care must be taken when employing linlog to certain datasets. In addition, it seems to indicate that there is a need to prove generalized kinetics methods upon artificial biological networks which contain higher degrees of complexity.

It was hypothesized that the convenience kinetics method would perform best overall due to its closeness in equation structure to that of the true rate equations. This hypothesis was not supported by the results of the current study. Surprisingly, generalized mass action kinetics performed the best when applied to the fully intact as well as when applied to isolated portions of the Claytor metabolic network. When applied to the isolated Claytor network metabolism, it also outperformed the parameter results estimated using the true rate equations. Interestingly, the true model and the convenience model performed similarly on the isolated Claytor network metabolism. Therefore, convenience kinetics is still a useful method for generalizing rate equations in this situation, as it gives results similar to those of the true model.

It was also hypothesized that the generalized mass action model would be the least affected by being restricted to the isolated metabolic portion of the Claytor network. Neither the generalized mass action nor the convenience kinetics model parameters was significantly affected by being thus restricted. However, the Wilcoxon p-value for the generalized mass action model was slightly larger, indicating that it was sightly less affected. Therefore, this hypothesis is supported.

Chapter 4: Reducing the Search Space in Parameter Estimation for Biochemical Networks

Introduction

One of the goals of systems biology is the representation of dynamic biochemical processes via the formation of a series of coupled ordinary differential equations (ODEs)(Albert 2007; Klipp et al. 2005b; Mendes 2001). This is accomplished via modeling strategies which can be classified as top-down (Stark et al. 2003), bottom-up (Teusink et al. 2000) , or a hybrid of the two. In top-down modeling approaches, these ODEs are reverse engineered by studying an intact system. By contrast, in bottom-up modeling methodologies individual components of the system are studied separately and later combined. Bottom-up modeling is the more biased of the two, as it incorporates *a priori* knowledge and conjectures about the system into the model. This knowledge is usually derived from previous non-system level experiments (usually *in vitro* experiments with purified molecules or cell-free extracts). Top-down modeling strategies use systems level experiments to create models in an unbiased manner (Mendes 2001). In most cases, a hybrid approach is taken to modeling that combines these two approaches as it is difficult to apply a method that is purely one or the other. Once a modeling strategy has been successful, a set of of equations which describe the system of interest results. Such a set of equations usually takes the following form:

$$\begin{aligned} \frac{dx_1}{dt} &= f_1(x_{1,}\ldots, x_n; k_{1,}\ldots, k_p) \\ \frac{dx_i}{dt} &= f_i(x_{1,}\ldots, x_n; k_{1,}\ldots, k_p) \\ \frac{dx_n}{dt} &= f_n(x_{1,}\ldots, x_n; k_{1,}\ldots, k_p) \end{aligned} \qquad 4.1$$

where each differential equation, dx_i/dt, represents the dynamic characteristics of x_i, one of n biochemical species in the network. The relationship between x_i and the other x_j members of the network is described via the functions, f_i . The functions f_i are linear combinations of the rate laws that specify the velocity of the reactions which affect the concentration of the chemical species of interest. These functions are also dependent upon a set of p parameters. Although the overall set of the rate law parameters for these equations may be large, each function is only dependent upon a smaller subset of

those parameters. In order to fully specify these equations, the values of these parameters must be known. The purpose of parameter estimation is the calculation of numerical estimates for these values (Moles et al. 2003). Top-down and bottom-up strategies differ in how this parameter estimation is applied. In top-down modeling approaches, the parameter values for all of the rate laws must be fit simultaneously, whereas in bottom-up modeling methods the parameter values for smaller pieces of the system are estimated separately, combined, and then undergo a final adjustment step (Mendes 2001). Both of these approaches have their difficulties. Simultaneous fitting of rate law parameters for a system is complex and computationally intensive. As the size of the system increases, applying a top-down parameter estimation approach without imposing restrictions on the ranges of the parameter value becomes less feasible. Because parameter estimation is applied to smaller pieces of the system in a bottom-up approach, the optimization problem is simpler than in the top-down approach. However, the bottom-up approach is limited by the quantity and quality of the pre-existing data. The amount of experimentally determined kinetic parameters is deficient for most systems and much of these have been determined *in vitro*. There can be wide discrepancies between *in vitro* kinetics and their *in vivo* counterparts (Bas Teusink 2000; Rizzi et al. 1997; Vaseghi et al. 1999; Wright & Kelly 1981). Since bottom-up approaches are dependent upon available kinetic data, these issues can confound its ability to construct accurate system models. Due to the differing limitations associated with top-down versus bottom-up modeling, the strategy employed should depend on both the size of the system (i.e. the number of reactions considered) as well as the type of experimental data available. In many cases, a hybrid of these two is the most useful modeling method. An ideal strategy would combine the tractability of the bottom-up approach with the non-biased systems-level approach of top-down modeling.

The focus of this chapter is the proposal of a method which is a hybrid of the top-down and bottom-up modeling techniques. The objective of this method is to decouple this set of ODEs by representing each chemical species as an independent function. This leads to the creation of a new set of ODEs, in which each component function, g_i, is independent from all of the other g_{n-1} functions. In order to accomplish this, the time dependent concentration of each chemical species of interest is substituted with a time dependent function, $x_i(t)$ which approximates its role. Thus, these new functions, g_n, are dependent only upon their respective parameters and these placeholder functions, $x_n(t)$. Since the $x_n(t)$ do not depend on any of the original parameters, the estimation of the (few) parameters of g_n is then

independent of the other parameters. The parameters for the functions g_n can therefore be estimated independently and used to restrict the parameter search space within the original coupled set of ODEs.

It is hypothesized that employing this method will result in two main benefits. First, it will allow the parameters of the functions f_n to be estimated in less time. It is thought that a reduced amount of time will be necessary to estimate the parameter values because the parameter space to search by the optimizing methods is much smaller dimension. Thus, it is believed that the more this space can be restricted the less time the parameter estimation will take. Secondly, it is hypothesized that in certain systems these values may be more accurate than those estimates performed without the benefit of a restricted search range. Depending upon the biochemical system of interest , the amount of data necessary to fully and accurately specify a model of it , as represented by ODEs, can be quite large. In cases where there is not enough data, the model becomes underdetermined (Albert 2007). In this situation, many parameter estimates will fit the available data, even if they are not near the correct values. By reducing the search space, it is thought that this problem will be minimized somewhat as there will be less search space for the parameter estimation methods to fall into one of these local minima.

Method

Overview

The goal of the proposed method is to decrease the time necessary to estimate the parameter values of a biochemical network by reducing the parameter search space. It is proposed that this may be achieved by transforming the representative set ODEs into an independent set of ODEs. This will allow the parameters on the right hand side of the new ODEs to be fit separately. After obtaining the estimates for the parameters of the new ODEs, this information can be used to inform the parameter estimation of the original system of ODEs.

This ODE decoupling is achieved via a process of substituting time dependent functions for each of the concentrations of the member biochemical species in each of the original f_n functions. These new functions do not need to represent structural properties of the underlying biochemical system, as do the

original. Rather, they only need to be time dependent and fit the available dynamic data. Thus, in the new functions, g_n, these concentrations are represented as being dependent upon time, but not of each other. In the auxiliary representation of the biochemical system, these new g_n functions on the right hand side contain different parameters from the original p parameters of their counterparts in f_n. Importantly, these parameters will have different values in each of the g_n functions, which will allow them to be assessed individually. These new representations take the general form:

$$\begin{aligned} x_1(t) &= g_1(t; a_{1,1}, \ldots, a_{1,l}) \\ x_i(t) &= g_i(t; a_{i,1}, \ldots, a_{i,l}) \\ x_n(t) &= g_n(t; a_{n,1}, \ldots, a_{n,l}) \end{aligned} \qquad 4.2$$

These functions g and their parameters a should be able to accurately reproduce the time series of the corresponding $x_i(t)$. Once these functions and their constituent parameters have been identified, then this information can be used to estimate the k parameter values used in f_i independent from the remaining k parameters used in f_{n-1} . In large biochemical systems this will result in a significant reduction in the search space of the optimization algorithms performing the fit, which should greatly improve execution time.

To achieve the objectives of this method, the functions g can be of any type that is capable of fitting the data exclusively as a function of time. Natural candidates are polynomial functions of time:

$$g_i = a_{i,0} + \sum_{l=1}^{q} a_{i,l} \cdot t^l \quad , \qquad 4.3$$

where q is the order of the polynomial. Another possibility is to decompose the time series through a truncated Fourier decomposition of order q:

$$g_i = \frac{a_{i,0}}{2} + \sum_{l=1}^{q} a_{i,l} \cos(l \cdot t) + b_{i,n} \sin(l \cdot t) \qquad 4.4$$

It is important to note that neither sets of functions depend on any of the x or k from the functions f, but only on t and the new parameters a_i or b_i. Other time dependent functions could also be considered as long as they do not depend on the remaining species concentrations and thus upon the other ODEs. Potentially attractive time dependent functions which could be applied within the method in the future are auto-regressive functions which combine low order polynomials with trigonometric functions (Eubank & Speckman 1990) and piecewise regression functions(McGee & Carleton 1970).

Determining the Auxiliary Parameters

Once the 4.3 or 4.4 representations are adopted, their respective parameters need to be determined; for both 4.3 and 4.4 this can be achieved through nonlinear regression, for example using COPASI (S. Hoops et al. 2006). In the case of 4.4 it could also be done through standard Fourier transform methods, such as Fast Fourier Transform (FFT) (Cooley & Tukey 1965) . All of the available data should be used in this step as there is no concern of over-fitting. However, each metabolite concentration trajectory that is used will need to be fit by an individual time dependent function, such as those in 4.3 or 4.4. The objective of this step is merely to be able to reproduce the data as a function of time. *No theoretical interpretations should be derived from these functions g. These are only auxiliary functions whose sole purpose is to improve the fitting of the functions f.*

Estimating the Main Parameters

After obtaining the parameters of functions *g*, it is then possible to carry out the estimation of the *k* parameters of the original biochemical system ODEs. For this step, one ODE is processed at a time, where the other ODEs are substituted with the appropriate equations of 4.2. Therefore only the few parameters *k* that are part of the ODE being processed at this time must be determined. This new system of equations can be represented as:

$$\begin{aligned} x_1(t) &= g_1(t; a_{1,1}, \ldots, a_{1,l}) \\ \frac{dx_i}{dt} &= f_i(x_1, \ldots, x_n; k_1, \ldots, k_p) \\ x_n(t) &= g_n(t; a_{n,1}, \ldots, a_{n,l}) \end{aligned} \quad . \qquad 4.5$$

At this stage the parameters *a* are already known and therefore do not need to be estimated. Only the much smaller set of parameters, k , of the single differential equation require estimation at this point. Since each ODE is processed independently, the problem of fitting the parameters of a set of *n* coupled ODEs has been reduced into the much more manageable problem of fitting the parameters of a single ODE. The search space of each problem is much smaller than the search space of the original problem. Since the computational time of global optimizations increases super-linearly, or even exponentially , with the dimension of the search space (Moles et al. 2003; Polisetty et al. 2006; Voit 1992) this method should result in a large reduction of the computational time necessary to estimate values for system parameters.

Final Adjustment

Because the general form of the ODEs of biochemical networks are linear combinations of some nonlinear terms, each parameter *k* appears in more than one ODE. This means that the procedure presented above will provide multiple estimates of these parameters. Depending on the coverage that the data provides, it is possible that the estimates of some parameters can be considerably divergent. To improve this, a final step is proposed where a global fit is carried out on the entire dataset using the original system of ODEs. In this final step, the rate law parameters are now limited to the interval of values obtained in the several independent fits. Obviously, if the intervals are extremely large there would have been no advantage of using this method. Therefore, it is not entirely clear whether or when this method will be advantageous. The success of this method depends upon on how well the data determines each of the ODEs. This, in turn, depends upon both how the experiments were designed (van Riel 2006) as well as the complexity of the model. On the other hand, this partition of the estimation problem into smaller ones of restricted dimensionality may result in a situation where some parameters can be identified unequivocally, but which would not be in the coupled system analysis. Therefore, there may well be advantages in this method even if all system parameters are not well estimated and this possibility will be further investigated.

The efficacy of this method will be investigated in a controlled way using a model where the actual parameters are known in advance. In order to test the hypotheses that this method will result in improved computation time and accuracy over parameter estimation applied in an unrestricted manner to the entire system, the parameters of this known model will be estimated using both the proposed and the control methods. In this case, the control method refers to parameter estimation on the fully coupled system without benefit of search space reduction.

Implementation

In order to demonstrate the application of this new method and also to investigate its performance, it was applied to a controlled example. Instead of using data from experiments of a real system, the method will be applied to a set of data obtained from a model with known parameter values. This has

three benefits: a) any experiment can be performed *in silico*, b) results can be interpreted with exact knowledge of true system, and c) the true values of the parameters are well known and therefore it is possible to measure the error of the estimates (P. Mendes et al. 2003).

Model

The model BIOMD0000000052 , which was obtained from the biomodels database (Le Novere et al. 2006) , was used as the test case in this study. BIOMD0000000052 is a model of the kinetics of heated monosaccharide-casein systems(Brands & van Boekel 2002). It contains 11 metabolites and 11 reactions. Each reaction follows irreversible mass action kinetics. A diagram of this model, as created in CellDesigner (Funahashi et al. 2003; Funahashi et al. 2008) can be seen in figure 4.1.

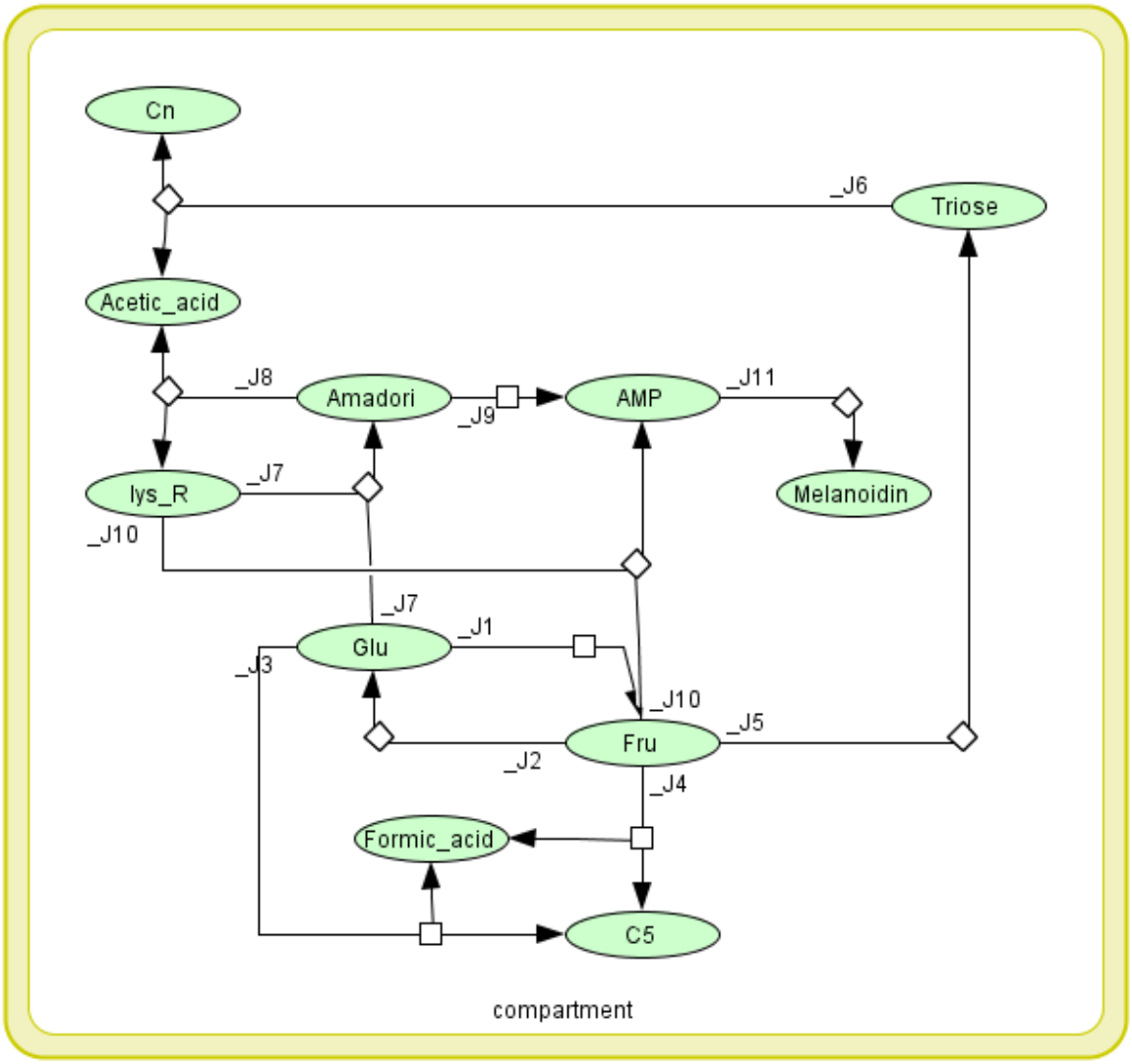


Figure 4.1 : Model of the monosacchride-casein system used in this study

Datasets

In silico experimental datasets were generated with the model using the COPASI (S. Hoops et al. 2006) biochemical network simulator. Two datasets were created, in the first experiment the initial conditions from the model were kept and in the second experiment a perturbation of the external Glucose concentration was performed. The initial metabolite concentrations for these two datasets are given in table 4.1.

Table 4.1 The initial concentrations of all metabolites is shown for experiments 1 and 2. These concentrations are the same except for the initial concentration of Glucose.

Metabolite	Experiment 1Concentration in mmol/L	Experiment 2 Concentration in mmol/L
Glucose	160	75
Fructose	0	0
Formic acid	0	0
Triose	0	0
Acetic Acid	0	0
Cn	0	0
Amadori	0	0
AMP	0	0
C5	0	0
Lys_R	15	15
Melanoidin	0	0

Mathematical Functions

In theory, any function which could properly fit the metabolite concentration curves as a function of time could be used successfully within this method. In this study, two types of functions were used to substitute for the kinetics of each metabolite (see eqs. 4. 3 and 4.4). COPASI was used to fit the parameters of the auxiliary functions of type 4.3 as well as to estimate the coefficients of the Fourier time series when applied. The resulting parameters of these curve fits were then used to interpolate values for the metabolite concentrations at times where they were not directly measured. The decision of which function to use was determined for each individual metabolite by how rapidly and properly a given function managed to fit the metabolite data. In general, polynomial functions tended to fit the less sharply curved datasets, while the Fourier series was able to better fit those datasets with sharp, initial peaks.

Fourier Series

The Fourier series decomposes a curve using a combination of sines and cosines or complex exponentials. It performs optimally when the signal being fit contains periodic elements (Fourier 1888).

The form of the Fourier series used in this study was composed of sines and cosines. It can be written as:

$$g(t)=\frac{a_{i,0}}{2}+\sum_{l=1}^{q} a_{i,l}\cos(l\cdot t)+b_{i,n}\sin(l\cdot t) \qquad 4.4$$

where *a* and *b* represent the coefficients, *q* represents the number of sine and cosine terms in the series and *t* represents the variable of interest, in this case time. As *q* approaches infinity, the Fourier series should be able to approximate any periodic signal correctly. For the purpose of this study, however, it was usually sufficient for *q* to be eight. In a few cases, eight was inappropriate and more terms were needed. The coefficients a_n and b_n are defined in the range $[-\pi, \pi]$. In the study, these coefficients were first initialized to a random number within this range. Parameter estimation was then performed on the coefficients to best minimize the mean square error of the estimate to the metabolite data.

Polynomial Function

Polynomial functions are a class of smooth functions of finite length which are composed of variables, constants, and exponents. These elements are combined via a selection of the mathematical operations of addition, subtraction and multiplication (Durbin 2009). Polynomial functions have been used extensively for nonlinear regression (McDonald 2008). Because they are smooth functions and thus have continuous derivatives up to a desired order, polynomials are useful for curve fitting of metabolite concentrations over time. In this study, polynomials were constructed according to the formula:

$$g(t)=a_{i,0}+\sum_{l=1}^{q} a_{i,l}\cdot t^{l} \qquad 4.3$$

where *q* represents the order of the polynomial being used, a_n represent the coefficients which must be determined and t represents the variable of interest, in this case time. The values of the coefficients may be either positive or negative. In practice, the coefficients of the higher order components tended have smaller magnitudes than those of the lower orders when fit to the metabolite concentration curves. These coefficient values were obtained by performing parameter estimation to minimize the mean square values of the estimates to the fitted data. In general, most metabolite concentration curves which were fit using polynomial functions could be approximated using fifth order polynomials, however in some cases higher orders were used to obtain a better fit.

Constructing Models Based in Mathematical Functions

As a starting point, the time-series data for each metabolite was fit using either a Fourier series or a polynomial function. In each case, the fitting function was initialized with a smaller set of terms. In the case of the Fourier series, eight terms were used as a starting point. The polynomial functions, by comparison, were initially set as fifth order polynomials The coefficients for each function were then estimated using the parameter estimation tools in COPASI (S. Hoops et al. 2006) . Depending on the quality of the fit, the utilized function was either maintained or altered. This alteration was enacted by either adding more terms or orders to the current function or by changing the function type. Each metabolite time course concentration curve was fitted separately in this manner. In the case where the two experiments are fitted simultaneously, the function which was used to fit the metabolite's concentration in experiment 1 was used as a starting point.

Determining Boundaries for the Parameter Search Space

After fitting each constituent metabolite of the given biochemical model using the mathematical functions, the second phase proceeded in the following manner. Using the resultant model where mathematical functions are substituted for the enzyme kinetics of each metabolite, individual metabolite concentrations were then separately defined in terms of the reactions in which they participated . The parameters in these reactions were estimated by fitting them to the concentration data of this metabolite. In each instance, the concentrations of the subset of other metabolites involved in the same reactions were interpolated using the previously defined mathematical functions. As would be expected, the accuracy of the parameter estimates for the rate laws of the fitted metabolite thus depended greatly on the quality of the fits of the previously defined time dependent functions to the participating metabolite concentrations. Parameter estimation for each rate law was performed as before within COPASI by fitting the time course data for a single metabolite concentration.

Because a single metabolite was fitted in a given parameter estimation run and all reactions were composed of at least two metabolites, each reaction had multiple estimates of their parameters. These resultant estimates were then used as the new boundaries for the final parametrization step.

Final Parametrization

In this step, the kinetic parameters for all of the model's reactions are estimated simultaneously using the previously determined ranges. The results from both the method where the experiments were fit simultaneously and where they were fit separately were compared to a version in which the parameter search space was not limited. The parameter range used in this control was 0 to 2000, and is based upon the observed parameter range in the BRENDA database (Chang et al. 2009; I. Schomburg et al. 2002). In all cases, both experimental datasets were used as targets for fitting in this final parameter estimation step.

Parameter Estimation

Parameter estimation at each step was performed using the tool COPASI (S. Hoops et al. 2006).A discussion of how parameter estimation methods may be performed in COPASI is given by Mendes *et al* (Pedro Mendes et al. 2009) . Different optimization algorithms were used at all stages , in the initial fitting of a metabolite's concentration to a mathematical function, in the fitting of reactions to individual metabolites, and in the final parameter estimation of the entire model. A description of the basic stages in the proposed method is given in table 4.2. A summary of the optimization methods used along with the stages in which they were applied is given in table 4.3.

Table 4.2 The basic stages of the proposed method

Method Stage	Description
1	Metabolite concentration fit to function which is only dependent on time
2	ODE for a given metabolite concentration is approximated by using time dependent functions from 1 to substitute for all other metabolite concentrations in original ODE. These ODEs are fit to the given metabolite concentration within the time course to obtain estimates for the rate equation parameters of reactions in which the given metabolite participates.
3	Final adjustment of all rate equation parameters is performed using parameter estimates obtained in step 2. Adjustment is performed by fitting all metabolite concentrations in all available timecourse datasets.

Table 4.3: A Brief description of optimization methods used and the method stage in which they were applied

Optimization Method	Stages Utilized	Brief Description
Particle Swarm	All	Stochastic method by which a group of particles attempts to find the optimum parameter values by performing multiple, simultaneous searches over the parameter space
Genetic Algorithm SR	All	Stochastic tournament selection with parallels genetic selection
Hook & Jeeves	1,2	Direct search method based on previously saved stepwise parameter moves
Levenberg - Marquardt	1,2	Gradient descent which combines Newton's methods with steepest descent

Results and Discussion

Time Dependent Functions Fit to Simulated Biochemical Data

Time dependent functions, see eqns 4.3 and 4.4, were fit to metabolite concentration data from two timecourses. In the first timecourse the initial concentration of Glucose is 160 mmol/ml, while in the second time course the concentration of Glucose was lowered to 75 mmol/ml. The concentrations of each metabolite within each of these timecourses is fit with a unique function which only depends on time. Therefore, each metabolite concentration is represented by two functions; one which fits the metabolite concentration in the first time course and the other which represents the metabolite concentration within the second time course. At this stage, it is very important that these functions fit the metabolite concentration time course data as perfectly as possible because all of the later steps in the method depend on the quality of this fit. In figure 4.2, fits of the time dependent functions to the concentrations of Fructose, AMP, Triose, and Acetic acid within experiment 1, where the initial Glucose concentration was 160 mmol/mL, are shown. The concentrations of Fructose and AMP were fit using a Fourier time series, while the concentrations of Triose and Acetic acid were fit using a polynomial function.

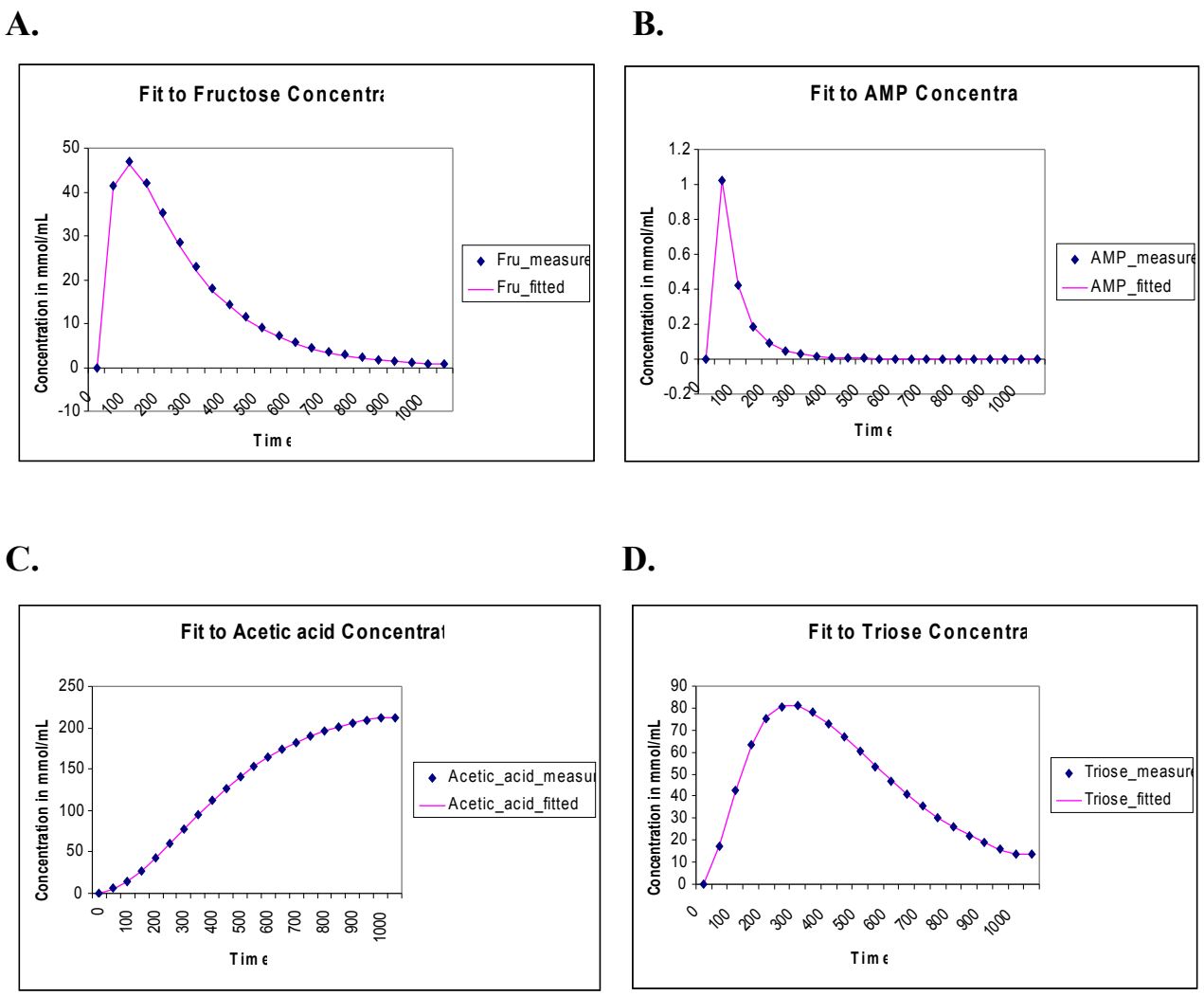


Figure 4.2 Fits of functions based on either 4.3 or 4.4 to metabolite concentration data. Functions of type 4.4 are used to fit the concentrations of **A**. Fructose and **B**. AMP. Functions of type 4.3 are used to fit the concentrations of **C**. Acetic acid and **D**. Triose

Change in Metabolite Concentrations with Respect to Time Using Time Dependent Functions to Substitute for Interacting Metabolites

In order to separate the initial set of ODEs for the system, such as those in eqn. 4.1, into ODEs which could be analyzed individually, the interdependence between metabolite concentrations was removed. These functions, see eqn. 4.5, were made independent by substituting all other metabolite concentrations with previously determined time dependent functions. These functions were based on close fits to the given metabolite's concentration time course data. Each of these new ODEs, which represent the dynamics of an individual metabolite, was fit to the relevant metabolite concentration

data in order to obtain estimates for the rate equation parameter values for all reactions in which the metabolite participated. In table 4.4, the quality of the fits of each of these new ODEs to the relevant metabolite concentration is shown using the quality bit score schema described in chapter 2. In this schema, a binary bit score is used to represent how well the given data is fit from a qualitative perspective, with '11111" being an ideal fit and '00000' being an unacceptable fit. Triose is related to Fructose via reaction J5 and to Acetic acid via reaction J6. The fits of the previously determined time dependent functions to the concentrations of Fructose and Acetic acid is acceptable, as can be seen in figure 4.2. Melanoidin is related to AMP via reaction J11. The fit of the previously determined time dependent function to the concentration of AMP is shown in figure 4.2. The time dependent functions used to substitute for the other metabolite concentrations in these ODE functions fit their respective metabolite concentrations well. Therefore, it is unlikely that the difficulty these ODE functions have with fitting their respective concentration data is due the quality of the time dependent functions used to represent the other metabolite concentrations in the reactions.

Table 4.4: The quality bit score for the fits of the ODEs of the type in eqn. 4.5 to their respective metabolite concentrations

Metabolite	**Quality Bit Score for Experiment 1: initial Glucose concentration = 160 mmol/mL**	**Quality Bit Score for Experiment 2: initial Glucose concentration = 75 mmol/mL**
Glucose	11111	11111
Fructose	11111	11111
Lys_R	11111	11111
Cn	11111	11111
C5	11111	11111
Amadori	11111	11111
Melanoidin	00100	01000
Acetic acid	11111	11111
Triose	00100	00100
Formic acid	11111	11111
AMP	11111	11111

As can be seen from table 4.4, most of the metabolite concentrations were fit quite well by the ODE functions created by this method. However, the ODE functions created for Triose and Melanoidin did not achieve ideal fits to their respective concentration datasets. The fits of these ODE functions to their metabolite concentration data in experiment 1 is shown in figure 4.3.

A. **B.**

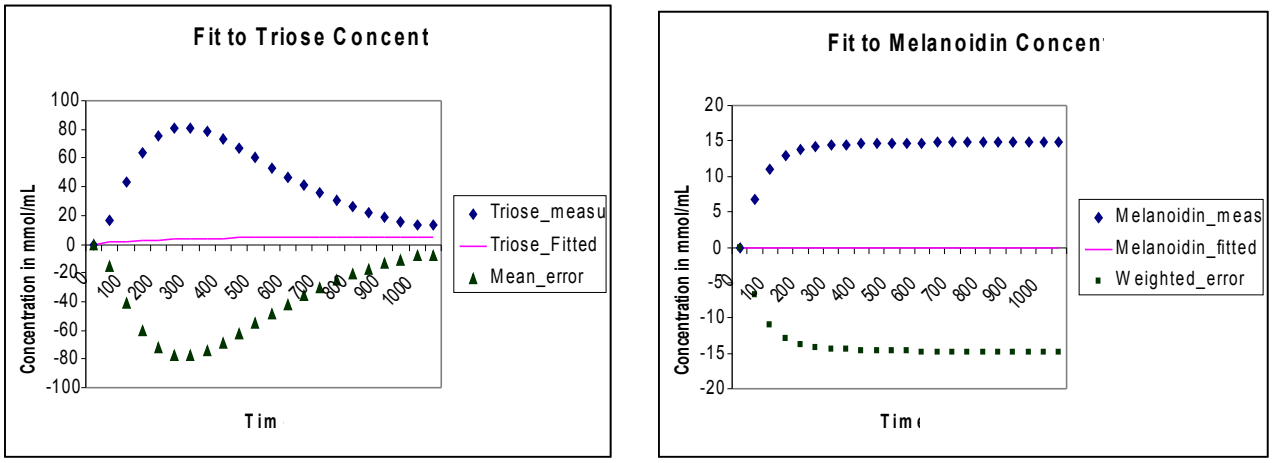


Figure 4.3: Fits of the ODE functions of the form in Eqn 4.5 to concentration data of **A**. Triose and **B.** Melanoidin.

Parameter Ranges Identified Via Proposed Method

In table 4.5, the true values of the kinetic parameters are given. In tables 4.6 and 4.7, the parameter estimation results obtained by fitting the auxiliary ODE functions, see eq.4.2 , created by the method to the relevant metabolite concentration data are given.

Table 4.5 The true values for each rate law parameter

Reaction	True Parameter Value
J1	1.00E-002
J2	5.09E-003
J3	4.70E-004
J4	1.10E-003
J5	7.12E-003
J6	4.39E-003
J7	1.80E-003
J8	1.11E-001
J9	1.44E-001
J10	1.50E-004
J11	1.25E-001

Table 4.6 : Parameter estimation results from fitting experiment 1 concentration data , where the initial Glucose concentration is

160 mmol/L

Reaction	Glucose	Fructose	Acetic Acid	Amadori	C5	Formic Acid	Cn	AMP	Melanoidin	Lys_R	Triose
J1	8.33E-03	1.20E+03									
J2	2.96E-165	7.60E+02									
J3	3.85E-306				9.88E-04	9.88E-04					
J4		5.21E+02			4.13E-03	4.15E-03					
J5		3.06E+02									7.50E-03
J6			4.57E-03				4.38E-03				4.55E-03
J7	2.13E-245			1.11E-04						2.13E-62	
J8			1.03E-142	7.10E-02						1.15E-01	
J9				1.30E-01				2.48E+02			
J10		4.01E+02						1.29E-19		8.53E-04	
J11								1.11E+02	1.25E-04		

Table 4.7 : Parameter estimation results from fitting experiment 2 concentration data, where the initial Glucose concentration is 75 mmol/L are shown

Reaction	Glucose	Fructose	Acetic Acid	Amadori	C5	Formic Acid	Cn	AMP	Melanoidin	Lys_R	Triose
J1	9.11E-03	2.00E+03									
J2	7.40E-50	3.30E+01									
J3	1.68E-46				1.01E-03	4.70E-04					
J4		3.43E+02			1.39E-03	1.10E-03					
J5		1.76E+02									1.15E-01
J6			4.72E-03				4.38E-03				2.02E-02
J7	4.01E-03			1.78E+00						1.64E-04	
J8			8.58E-68	8.03E+02						6.62E-02	
J9				1.78E+03				2.72E+02			
J10		3.46E+02						0.00E+00		5.73E-303	
J11								1.21E+02	5.74E-06		

Final Parametrization Results

The results of the final parametrization step are shown in table 4.8. As was expected, by restricting the interval size of the parameter search space, the resulting parameter estimates for the rate equations of the system were close to the true values.

Table 4. 8 : True and estimated values for the rate law parameters of each reaction obtained from applying the proposed method

Reaction	True Value	Estimated Value	$(p-\hat{p})^2$
J1	1.00E-002	8.33E-003	2.79E-006
J2	5.09E-003	3.04E-003	4.20E-006
J3	4.70E-004	3.93E-017	2.21E-007
J4	1.10E-003	1.89E-003	6.24E-007
J5	7.12E-003	7.50E-003	1.44E-007
J6	4.39E-003	4.38E-003	1.00E-010
J7	1.80E-003	2.99E-004	2.25E-006
J8	1.11E-001	1.19E-001	6.40E-005
J9	1.44E-001	1.99E-001	3.03E-003
J10	1.50E-004	0.00E+000	2.25E-008
J11	1.25E-001	1.11E-001	1.96E-004
Sum of Squares of the Residuals			3.30E-003

In figure 4.4, the fits of the final model obtained via the proposed method to the metabolite data of experiment 1 are shown for two representative reactions, J6 and J9. These reactions were chosen because they represent the rate law parameter estimates where the square of residuals was the smallest (J6) and the largest (J9). In both of these cases, the fits of the model to the metabolite data of interest is good.

A. **B.**

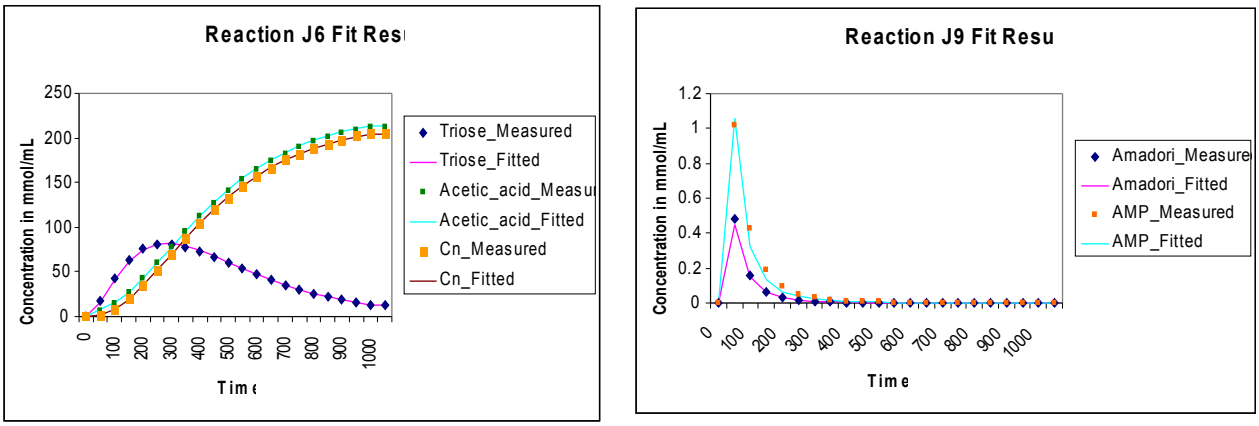


Figure 4.4 : The fits of the model, estimated using the proposed method, to the metabolite data for representative reactions are shown for experiment 1. The fits to : **A**. J6 and **B.** J9 are displayed in terms of their constituent metabolites. The parameter range in this instance is based upon the estimates obtained via the proposed method.

Control Model in Which No Range Boundaries Are Imposed on Final Parameter Estimation

Table 4.9 The true and estimated values for all rate law parameters where no addition range limitations beyond those derived from BRENDA are imposed

Reaction	True Parameter Value	Estimated Parameter Value	$(p-\hat{p})^2$
J1	1.00E-002	3.83E+002	1.47E+005
J2	5.09E-003	9.39E-001	8.72E-001
J3	4.70E-004	6.23E+001	3.88E+003
J4	1.10E-003	1.88E-005	1.17E-006
J5	7.12E-003	5.48E-135	5.07E-005
J6	4.39E-003	1.74E+000	3.01E+000
J7	1.80E-003	2.00E+003	4.00E+006
J8	1.11E-001	1.95E+001	3.76E+002
J9	1.44E-001	0.00E+000	2.07E-002
J10	1.50E-004	3.99E-002	1.58E-003
J11	1.25E-001	2.00E+003	4.00E+006
Sum of Squares of the Residuals			8.15E+006

In figure 4.5, plots of the fits of the model obtained using the control method to the metabolite data

from experiment 1 are shown for representative reactions, J4 and J11. The estimated parameter value for the rate law of J4 had the lowest residual square value, while the estimated parameter for J11 had one of the highest residual square values. The fits for the metabolites which participate in reactions J4 and J11 are not particularly good. However, the fits to the metabolite concentrations in reaction J11 seem better than in J4, even though the parameter value estimated for J11 was much worse than that estimated for the rate law parameter of J4.

A. **B.**

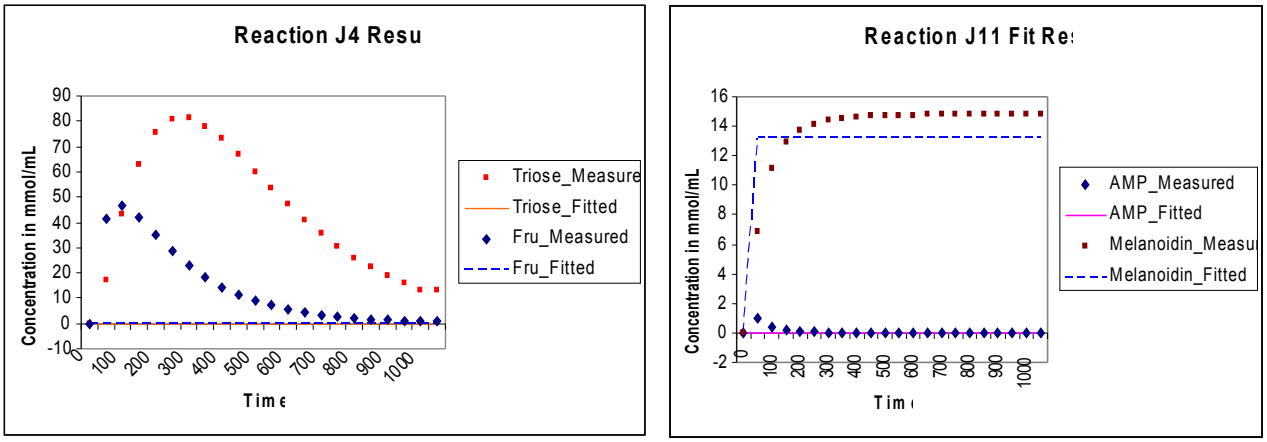


Figure 4.5 : The fits of the model, estimated using the control method, to the metabolite data for a representative set of reactions are shown for experiment 1. The reaction fits: A. J4 and B. J11 are displayed in terms of their constituent metabolites. The parameter range used in the control method were based upon those obtained from BRENDA.

Conclusions

The method proposed in this chapter demonstrated a strategy by which a system of ODEs, representing rate laws of a set of interacting metabolites, could be analyzed separately. This was done by substituting time dependent functions, such as those of eqns. 4.3 or 4.4 , for other metabolite concentrations not directly represented by the given ODE. The parameter estimates which resulted from fitting each individual ODE function, see eqn. 4.5, to the concentration data for its respective metabolite were used to restrict the parameter search space for the original set of ODEs.

The results of applying this method were obtained by using an artificial biochemical network. By using

an artificial biochemical network, it was possible to determine the difference between the estimated values of the rate law parameters, obtained in the proposed and control methods, and the true value of these parameters. The sum of squares of the residuals between the estimated and true values of parameters was used to compare the proposed method with the control method.

It was hypothesized that employing this method would have two main benefits over the control method; improved speed and accuracy. In this study, the sum of squares of the residuals for the differences in the parameters estimated using the proposed method is much lower than than of the control sum of squares. The final adjustment step, where parameter estimation is done on the full system, of the proposed method also took much less time to converge than did the parameter estimation of the control. Thus, the results of this study support both hypotheses concerning this method. Further studies should concentrate on different models, to investigate the generality of the method, as well as data that contains increasing levels of noise to investigate how noise may affect the procedure. In case it is demonstrated that this is a robust procedure, software could be developed to automate the entire process.

Chapter 5: Conclusions and Future Directions

Introduction

The purpose of this work was to investigate the effect that missing and incomplete knowledge have on systems biology studies, and to propose methods of accounting for this deficit. Although missing and incomplete knowledge affects all levels of scientific inquiry, its presence in systems biology is especially pervasive. A goal of systems biology is to build models which accurately reflect the underlying complex set of interactions responsible for an organism's ability to respond to environmental stimuli (Mendes 2001). However, the amount of knowledge necessary to create these models is generally unavailable. Thus, in many cases systems biology studies must attempt to recover the whole with only partial information.

Conclusions from Research

The Influence of Partial Network Information on Obtaining System Parameters

The full underlying biochemical network of interactions is generally not known for most organisms. In chapter 2, an analysis was performed to determine how this incomplete network knowledge might affect the ability to recover systems level information. Specifically, the focus was on how studying only the metabolic portion of an artificial biological network might skew the estimations for kinetic parameters of the studied reactions. It was hypothesized that the rate law parameters for those reactions which were under greater transcriptional control would be most affected by a study in which only the metabolome was considered. In order to test this hypothesis, a method was proposed to test the strength of association between reaction species and pre-identified regulatory components. These regulatory components were classified as either contributing to metabolic or hierarchical control depending upon both their identity and the context of their relationship to the metabolic species. For example, in some contexts a protein was classified as exuding transcriptional control, while in other instances it was considered an aspect of metabolic control.

The results from chapter 2 indicate that under those specific conditions, there were significant differences overall, as determined by the Wilcoxon signed rank test (Whitley & Ball 2002), between the true kinetic parameters and those estimated using the only metabolic portion of the Claytor network. However, the significance of the differences for the most part disappeared when the reactions were assessed individually. While none of the differences for individual reactions was significant at the $\boldsymbol{\alpha} = 0.05$ level, one reaction was closer to having significantly different parameter estimates from its true values than the rest. This reaction was compared to all of the others in terms of its association with pre-defined transcriptional and metabolic regulatory species. In most of the experiments where the association with either the transcriptional or metabolic control types for this reaction was assessed, the association was stronger with the metabolic control type. However, in one experiment, a gene knock out, this reaction was slightly more associated with the hierarchical control type. Therefore, it was concluded that the significance of the differences between the true and estimated kinetics were likely due to the combined effects of non-metabolic regulation on each reaction, rather than on specific reactions. However, it was noted that a future study should be done to check the association of each reaction with translational control.

Several observations resulted from calculating the association of each reaction and its component metabolic species with the metabolic and hierarchical control types. For one, it was observed that the experiment which was used to calculate kinetic parameter estimates contained the most information. Here, the amount of information in an experiment refers to the number of metabolic species whose concentrations underwent alterations due to the experimental conditions. In the other two experiments, there were several metabolic species whose concentrations were unaffected by the given perturbations. Because of this lack of information in these experiments, there were several instances where the control type of a given metabolic species could not be determined. In addition, it was noted that when association with a control type could be predicted, it was most often predicted as being associated with metabolic control. These observations indicate that the effect of studying the metabolic reactions in isolation from transcription and translation will depend on both what the predominant control type of these reactions is as well as the experimental dataset which is used to estimate the kinetic parameters. In this study, because most of the reactions were predicted as being predominately under metabolic control, it is likely that parameters estimated using only the metabolic portion of the network were far

less detrimental than in an experiment where hierarchical control was more dominant. It also indicates that the quality of the parameter estimates determined depends greatly on the amount of information which can be gleaned from the experiments which they are based upon. If one of the other experiments had been used to calculate parameter estimates in this study, it is likely that the differences between these and the true parameter values would be much more significant.

Comparing Methods for Forming General Kinetic Rate laws

Unknown kinetic rate laws are often a confounding factor in constructing accurate models of biochemical systems. In order to circumvent this lack of knowledge, several methods have been proposed which can create generalized rate laws. In chapter 3, three of these methods were compared: generalized mass action (Guldberg & Waage 1879; L. Segel & John J. Tyson 1992), linlog (Visser & J. J. Heijnen 2003b), and convenience kinetics (Liebermeister & Klipp 2006b). For the purposes of evaluation, the Claytor artificial biological network was used. The generalized rate law methods were compared based on how well they managed to fit concentration data from time-series experiments performed on the Claytor network as well as the number of parameters they employed to obtain this fit. The criteria for model selection used was the Akaike's Information Criterion (AIC) (Akaike 1974) , which takes both of these factors into account. These methods for generalizing rate laws were further compared based on how sensitive they were to the availability of network knowledge. This sensitivity was assessed by creating kinetic models by each approach and then estimating the resultant parameter values when these metabolic reactions were integrated into the full network and then again when these reactions were separated from the full network. It was expected that convenience kinetics would perform best overall because the form of its equations is most similar to that of the true kinetic rate laws. It was further predicted that the generalized mass action kinetics were those who produced the most accurate results after isolating the metabolic reactions from the full Claytor network .

Surprisingly, the method which performed best in this study was generalized mass action. In fact, when these methods were implemented on the metabolic portion of the Claytor network isolated from the transcriptional and translational reactions, the generalized mass action method resulted in a better AIC value than even the true rate laws. Two possible reasons for this are : 1) generalized mass action has

significantly fewer parameters than the other methods 2) certain features of the experimental dataset made it more amenable to analysis by generalized mass action . The generalized mass action method did not fit the data quite as well as the true kinetic rate laws, as evidenced by the sum of squares of the residuals. However, the true kinetic rate laws contain many more parameters than does generalized mass action. Because the quality of fit for the more complicated model based on the true rate laws is not significantly better, the simpler model was preferred by AIC. In addition, it is likely that the experimental dataset being fit was of a type which is more easily fit by generalized mass action. This experiment was the same as the one used in the study in chapter 2, where it was predicted to be under predominantly metabolic control. It is possible that generalized mass action was able to fit the data well because the effects of the modifiers were minimal as compared to the control exerted via metabolism. If the enzyme concentrations had been altered greatly, via transcriptional regulation, in this experiment, then it is likely that generalized mass action would not have performed as well.

Convenience kinetics performed essentially the same as the true kinetic rate laws when the metabolic reactions were separated from the full Claytor network. Thus, convenience kinetics is still likely an excellent approach for generalizing rate laws when the true kinetic equations are unknown. By contrast, models based on linlog rate law approximations failed to converge. In two cases, even replacing the rate laws of a single reaction with linlog caused a failure to converge. This failure indicates that for these reactions the linlog parameters were unidentifiable, most likely due to certain features of the equation. Thus, although it has been shown to be effective for the purposes of other studies, linlog was not not useful in this study.

A Data-Driven Approach to Reducing the Parameter Search Space in Biochemical Systems

In chapter 3, a method is proposed for reducing the parameter search space in biochemical systems. In the proposed method, the set of ordinary differential equations (ODEs) which represent the kinetic rate laws of the system are presumed to be known. Calculating estimates for the parameters of systems of ODEs becomes increasingly difficult as the dimension of the system increases. One way of diminishing this difficulty would be to somehow estimate the parameter values for the individual ODE functions separately. In order to accomplish this goal, the equations would need to be made independent from each other. I proposed a method that accomplishes this aim by using functions which only depended on

time; the empirical parameters of these equations have to be estimated from the data, however this is done very efficiently. These functions were not assumed to contain any structural properties which related them to the biochemical system, the only requirements were that they fit the concentration time-course data well and only depend on time – and therefore are decoupled. The functions used in this case were the polynomial (Durbin 2009) and the truncated Fourier series (Fourier 1888) functions. These time-dependent functions were used to create auxiliary ODEs, where each ODE function for the concentration of a given metabolic species was independent from that of every other ODE function in the model. By employing this method, it was possible to estimate the parameters of each auxiliary function separately. The resultant values were then used to restrict the interval size of the parameter search space for the original system of ODEs.

It was hypothesized that this method will greatly improve on the computational time necessary to obtain estimates for the parameters of the kinetic rate laws of biochemical systems. It was also proposed that by restricting the search space there would be some scenarios in which the method would obtain parameter estimates close to the true values, while a global optimization applied to the entire system with the coupled equations would not obtain accurate values for these parameters, at least not in a feasible computational time.

The proposed method was tested in a controlled manner by applying it to an artificial biochemical network obtained from the curated BioModels database (Le Novere et al. 2006). As a control, parameter estimation was performed on the intact system of ODEs. In this case, the parameter ranges were bounded to the values existent in the kinetic parameters database BRENDA (I. Schomburg et al. 2002). The control case and the proposed method were compared in terms of how long the final parameter estimation took and also on how accurate the resulting values were. In both cases, the proposed method outperformed the control case. Therefore, the hypotheses that the proposed method would improve on both computation time and accuracy were supported in this study.

Future Directions

Simplification

It was mentioned in the introduction to this thesis that in some cases, certain types of missing information might not be detrimental to the formation of a useful model of the system. This leads to an interesting possibility for future research. Namely, it may be possible to instead look at the problem of missing information from a reverse standpoint. By identifying information which is not as necessary for the formation of the model, it may be possible to simplify a complex model into one which is more tractable while still describing the known data fairly well. This could be especially useful when studying large biological networks. It is often difficult to carry out analyzes of these networks because of their size. In many cases, a researcher will not be interested in the whole network, but only in a subset of it. By identifying the critical qualities of the network surrounding the subnet of interest, it may be possible to include only that outside information which is necessary. Ultimately, this non-focal information could even be substituted by non-mechanistic functions, in a manner similar to how the concentrations of dependent metabolic species were substituted with time-dependent functions in Chapter 4. By simplifying the network in this fashion, it may be possible to improve the ease of analysis for the subnetworks of interest while still including all relevant information.

Integration of 'Omics

In chapter 2, a method was introduced to calculate the association between metabolic species and potential regulatory species. This method could also be adapted to aid in the integration of different levels of 'omics data, such as metabolomics and transcriptomics. In spite of the recognition of its importance, simultaneous study of multiple 'omics levels is still relatively rare. One of the difficulties involved is ameliorating the discrepancies in scale which can exist between these levels, and in some cases, within the same level. By employing Z-scores based on correlation, the wide possible differences in scale would no longer be a confounding issue.

Prediction of Missing Biological Interaction Network Knowledge

Another possible future direction indicated by this work is the prediction of pieces of the network which contain missing information. In chapter 2, the fit of the metabolic model of Claytor network to the concentration data was imperfect in several cases. The poor fit of a model to the data indicates that there is missing knowledge of the underlying model. Especially if more data were available, it might be beneficial in these cases to see if these sub-optimally fit species concentrations were correlated to other species not codified into the underlying model. In this way, it might be possible to predict both the location of a gap in information and to suggest relationships which might fill it.

Conclusion

The focus of this work has been to investigate the effect that different types of missing information or knowledge can have on a systems biology study. Systems biology aims to construct models which take into account the underlying network of interactions that make it possible for an organism to respond to its environment. Because of this, much more information is necessary than in a reductionist approach. Indeed, the amount of information which is required for a systems analysis of an organism is such that incomplete information is likely to be a consistent reality for these studies. In addition, systems biological studies must often deal with scenarios where there is incomplete knowledge. Here knowledge is indicated as separate from information, and refers to information which has been codified into systems biology models at some level (mechanisms). Missing information and partial knowledge are interrelated. When information is missing, it is impossible to form models which take this information into account. Thus, incomplete information leads to incomplete knowledge.

The relationship between hypotheses and experimental studies was discussed in the introduction (Kell & Oliver 2004). Namely, there is a cyclical association between the formation of valid hypotheses, or models, of a biological system and experimental evidence. New experimental evidence suggests new hypotheses, which subsequently suggest new experiments. The relationship between missing information and incomplete knowledge may be thought of as an aspect of this cycle. Identifying a situation where there is incomplete information suggests that the underlying knowledge of the system itself may be incomplete, which suggests the necessity for more experiments. Methods to suggest which experiments to perform in this situation would be a major advance in the scientific methodology.

It is hoped that the results presented in this thesis make a small, but significant, contribution towards this goal.

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