Discrete modelling of the energy metabolism regulation of eukaryotic cells and formal validation of its dynamics

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Modélisation discrète de la régulation du métabolisme énergétique des cellules eukaryotes et validation formelle de sa dynamique

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UNIVERSITÉ DES MASCAREIGNES
SAVOIR, C'EST POUVOIR

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Abstract

We present a formal model of the regulation of the energetic metabolism in eukaryotic cells. The main originality of this model is to consider explicitly an abstraction of the main metabolic processes that pilot this metabolism, thereby greatly reducing the number of variables in the model. Moreover, the modelling framework proposed by Réné Thomas is particularly well suited for a qualitative view of regulatory networks resulting in a model with 14 variables and 112 parameters, with integer values. However, the model contains a lot of feedback loops which are intricately linked and which makes the dynamic of the system very complex. As in all complex system modelling, the main difficulty is to identify the value of all parameters in a coherent way with respect to known dynamic behaviours.

The identification of parameters has been smoothed due to a large repertoire of knowledge in molecular biology, and the validation of the proposed model has been done by model checking, in more than 160 temporal logic formulas (including the main metabolic phenotypes, notably the Warburg effect). It has been a meticulate process which has been successful by putting in place a solid and pluridisciplinary method of modelling together with a software platform (DyMBioNet), both pivotal for this thesis. The model has been conceived to be used as a backbone, which can be plugged with other regulatory networks like the cell cycle or the circadian clock, for potential applications to cancer or chronotherapy. The DyMBioNet software is bundled with three main functionalities including verifying system properties with CTL, simulation as well as visualisation of a complex system. Furthermore, this well-defined methodology, and its software platform DyMBioNet, would be useful to directly construct other formal regulatory networks of large size.

Keywords: Modelling, Biological networks, Formal logics, Metabolism

Modélisation discrète de la régulation du métabolisme énergétique des cellules eukaryotes et validation formelle de sa dynamique

Résumé

Nous présentons une modélisation formelle de la régulation du métabolisme énergétique de la cellule eucaryote. Le choix original de cette modélisation est de considérer explicitement des abstractions des principaux processus cellulaires qui pilotent ce métabolisme, réduisant ainsi considérablement le nombre de variables à prendre en compte dans le modèle. De plus, le formalisme de modélisation introduit par Réné Thomas est particulièrement adapté à une vision qualitative des phénomènes de régulation, de sorte que le modèle repose sur seulement 14 variables et 112 paramètres entiers. En revanche, le modèle possède de nombreux cycles de retroaction fortement intriqués, qui rendent la dynamique du système très complexe. Comme dans toute modèlisation de système complexe, la difficulté majeure est l'identification des valeurs des paramètres de manière cohérente avec les comportements dynamiques connus.

L'identification des paramètres a été effectuée sur la base d'une abondante connaissance biologique moléculaire, et la validation du modèle a été effectuée par model checking sur plus de 160 formules temporelles (incluant les principaux phénotypes connus, en particulier l'effet Warburg). Il s'agit d'un travail minutieux qui n'a pu être mené à son terme qu'en mettant en place une méthode pluridisciplinaire de modélisation et une plateforme logicielle (DyMBioNet), qui constituent également une contribution importante de la thèse. Le modèle achévé a été conçu comme un "noyau formel" réutilisable en connexion avec d'autres réseaux de régulation comme le cycle cellulaire et l'horloge circadienne, par exemple en vue d'application au cancer ou à la chronothéraphie. L'outil DyMBioNet présente plusieurs fonctionnalités incluant la possibilité de faire des preuves en CTL, la simulation ainsi que la visualisation d'un système complexe. Enfin, la méthodologie définie ici et son outillage DyMBioNet pourront être réutilisés directement pour construire d'autres modèles formels de régulation de grande taille.

Mots-clés: Modélisation, Réseaux biologiques, Logique formelles, Métabolisme

Dedications

To my parents, for giving me the most powerful tool in my life, Education

To my wife and kids, for bearing with me, and for being my source of motivations

To my supervisors, always there for helping and guiding me

To my University, for providing me all forms of support

To all those people, who have always underestimated me

To God, for this beautiful life on Earth !

Contents

CHAPTER 1

INTRODUCTION

Cellular metabolism or central carbon metabolism is the economy of the cell: how to transform resources (nutrients) into energy and building blocks (catabolism) to produce biomass (anabolism) for cell proliferation. Two main regimes exist for catabolism: a slow but very efficient metabolism known as *respiration* and an inefficient but fast metabolism known as *fermentation*. Mammal cells and facultative microorganisms adapt their metabolisms to the environment especially when nutrient is scarce or abundant. Cells, in general, favour the respiration pathway when nutrients are abundant and shift to the fermentative mode when nutrients are scarce. This cellular adaptation (respiration-fermentation shift) to the environmental milieu is known as *regulation* and it is exactly the politics of the cell on how to manage this economy.

The goal of this thesis is to model the mechanism of the respiration-fermentative shift of cells with facultative metabolism; that is cells that have the option to shift between respiration and fermentation processes. This shift is triggered by high intake of glucose and occurs even in the presence of oxygen. It is quasi irreversible in cancer cell lines and is known as the *Warburg effect*. In fermentation process (for example wine production), this shift is reversible and is known as the *Crabtree effect* in hommage of H.G Crabtree who studied this effect in bacteria and cancer cells. In this text, we will refer to this effect in the most generic term as the Warburg/Crabtree effect.

A major contribution of this thesis is the use of Réné Thomas qualitative modelling approach to the regulation of energetic metabolism. R.Thomas pioneered this approach to genetic networks in which variables correspond to genes and regulation signals are triggered by molecular activators and inhibitors. In the case of cell metabolism, we were able to use an abstract view of metabolism by exchanging genetic variables to phenotypic variables (metabolic pathways for which regulation signals, mechanisms and functions are well known from biochemistry literature). Parameters identification, the crucial problem in biological networks became, in that case, possible and such qualitative parameters are even compliant *in vivo*.

This abstraction effort from gene or enzyme to biological pathways is made by changing the activation / inhibitor standard interpretation to the meaning of "is consuming" or "is providing" the resource in regulatory interactions (we will see later on how we managed linear vs. non linear interactions). As we are interested in behaviour, including potential therapy on the Warburg/Crabtree effect to reverse the cancer fermentative metabolism to respiratory metabolism, we use Computation Tree Logic (CTL, which is a temporal logic) to encode the asymptotic behaviour of this metabolic shift (with a useful macro-language to handle fairness properties).

This introductory chapter presents the important features of our application of R.Thomas framework to energetic metabolism. The first section describes the pertinent characteristics of our complex biological system made of intricate biological cycles. Section 2 describes how Thomas approach is well suited to tackle such complexity. Section 3 presents the main steps of the methodology we have developed for abstracting biological network in the case of energetic metabolism. In Section 4, we describe the DyMBioNet software to investigate a given Thomas model through dynamic simulations and proof checking techniques. The last section gives a roadmap of the thesis.

1.1 Abstract view of metabolism

Cells regulate metabolism through a balance between the two complementary subunits that are tightly regulated: catabolism and anabolism. These subunits regroup four main processes namely glycolysis, fermentation, Krebs and oxidative phosphorylation which are intricately linked by positive and negative

loops. As we will make more precise later on, negative loops may generate oscillatory behaviour leading to homeostasis and positive circuits may generate a multiplicity of attraction basins. Taking into consideration the complexity of these loops, we needed a more systemic and abstract overview while mirroring the major metabolic processes in order to preserve important regulatory information. At the same time, this prevents us from infringing into molecular or genetic details.

To address this level of abstraction, it is important to consider only metabolic components for which most of the information are available from in-vivo experiments. Chemical reactions and genetic regulations lack these kind of precise information and can be misleading if we integrate them in this abstraction exercise. We have adopted a coarse-grained strategy as applied in physics, in which large components in a system can be simplified by removing fine-grained components while maintaining the physical and behavioural properties of the whole system. In a similar vein, the metabolic network has been constructed in a way where fine details (genes, enzymes, sub processes) are smoothed over. In other words, we have a compact description of the regulation of the metabolic network in which fine-grained details are compressed and hidden in larger components while preserving the biological aspects of the system: energy and biomass production. This "lossy but adequate" property is one factor that distinguishes coarse-graining from other types of abstraction [1].

In this research work, we confront this problem by constructing a coarse-grained equivalent of the whole metabolic network using a formal approach and focusing only on the regulation of energy and biomass metabolism. As such, only metabolic pathways, cofactors, nutrients and input resources for which the kinetic parameters are available in biological literature, have been considered. They are chosen with respect to the question we want to address: the shift between respiration and fermentation in cancer, commonly known as the *Warburg effect*. It is important to note that a model emerges from a given question to be addressed: questions imply dedicated models, and no question mostly means useless model.

In the next section, we showcase how our chosen formalism helps us leverage this abstraction and regroups these metabolic actors to address this metabolic shift, and how we customise the metabolic network for studying the given phenotype, the *Warburg effect*.

1.2 Choice of formalism

The energetic metabolism can be characterised by its main metabolic cycle: oscillation between anabolism and catabolism. When the cell shifts to fermentation, a new cycle occurs with NAD+ which is reduced during glycolysis and regenerated during fermentation (by oxidising NADH+). Such intricate cycles also occur with ATP/ADP and oxygen in nearly, if not all, enzymatic pathways. Modelling the intricacy of such cycles needs a detailed information on the force and priority of the activation and inhibition signals. In the Thomas "language", this starts with the value (threshold) above which a variable starts to be a resource or not.

The formal definition of the R. Thomas framework, together with model checking using CTL [2, 3], offers the ability to question biological models on any long term asymptotic behaviour of a given variable of interest (in our case respiration or fermentation). This makes the R.Thomas framework attractive from a computational point of view. It is the role of formal proof techniques to assess the pertinence of therapeutic actions on the metabolic model to control the shift between respiration and fermentation.

Finally, this formalism leads us to a generic model that is configurable to adapt to any metabolic scenario: that is, a reusable model with relatively minor reorganisation. Such reorganisation will allow a sort of "plug and play" of external modules or variables with this model. Surprisingly, the Thomas framework which has long been used for gene regulatory networks (genes activation / inhibition above a certain threshold), has proved its versatility for the modelling of this large metabolic network: a major contribution of this thesis. From our knowledge, it is the largest model constructed using this framework. To achieve this level of abstraction without deviating too much, the best way was to put in place a rigorous methodology, discussed in the next section.

1.3 An indispensable methodology

Perhaps the most critical point is to conceive a methodology not only as a set of practices but as a way of approaching the subject matter of interest. This generic methodology, which we have developed, is ideal

for discrete and formal modelling of any biological network of any size and is divided into two parts:

1. Thomas modelling framework which is based on three well-defined steps:

   a) choice of variables
   The first step is to build a repertoire of variables abstracted at a coarse-grained level.

   b) inventory of interactions and the relative needed resources for influence (threshold)
   The second step is to build the interaction between the variables and identify any cooperative action which is abstracted into "multiplexes". Next, we find the thresholds for each variable and their actions in each multiplex which we transform into logical formulas.

   c) determination of the parameters describing the long term asymptotic evolution of the state variables
   Once we have the interaction graph in hand, the next step is to determine the kinetic parameters that are key concepts for observing the dynamics of the whole network.

2. Implementation of Computation Tree Logic and proof checking techniques in Thomas' framework provide three additional steps in our proposed methodology:

   a) building a validation matrix to check for the recovering of known phenotypes
   The classical knowledge of metabolism has allowed us to implement a validation matrix with all the cellular conditions and their expected outcomes even before we finalised the whole metabolic network. This is inspired from the software requirements matrix for verifying functional properties in software engineering.

   b) simulations of dynamics to track unexpected biological features
   Equipped with the interaction network and kinetic parameters, the next useful step is to simulate and observe known phenotypes and learn from the network.

   c) hypothesis checking using CTL
   To validate the model with known metabolic traits, we needed to formalise these behavioural knowledge. This is where formal methods, especially temporal logics, have been used. By using temporal logics (here CTL, short for Computation Tree Logic), one can express behavioural properties (checking a given property along a trajectory in a short as well as long term). It is important to pinpoint that due to complexity of the proposed model and the large number of variables, there are some states that might not be reached due to cyclic phenomena. To solve this issue, we have introduced a certain degree of path fairness to ensure reachability: called fair path CTL, which is described in Chapter 5 (Section 5.7).

Finally, to implement all these functionalities, we have developed a software platform which is critical for model simulation and validation, as we showcase in the next section.

1.4 DyMBioNet platform

Simulation of the model under various perturbations can generate novel hypotheses and motivate the design of new experiments. We already have the SMBioNet tool suitable for the formal study of a network behaviour but it can handle exclusively proofs using CTL [2]. With a large model in hand and the endless validations to carry out, it is imperative to visualise the evolution of the network.

Another contribution of this thesis is the development of a software platform, called DyMBioNet (short for **Dy**namic **M**odelling of **Bio**logical **Net**works) to accompany the modeler with all the tools necessary for complex and discrete modelling of biological networks. DyMBioNet is a graph-based software which has assisted us with the development of our proposed methodology and includes the following features:

- Human Computer Interface
  We have developed a GUI interface which allows us to design the interaction graph and configure easily the kinetic parameters. There is also a charting feature with the possibility to view how the variables progress with time. This allows a fast debugging of the network. The software also integrates easy parameterisation of the external environment (called input variables) and can even generate metabolic phenotypic results for all possible combinations of these input variables, all at one go.

- Integration of SMBioNet
  DyMBioNet benefits from the integration of SMBioNet for model checking purposes. The model,

in XML format in DyMBioNet, is transformed into textual equivalent for input in SMBioNet. A reverse transition, from SMBioNet to DyMBioNet, is possible. This applies solely for existing text-based models in SMBioNet which can then be visualised graphically.

- Implementation of fair path CTL
  For any fair path CTL formula, the equivalent translation to classic CTL is integrated in the software. This guarantees that no transition in the transition graph is ignored if it can be fired an infinity of times.

1.5 Thesis roadmap

We end this chapter by giving a brief explanation about the roadmap of the thesis, which is organised as follows:

Chapter 2: We give a detailed overview of metabolism and the underlying network of regulations with respect to the production of energy and biomass. All the catabolic and anabolic processes are differentiated with a focus on the interlink between the main actors, cofactors and nutrients which form the coarse-grained model. Only the input of nutrients are considered as external factors on which we have control and which are equally useful for representing the cellular environment.

Chapter 3: We discuss briefly some of the classical formalisms that have been attempted to model the metabolic network at the genetic and molecular levels. Quantitative methods are compared to their qualitative counterparts and we provide justifications why we opt for the R.Thomas formalism.

Chapter 4: Thomas modelling framework and its adaptability to this large regulatory network is prioritised in this chapter. All the technicalities of the concerned formalism are explained in detail and we show how we can use powerful formal verification techniques to validate well-studied phenotypic properties.

Chapter 5: We inspire ourselves from methodologies in software engineering to build a methodology for the design of biological regulation network, dedicated to Thomas framework. In this chapter, we list all the steps and give examples of how we proceed with each step. We give a narration on how we identify the important variables and their classification into diverse categories. Both static and dynamic modelling procedures are listed which help to extract useful information from the biologists. Once the static and dynamic representation of the biological network are completed, we will see how to use verification techniques like simulation and model checking to validate the model. This chapter is used as a methodological backbone for the rest of the thesis.

Chapter 6: As we mentioned earlier, we want to be assisted by computer-aided tools for verification and validation purposes. In this line, the DyMBioNet tool is showcased in this chapter. The key functionalities of the software are displayed with simulated examples to explain the importance of the software. It integrates also a model checking tool called SMBioNet (Symbolic Modelling of Biological Networks) which acts as the bridge between DyMBioNet and model checking.

Chapter 7: Static modelling of the energy metabolism network is portrayed in this chapter, showing the regulations between the main actors and the cofactors, nutrients and external inputs comprising input of NCD (Nitrogen-Carbon donors), FA (Fatty acids), oxygen and glucose. We also show the interaction graph with a particular attention to the use of multiplexes integrating useful metabolic information, and we equally give their biological relevances. Explanations on the different thresholds for each variable are also justified in this chapter.

Chapter 8: Time-dependent states which are abstracted as state-transitions are depicted in this chapter with emphasis on kinetic parameters for all variables of the energy metabolism regulatory network. Time is abstracted as discrete transitions. The value toward which each variable is attracted is cautiously presented. On the whole, we present 112 parameters for this coarse-grained energetic metabolic model, all obtained from a rich bibliography.

Chapter 9: Useful validations of the coarse-grained model are carried out. We illustrate how we produce the two main metabolic phenotypes: respiration and fermentation, and how we can regulate the model with control over external nutrients which are crucial for cell survival. We also give a hint on the use of fair CTL, which shows equity in terms of computational paths. Some remarkable results are exposed in

this chapter with simulated screenshots.

Chapter 10 is the conclusion: We have examined a coarse grained representation of the metabolic network focusing on classical processes, and it is evident that this is not a final representation of the energy metabolism regulatory network. We are aware about other external factors that can be integrated if we have to design a complete robust model. Factors like drugs, growth factors and exercise (consumption of ATP) can form an integral part of this model if we need to cover all the tantalising possibilities of this regulatory network. This remains areas of investigation and represents future directions of this research work that will need more attention and collaboration with diverse stakeholders in systems and synthetic biology. Some of these future works are mentioned in this concluding chapter.

CHAPTER 2

REGULATION OF THE CELL ENERGY METABOLISM

2.1 Introduction

Metabolism, which is central to microbial (and macrobial) life, is a dynamical oscillations between two exclusive phases: catabolism and anabolism (see Figure 2.1). Catabolism is the degradation of molecules (sugars, lipids and other macromolecules) to release energy to sustain cellular activities including growth, reproduction, proliferation and maintenance. Anabolism does the reverse : it synthesises building blocks (proteins and fatty acids) for growth. Energy (in the form of ATP) and building blocks (amino acids, nucleotides, etc) produced by catabolism are used by anabolism to form biomass. Depending on the state of the cell (quiescence or proliferation), these two metabolic activities are tightly regulated to avoid futile activities. We are interested in the regulation of central carbon metabolism and especially in understanding the metabolic shift between respiration and fermentation: a tradeoff between slow but efficient glucose oxidation for producing ATP (respiration) versus an inefficient (extracts less ATP for the same amount of nutrients) but fast process for producing ATP and building blocks (fermentation) to support high rate cell proliferation.

This chapter describes the essential components of the central carbon metabolism and the underlying regulations between catabolism and anabolism. In section 2, we detail the different catabolic processes which generate the necessary precursors to fuel the anabolic pathways. The focal point here is carbohydrate catabolism which integrates most of the components of the catabolic pathways for energy production. Section 3 focuses on the anabolism, i.e. production of building blocks for lipids, proteins and nucleic acids. Given the complexity of the metabolic network (genes, enzymes and chemical reactions), it is a challenge to understand the whole regulatory mechanisms at this level. As such, in section 4, we give only a panoramic view of the regulation of metabolism at a coarser grained level with a central focus on energy and biomass production. In between, we try to differentiate metabolism in normal and cancerous cells. We complete this chapter with some metabolic shuttles important to facilitate the smooth exchange of metabolites between the different metabolic processes. These notions will be helpful to justify the construction of our proposed model in chapter 7 (section 7.1) and 8 (section 8.1).

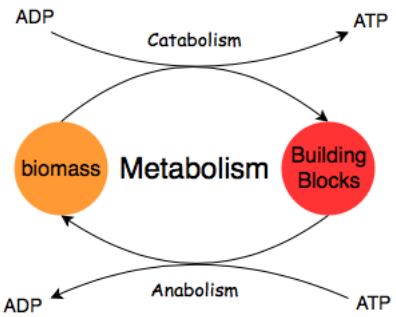


Figure 2.1: Anabolism and catabolism are mutually exclusive: catabolism degrades biomass to produce energy and anabolism does the reverse by consuming ATP.

2.2 Catabolism

Catabolism is a trade-off between the production of ATP and the production of building blocks. Dealing with this trade-off is a matter of whether the cell is in quiescence or proliferative modes. Cells have two possible pathways for ATP production and generating building blocks: respiration and fermentation. Respiration occurs only in the presence of oxygen. It is a slow machinery that generates the majority of ATP the cells require. The fermentative pathway has a double meaning: it can occur in the absence of oxygen but it can also occur in the presence of oxygen under other cellular conditions. Moreover, it is faster process with a smaller yield of ATP production than respiration. Cells have the necessary mechanisms to switch between respiration and fermentation. This metabolic plasticity enables cell to survive in stressful conditions. In this text, the main nutrients that we use take the form of glucose, fatty acids and nitrogen & carbon donors, and they are discussed in next section.

2.2.1 Nutrients

2.2.1.1 Dual view of nutrients

Nutrients captures a different meaning as we pass from the cell metabolism description to understanding of human physiology in which case, nutrition and diets are more suitable terms. They capture the first notion of fuel for the organism for which cellular central carbon metabolism is the core machinery. As regulation is concerned whether at the cellular level or at the physiological level in case of evolved organisms such as humans, nutrients refer also to molecular controllers (e.g. minerals, vitamins, neutraceutics, drugs) that regulate the whole human physiology. The understanding of the interconnection between the various hierarchical levels from cell metabolism to the control of homeostasis is the core of systemic pharmacology. In this context, nutrients play the role of molecular bio-indicators of various cell metabolic regimes as well as dysregulated homoeostasis that explain the appearance of disease-related phenotypes such as glucose level in diabetes and cancer. This is possible with PET-scan (PET) which allows the visualisation of glycolytic activity in vivo [19] and helps the diagnosis of cancer and tumors. As the understanding of these inter-hierarchical relations is beyond the scope of this study, we consider nutrients in this chapter as cellular food for energy and biomass production.

Two primary elements crucial for cells are carbon and nitrogen, which in our study, come at different levels, in the form of glucose, fatty acids and nitrogen/carbon donors. These nutrients contribute to most of the cellular carbon sources used for biogenesis [4]. Glucose catabolism generates ATP, NADPH and other biomasses for reductant biosynthesis and ROS detoxification. In association with the TCA cycle, glutamine (a major nitrogen & carbon donor) metabolism provides not only a carbon source but also NADH, NH3+ (nitrogen sources) and other essential intermediates for lipid biosynthesis, amino acid synthesis [4], and cellular acid detoxification. Fatty acids are precursors for lipid synthesis. Therefore, glucose, NCD, AA and FA seems to universally be the most critical nutrients for the growth and proliferation of both normal and cancer cells. These nutrients are explained in this section and their metabolic importance are highlighted.

2.2.1.2 Sugar, lipids and amino acids (glutamine) as carbon sources

At the cellular level, the primary element for biomass production is carbon for which sugars and lipids are the more abundant sources. Central carbon metabolism is perfectly adapted to produce energy from these nutrients. Proteins are also carbon sources, and secondary pathways exist to replenish primary oxidative pathways that oxidize glucose into CO_2 to produce energy (ATP).

Sugars and lipids represent a reservoir of electrons and protons through the associated hydrogen atoms. The electron content of carbon within sugar and lipids or any molecules is a quality index for these molecules to produce ATP from electro-chemical energy. This can be estimated as the average of the redox states of each carbon atom of the molecule that ranges from -4 for CH_4 to +4 for CO_2. Glucose and lipids have an average redox state of 0 and -2 respectively. Lipids are therefore more energetic per atom of carbon. This explains the energetical yield of these two molecules after complete oxidation of +34 ATP produced per molecule glucose and +143 ATP per molecule of palmitic acids.

2.2.1.3 Glutamine as a major nitrogen and carbon sources

The degradation of glutamine to provide nitrogen and carbon sources follows two possible pathways: an oxidative pathway representing the normal Krebs cycle (&Keto-Glutarate to malate) and a reductive pathway where glutamine is reduced to glutamate to do a reverse Krebs cycle to convert &Keto-Glutarate to citrate. The second most important atom after carbon is nitrogen which is, in particular, useful for the composition of nucleic acids and alpha amino acids. Glutamine is the most abundant amino acids in plasma (20%) and in muscle (40%), and represents the major source of nitrogen for purine and pyrimidines biosynthesis in organs [5]. In cultures, tumor cells metabolise glutamine faster than any other amino acids. However, only a small fraction of glutamine is used for nucleotides synthesis [6]. It plays a crucial role in lipid synthesis as well, which makes glutaminolysis the major component of the metabolism of proliferating cancer cells [7]. In this text, we use a more global name, NCD, to regroup all forms of nitrogen and carbon donors.

2.2.1.4 Other nutrients

Other atoms enter into the constitution of cells and may play a crucial role in redox, pH homeostasis as certain ions in osmosis regulation. These take the form of proteins, vitamins, minerals and water. The systemic understanding of the different homeostasis is certainly explained through physiochemical considerations through thermodynamics equilibrium and kinetics constants, and the regulation rules refer to a much finer granularity which is also beyond the scope of this study. These effects are therefore not explicitly taken into account in our model. All these essentials atoms or constituents are however not considered as a cell carburant as sugars and lipids does, unless in certain bacteria in which ammonium ions (reduced form of nitrogen) serve as reservoir of electrons.

2.2.2 Glycolysis

The degradation of the 6-carbon glucose in the cytosol inside cells is a quintessential step of glycolysis, which is a precondition for either fermentation or respiration. After its intake by enzymes (GLUT1 and GLUT2; also called glucose transporters; in neurons, GLUT3 does this) through cell membranes, glucose undergoes a series of oxidative-reductive steps to be finally transformed into the 3-carbon molecule, pyruvate (see Figure 2.2).

2.2.2.1 A global view

Pyruvate is at the bifurcation point of two main pathways of carbon metabolism: fermentation and respiration. Under the presence of oxygen, pyruvate enters the TCA (TriCarboxylic Acid) or Krebs cycle to complete its oxidative degradation via the respiratory pathway. In hypoxia (<2% vol of oxygen), pyruvate is reduced into lactate or ethanol depending on the organism. The fermentation pathway in our semantic is constituted of a single enzyme, Lactate Dehydrogenase (LDH) in humans for example or Alcohol Dehydrogenase (ADH) in Saccharomyces Cerevisiae, for example.
Cells obtain their initial dose of energy through this pathway which is equivalent to a net of 2 ATP (2 molecules are consumed initially and 4 ATP are produced at the end of the glycolytic pathway). This is accompanied by a parallel production of 2 NADH where the compound Glyceraldehyde 3-phosphate(G3P) is converted in a multitude of steps to produce pyruvate. The enzyme Glyceraldehyde 3-phosphate dehydrogenase (commonly abbreviated as GAPDH) is involved during this conversion of NAD+ to NADH.

GAPDH plays a pivotal role in glucose metabolism and is central for the homeostatic reservoir of NAD+/NADH. In conditions of oxidative stress, GAPDH plays a non-glycolytic role by a catalytic conversion of NADPH to NADP+ in the Pentose Phosphate Pathway (PPP; see Figure 2.3). GAPDH is overexpressed in multiple human cancers, such as cutaneous melanoma, and its expression is positively correlated with tumor progression [22, 23, 24]. Its glycolytic and anti-apoptotic functions contribute to proliferation and protection of tumor cells, promoting tumorigenesis. The degradative process of glucose is closely regulated to match the cell's demand for energy and has two potential destinations: either used as building blocks in PPP or for energy production in respiration and fermentation. This depends on the status of the cell (quiescence or proliferation). If excessive glucose enters the cell, the high level of ATP will allosterically inhibit the enzyme PhosphoFructoseKinase (PFK1), responsible for the conversion of fructose-6-phosphate to fructose-1,6-biphosphate. PFK1 represents the most important regulatory enzyme of glycolysis and it catalyses the first irreversible part of glycolysis. Its action ensures that both glycolysis and gluconeogenesis (producing glucose from non-carbohydrate sources) do not overlap. As soon as the energy charge in the cytosol decreases (the ratio of ATP/ADP is low), this simulates the allosteric activity of PFK1 which "restarts" glycolysis. Indeed, the PPP (see in 2.2.2.3) that synthesises

the precursors of biomass (nucleic acids, amino acids) has its root just above PFK1, using G6P as a starting point. Therefore, PFK1 regulates the cellular fate between production of precursor or ATP generation for glycolysis. As long as cells receive glucose nutrient, they will churn out ATP endlessly for sustainability reasons.

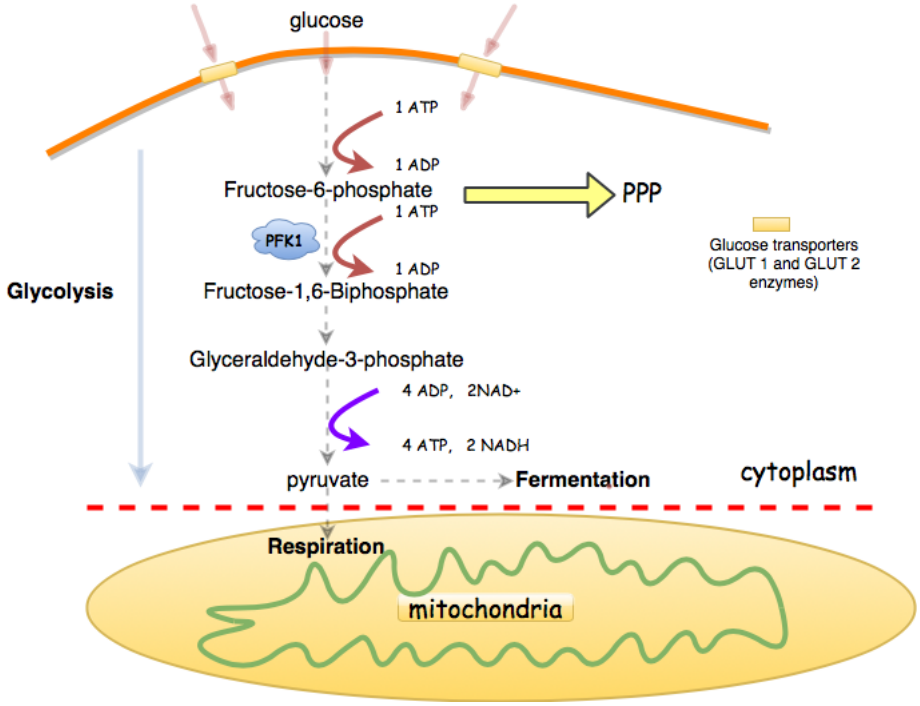


Figure 2.2: Glycolysis: A catabolic process producing ATP, NADH and pyruvate, and some precursors for Pentose Phosphate Pathway (PPP)

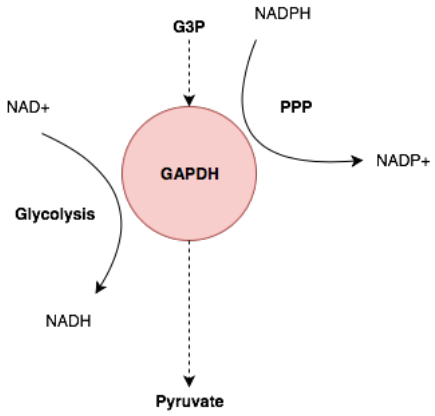


Figure 2.3: GADPH plays a double role in cytoplasmic metabolism: Glycolytic (conversion of NAD+ to NADH) and non-glycolytic (conversion of NADPH to NADP+)

In tumour cells, fermentation occurs even in presence of oxygen and under high supply of glucose as observed by O. Warburg in 1922 and later in 1929 by H. Crabtree for bacteria. This transition from respiration to fermentation is referred to as the Warburg/Crabtree effect. We will not differentiate in this study the long term irreversibility and the short term reversibility character of these two effects. The reversibility can be observed in facultative fermentative aerobes (e.g. Saccharomyces Cerevisiae) when changing from rich milieu to deficient glucose supply. Beside the production of ATP, glycolysis provides the intermediate metabolites for building blocks synthesis that will be used during anabolism for biomass production. The two main anabolic pathways directly connected to glycolysis is the PPP with glucose-6-phosphate (G6P) as entry point and the serine biosynthesis pathway which takes place at the level of 3-phosphoglycerate.

The use of glucose for building block synthesis is favored in tumour cells by an increased intake of glucose through over expression of glucose receptors (GLUT) and by increasing glycolytic flux, and divergence towards anabolic pathways by modifying the thermodynamics balance through metabolite accumulation.

2.2.2.2 ATP production

In the first part of glycolysis, cells invest energy dispense that prevent glucose efflux: a first phosphorylation, consuming 1 ATP is realized by hexokinase. It prevents glucose exit by diffusion into the membrane. A second phosphorylation consuming also 1 ATP is realized by Phosphofructokinase (PFK1,2). These two phosphorylation on alcohol groups are expensive and are made at the expense of 2 ATP. The fructose 1,6-biphosphate is then cleaved into two C3 molecules that are half phosphorylated. Here comes the nice trick of glycolysis to make ROI (Return of Investment): to be able to produce 2 ATP per C3 intermediates, the cell phosphorylates each intermediate with an additional inorganic phosphate. For this cheap reaction to happen in terms of electrochemical energy, alcohol is first oxidized into aldehyde (a toxic intermediate) with ionization state of aldehyde carbon = +1 which favors the oxidation by organic phosphate. The last step of glycolysis is the energy production stage: the two Glycerate 1,3 biphosphate are now the substrate for ATP production, 2 ATP per intermediate, that is, 4 ATP in total per molecule of glucose. The second phosphorylation with inorganic Phosphate (Pi) is less costly energetically than phosphorylation of alcohol group. The coupled reduction of NAD+ into NADH+ provides the necessary electrochemical energy for this phosphorylation. As this reaction consumes 2 protons, the energetic yield of glycolysis is expected to be favoured under acidic conditions.

2.2.2.3 A building block synthetic pathway: PPP

The Pentose Phosphate Pathway is a maintenance pathway that occurs in the cytoplasm and accounts for the formation of the reducing agent NADPH and precursors for the production of biomass. PPP is tightly connected to glycolysis (see Figure 2.4). Some glucose-6-phosphate molecules "leak" from the glycolytic pathway to be used as a first substrate in PPP. It undergoes two types of metabolism in the PPP: oxidative and non-oxidative branches. The oxidative branch includes a series of non-reversible conversions of glucose-6-phosphate to ribulose-5P essential for nucleic acid synthesis. At the same time, NADP+ is converted to NADPH, which has a crucial role in cancer cells as it relieves them from oxidative stress during ROS (using glutathione reductase to maintain redox state of cell). Thus, NADPH is a good scavenger but is also known for participating meagrely in fatty acids synthesis. NADPH homeostasis is critical for cancer cells in starved microenvironments. The non-oxidative segment produces glucose derivatives like fructose-6-phosphate and which are reused in glycolysis. Interestingly, the potentiality of PPP has been demonstrated in disease like sleeping sickness [8, 9].

It has become clear that the PPP plays a critical role in regulating cancer cell growth by supplying cells with not only ribose-5-phosphate but also NADPH for detoxification of intracellular reactive oxygen species, reductive biosynthesis and ribose biogenesis. Thus, alteration of the PPP contributes directly to cell proliferation, survival and senescence. Dysregulation of PPP flux dramatically impacts cancer growth and survival. PPP is both positively and negatively regulated by numerous factors as shown in Figure 2.5. Therefore, a better understanding of how the PPP is reprogrammed and the mechanism underlying the balance between glycolysis and PPP flux in cancer could be valuable in developing therapeutic strategies targeting this pathway.

The tumour suppressor, p53, is the most frequently mutated gene in human tumours and has been shown to inhibit PPP. Through the PPP, p53 suppresses glucose consumption, NADPH production and biosynthesis. Tumour-associated p53 mutants lack the G6PD-inhibitory activity. Therefore, enhanced PPP glucose flux due to p53 inactivation may increase glucose consumption and direct glucose towards biosynthesis in tumour cells [11, 12]. p53 deficiency reduces the expression of TIGAR, which has a role in suppressing glycolysis by lowering intracellular levels of fructose-2,6-bisphosphate (F-2,6-P2). F-2,6-P2 is a strong allosteric activator of phosphofructokinse-1 (PFK1), and the reduction of F-2,6-P2 results in decreased PFK1 activity and glycolytic flux [11]. p53 suppresses the PPP by directly binding to G6PD and repressing its enzyme activity. However, the ability to inhibit G6PD is restricted to wild type p53. To conclude, it can be hypothesized that in cancer cells, p53 mutations may liberate G6PD and activate PFK1, causing increased PPP flux and glycolysis.

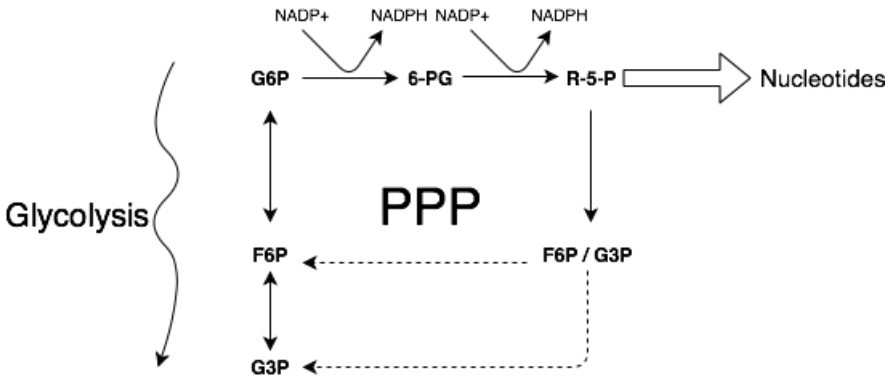


Figure 2.4: A brief summary of PPP: Oxidative decarboxylation occurs where G6P is converted in intermediate steps to Ribose-5-Phosphate (R5P). Depending on cell demands, R5P can either route to the oxidative branch to support biomass production through nucleotide synthesis. In starve conditions, R5P is converted into intermediates F6P and G3P to be reused in reverse glycolysis.

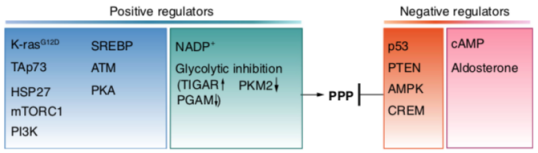


Figure 2.5: Positive regulation on the left and negative regulation on the right [10]

2.2.3 Fermentation

When oxygen supply is low in cells (e.g smooth muscular cells during a sprint), pyruvate, the end-product of glycolysis is transformed into lactate in mammalian cells or alcohol in certain microorganisms like yeast S.C. Like other molecular transformation which are catalysed by enzymes, this process is catalysed by Lactate Dehydrogenase (LDH) or enzyme pyruvate decarboxylase to produce lactate. Some organisms even earn their living through fermentation only (strict fermentative microorganisms). Others can do both such as yeast which have commercial applications such as bread making and wine production. A good example is the manufacture of bioethanol which is the world's leader biofuel and it is produced by fermentation from glucose feedstocks.

This reduction process of pyruvate to lactate or ethanol needs a donor of electrons and protons, NADH+, which is converted to the oxidised form NAD+. Fermentation is a way to refuel the oxidised cofactor NAD+ that is necessary to run glycolysis. We called this the glycolytic-fermentation loop and is shown in Figure 2.6. This loop is hyper active in all cancer cell lines.

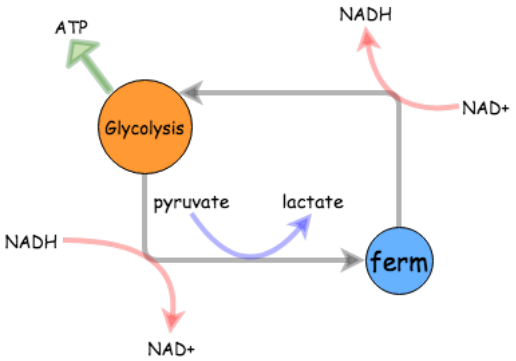


Figure 2.6: Metabolic loop between glycolysis and fermentation in mammalian cells

2.2.4 Respiration

Respiration is an efficient energy production machinery which works in the mitochondria. Its main input substrate is pyruvate, the end product of glycolysis which is entirely decomposed to produce energy

(ATP). The side product is CO_2 and H_2O, an important catalytic solvent for the cell. The respiration is composed of two steps: Krebs cycle and oxidative phosphorylation.

The metabolic fate of pyruvate depends on the type of organisms and on certain cellular conditions. In the presence of oxygen, the second metabolic destination allows pyruvate to flow into the mitochondria, a process called *aerobic respiration*. This process consists of the Krebs cycle and oxidative phosphorylation, which are two processes intertwined to allow the flow of electrons and cofactors necessary for their metabolic functioning. Pyruvate, which is a 3-carbon molecule, is first converted to acetyl-CoA to enter the Krebs cycle, where it undergoes a series of oxidative steps. The ultimate function of the Krebs cycle is to provide the necessary cofactors, NADH and $FADH_2$, which are effective electron carriers that traverses the mitochondrial membrane to stimulate the oxidative phosphorylation process. At the same time, carbon elements extracted from pyruvate combine with oxygen molecules to be excreted as carbon dioxide (CO_2) from the Krebs cycle. Pyruvate is therefore an important intermediate central for the carbon cycle (from glucose to carbon dioxide).

However, in cancer cells, there is a metabolic impairment of the respiration phase which is followed by excessive production of reactive oxygen species (ROS), considered harmful for cells. Under this stressful conditions, cancer cells undertake the fermentation phase even in the presence of oxygen, a process called *Warburg effect*.

2.2.4.1 Oxidative Krebs

Krebs cycle, also known as the TCA (TriCarboxylic Acid cycle), is the alternate catabolic route to metabolise pyruvate. It occurs in the mitochondrial matrix when oxygen regime is adequate in the cells. Pyruvate substrates (which is the master carbon fuel input) are converted to acetyl CoA prior to entry in the mitochondria. Seemingly, proteins and fatty acids use the Krebs cycle as their final metabolic pathway by transforming into Acetyl-CoA. This protein is composed of three enzymes: a decarboxylase, an acylTranferase and an oxydoreductase associated. Each enzyme uses cofactors: Thiamine pyrophosphate (TPP) for the first, lipoamide/dihydrolipoamide Coenzyme A (CoASH) for the second and Flavine Adenine Dinucleotide (FAD) and Nicotinamide adenine dinucleotide for oxydoreductase. The absence of these cofactors plays the role of inhibiting regulation signals. The nine enzymatic steps of Krebs cycle are shown in Figure 2.7.

The main function of this catabolic route is to replenish the molecular pool (NADH+ and FADH2) of electrons and protons that are needed for the respiratory chain. FAD+ is the cofactor of succinate Deshydrogenase which is part of the complex II of respiration and embedded in the mitochondrial membrane. This connects the oxidative mode of Krebs to oxidative phosphorylation. The electrons and protons of FADH2 are immediately transferred to ubiquinone (a cofactor of complex II) which is reduced into ubiquinol and FADH2 is reoxidized into FAD+. To summarise, the Krebs cycle is turned on by high ratios of either ADP/ATP or NAD+/NADH which indicate that the cell has run low of NADH or ATP. Many of the intermediates of the Krebs cycle are used as precursors for synthesising biomolecules. Citrate, for example, can be exported out of the mitochondria into the cytostol where it is partly converted to acetyl-CoA. The Acetyl-CoA produced is a precursor for fatty acids. Many other types of amino acids are also produced.

The oxidative Krebs is classic for normal cells but in cancer cells, and other highly proliferative cells, a reductive form of Krebs is possible. Reciprocal arrangements, called anaplerotic reactions, are put in place by cells, to replenish the intermediates removed from the citric acid cycle for biosynthesis.

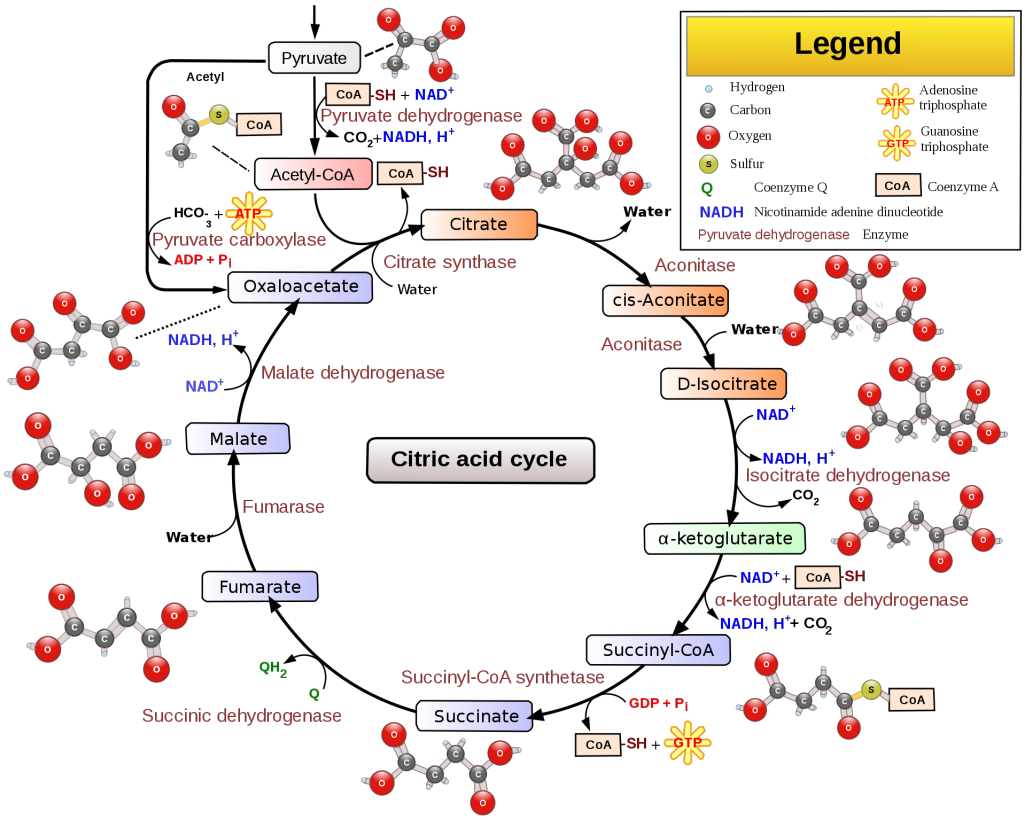


Figure 2.7: Summary of the different steps in oxidative krebs. Source: [27]

2.2.4.2 Reductive Krebs

In conditions of nutrients deprivations (absence of oxygen, glucose and other nutrients), cells can survive. To maintain constant energy and biomass production, critical for cell survival, intermediates enter the Krebs cycle to keep it running. For example, in hypoxic conditions, glutamine metabolism uses the Krebs cycle in a reverse way. Glutamine, transported from cytosol, is first hydrolysed in the matrix to glutamate, a process called *glutaminolysis*. Glutamate plays a very important role in the movement of nitrogen through cells for biosynthesis. It is further converted to αKG and the whole conversion is reversible.

In highly proliferative milieu, for example in cancer cells, the lipids demand is high. This stimulates the synthesis of citrate from αKG which is excreted from mitochondria to be converted into acetylCoA to fuel lipogenesis in the cytosol. It is important to note that IDH1 and IDH2, which are key metabolic enzymes to generate αKG from citrate while reducing NADP to NADPH [31], are mutated in tumoral environment. Therefore, this explains how cancer cells adapt themselves and reconfigure their metabolic activity to survive stress. The double implication of αKG shows possible therapeutic potential and cannot be downlooked.

2.2.4.3 Oxidative phosphorylation

Oxidative phosphorylation occurs in the mitochondria where it takes NADH+ from the Krebs cycle to separate charges (protons and electrons) along the respiratory chain. This respiratory chain is composed of four membrane protein complexes which oxidize NADH to provide the necessary protons for the reduction of oxygen into water. The four complexes are linked by low molecular weight mobile carriers which ferry the reducing equivalents from one complex to the next. Except for succinate dehydrogenase (complex II), all these complexes pump protons from the matrix into the interstice mitochondrial membrane as they transfer electrons or protons (reducing equivalent) to the next complex. This proton gradient is created thanks to the pools of reduced cofactors NADH+ that is produced during Krebs cycle.

In this part of the mitochondria, a cascade of redox reactions make best use of these rich energy electrons. At each step of the reaction cascade happening between complex I to IV (see Figure 2.8), a small but sufficient portion of electron redox energy is used to transfer one proton in the membrane

interstice against its proton gradient.

As the consequence of a separation of charge between protons and electrons, an electro-chemical energy conversion is possible. In the last complex V, the enzyme ATP synthase uses this proton gradient to produce energy in the form of ATP from ADP. At the end of the electron transfer from complex I to complex IV, a strong electron acceptor (usually O_2 but not necessary) is able capture the low energy electron to create water with the protons that were transferred through ATP synthase according to the reaction: $O_2 + H+ + e- \leftrightarrow H_2O$. The process is summarized in Figure 2.8.

2.2.5 Energy yields

The energy yield of a central carbon metabolism is the ratio of ATP produced per molecule of substrate consumed. This yield depends on the environment as cells shift their metabolism according to the abundance or deficiency of nutrients, especially glucose.

How this yield control cell growth rate during proliferation is a key aspect in optimizing the production of biomass or high added value metabolites. In our central carbon metabolism description, we consider two substrates: glucose and lipids. A total of 4 ATP molecules are produced during the glycolytic conversion of glucose to pyruvate among which 2 ATP are used for converting fructose and its intermediates. This is a fast process after which pyruvate has then two destiny: fermentation or respiration.

In the respiration mode, high energy electrons are transferred from both $NADH$ and $FADH_2$ to other suite of electron carriers present in the mitochondrial membrane. This membrane is equipped with three protein complexes (I, II and III) with specific roles. During the oxidative phosphorylation phase, hydrogen atoms are extracted from NADH (by complex I; $NADH \rightarrow NAD + H^+ + e^-$) and $FADH_2$ (by complex II) to form a proton gradient (called proton motive force) in the internal membrane of the mitochondria.

These two distinct and successive flow of protons and electrons are coupled, and as a result, the energy released during electron transport is stored in the form of an electrochemical gradient of protons across the membrane. When the concentration of H+ ions accumulates, they flow from a high potential redox to a low potential redox inside the mitochondrial matrix. As soon as the reservoir inside the matrix is full, the opposite shuttling of H+ ions is favoured. This maintenance of H+ ions level inside the mitochondria is called *chemiosmosis*, which powers ATP synthesis by the enzyme ATP-synthase.

From the standpoint of ATP synthesis, a significant amount of 36 molecules of ATP (+ 2 ATP from glycolysis) are manufactured by this electron transport chains (respiration) with the recycling of NAD+ for continual running of glycolysis (see Figure 2.9). Excessive H+ ions are finally transferred to their terminal acceptor, oxygen, which is a strong oxidising agent, to form water molecules ($2H^+ + O + e^- \rightarrow H_20$).

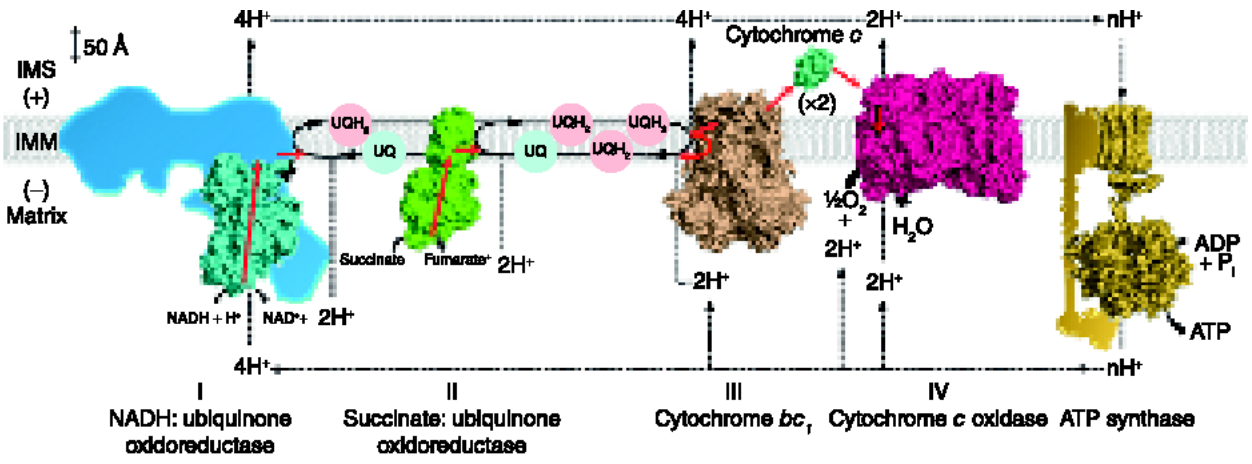


Figure 2.8: Proteins complexes (I to IV) transferring electrons in Oxidative Phosphorylation. [28]

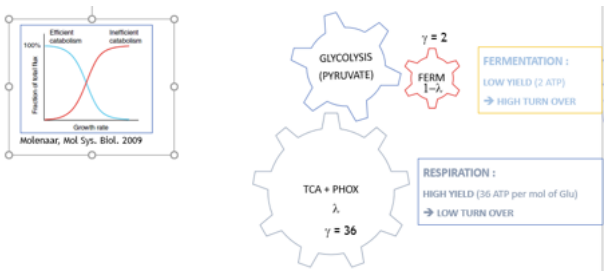


Figure 2.9: Efficient (respiration) versus inefficient catabolism (fermentation) [29]

2.2.6 Alternative Catabolic Pathways

Other catabolic pathways that are part of central carbon metabolism are lipid degradation or β-oxidation. Fatty acid oxidation is the mitochondrial aerobic process of breaking down fatty acids into Acetyl-CoA units. Fatty acids move in this pathway as CoA derivatives utilizing NAD and FAD [30]. The energy yield from fatty acid oxidation is larger than glucose.

2.3 Anabolism: from energy to biomass

2.3.1 Introduction

The repartition of energy and precursor synthesis during catabolism depends on the demand of the cell: quiescence or proliferative mode. Biomass production depends therefore on the growth rate of the cell which itself is highly dependent on the external milieu, whether rich or poor in nutrients. This establishes the link between metabolism and proliferation. In this text, we ignore the interlink between cell cycle and metabolic clock. We will therefore assimilate biomass production and cell proliferation in this thesis.

Building blocks for cell repair, reproduction and proliferation take the form of proteins and fatty acids which are manufactured from simpler compounds. In the cytosol, the only pathway building materials for nucleotides (DNA) is the PPP. In the mitochondria, the Krebs cycle assumes this biosynthetic role by providing a partial group of amino acids but mostly supplies Acetyl-CoA for the synthesis of fatty acids (see Figure 2.10). These two biosynthetic reactions require a full reservoir of electrons and protons which are partly stored in NADPH+ cofactors and precursors from the Krebs cycle. Depending on the status of the cell, there is a constant equilibrium between biomass and energy production to match the cell's demand. The production of biomass takes the form of protein and lipids synthesis. These two anabolic pathways are discussed in this section.

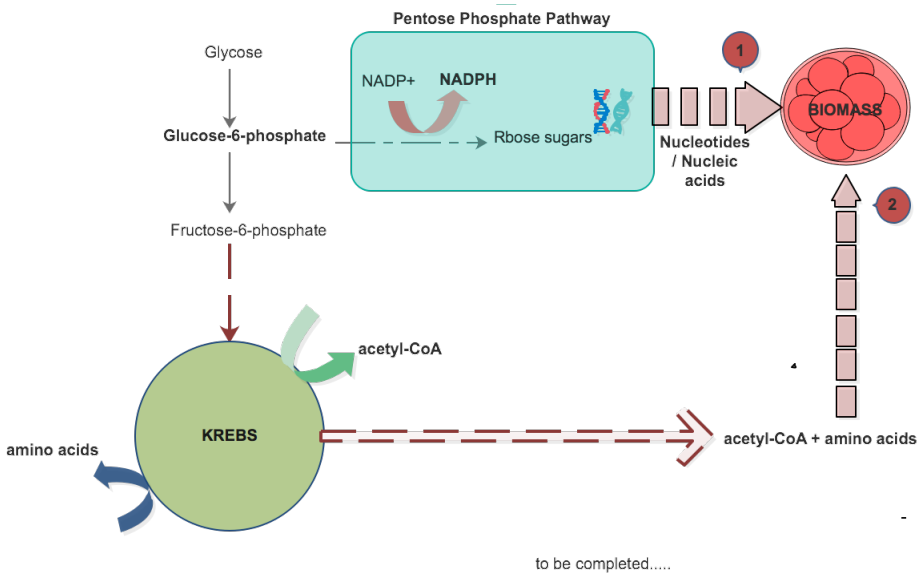


Figure 2.10: Overview of anabolic activities. Biomass synthesis from 1) Nucleotides for DNA and 2) Acetyl-CoA for fatty acids

2.3.2 Protein synthesis

Proteins are the workhorses of the cell, controlling virtually every reaction within as well as providing structure and serving as signals to other cells. Protein metabolism is the most energy intensive activity occurring inside cells because of the complex translation machinery [32]. Translation guides the expression of genes and its dysregulation has been observed in many forms of cancers. Many protein precursors are renewed in the Krebs cycle and generate the necessary amino acids for protein synthesis. A host of essential amino acids are synthesised in the Krebs cycle (includes α-ketoglurate to glutamate by reductive amination, pyruvate to valine and leucine, and aspartate to methionine [33]) and part in PPP (ribose-5-phosphate to Histidine for example). Other essential amino acids must be obtained from diet.

Due to a restrictive diet in the cell's milieu, cancer cells adapt themselves and adjust their protein synthesis pathways using various genes to survive. mTOR is one major stimulator of protein synthesis and other anabolic activities. While mTOR regulates protein synthesis, it is a major negative regulator of autophagy. Nutrients deprivation which causes mTOR inhibition, also induces autophagy. This action supply free amino acids needed for the synthesis of crucial proteins. To maintain good energy balance, autophagy generates amino acids for protein synthesis, which consequently consumes them [32]. But, in highly proliferative mode, lipid synthesis is more expressed than protein synthesis.

2.3.3 Lipid synthesis

The TCA cycle serves as a convergence point in the cellular respiration machinery, which integrates multiple fuel sources derived from the diet including glucose, glutamine, and fatty acids [34].
The third type of fuel source in cancer cells is fatty acids, which enter the TCA cycle after undergoing β-oxidation to generate Acetyl-CoA intermediates. Acetyl-CoA is the substrate for both the fatty acid synthesis pathway and the TCA cycle, making lipogenesis an important convergence point for TCA cycle flux and cellular biosynthesis [36]. This process generates more Acetyl-CoA per molecule than does either glucose or glutamine [35]. De novo synthesis of fatty acids is critical to supply lipids for cell membrane formation in rapidly proliferating cells, and is regulated by fatty acid biosynthetic enzymes: adenosine triphosphate citrate lyase (ACLY), Acetyl-CoA carboxylase (ACC), and fatty acid synthase (FAS). ACLY converts citrate to oxaloacetate and cytosolic Acetyl-CoA.

While enzymes regulating lipid synthesis are often expressed in low levels in most normal tissue, they are overexpressed in multiple types of cancers. ACLY is overexpressed in non-small cell lung cancer, breast cancer, and cervical cancer among others [36, 37]. ACC is upregulated in non- small cell lung cancer and hepatocellular carcinoma ([38]). FAS is overexpressed in prostate and breast cancers ([39, 40]). In tumor cells where the demand is much greater, lipogenesis occurs via these overexpressed enzymes. The increased activation and overexpression of these enzymes in tumors correlates with disease progression, poor prognosis, and is being investigated as a potential biomarker of metastasis [37].

Therapeutically targeting the TCA cycle function in cancer is an attractive strategy to treat cancer. Many tumors utilize glutamine as a fuel source for the TCA cycle, thus suppression of glutaminolysis through small molecule inhibitors is an attractive approach to therapeutically target these tumors ([41, 42]). Additional studies have demonstrated that glutamine limitation, through either depletion of glutamine in the plasma (L-aspariginase) or blocking glutamine transport (sulfasalazine), can provide therapeutic benefit ([43, 44, 45].
Therefore, the TCA cycle is a critical metabolic pathway that allows mammalian cells to utilize glucose, amino acids, and fatty acids. The entry of these fuels into the cycle is carefully regulated to efficiently fulfill the cell's bioenergetic, biosynthetic, and redox balance requirements. This forms part of one of the objective of this thesis to implement the dynamics of this regulation. Multiple types of cancer are marked by drastic changes to TCA cycle enzymes, which result in characteristic metabolic and epigenetic changes that are correlated with disease transformation and progression. As a result, several components of the TCA cycle may be exploited therapeutically for the treatment of diseases. However, due to the importance of the TCA cycle in normal cell development, high toxicity is a concern of this approach.

It is important to highlight that TCA cycle anions that are removed from the cycle, must be replaced to permit its continued function. This process is termed *anaplerosis*. Pyruvate carboxylase, which generates oxalacetate directly in the mitochondria, is the major anaplerotic enzyme. The principal source for anaplerotic is the metabolism of amino acids and in particular that of glutamine during glutaminolysis. Conversely, 4- and 5-carbon intermediates enter the TCA cycle during the catabolism of amino acids. Because the TCA cycle cannot fully oxidize 4- and 5-carbon compounds, these intermediates must be

removed from the cycle by a process termed *cataplerosis*. Cataplerosis may be linked to biosynthetic processes such as gluconeogenesis in the liver and kidney cortex, fatty acid synthesis in the liver, and glyceroneogenesis in adipose tissue.

In the light of this text, we focus only in the regulation of the main carbohydrate metabolism pathway and as such in the next section, we explain how cells maintain such regulations.

2.4 Regulation of metabolism

Cells are tightly regulated by enzymes to maintain homeostasis, energy balance and nutrients uptake. These happen at the molecular level and the same type of regulation can be observed at the granular level. An increase in glucose uptake will increase the velocity of glycolysis which will have a cascade increase in ATP:ADP as well as NADH:NAD concentration ratios. Cells are highly sensitive to these concentration levels and react accordingly to prevent damage.

If the energy demand is low, high ATP levels reduces the affinity of PFK1 and this has the effect of blocking glycolysis. When the AMP level increases, that is energy is depleted inside cells, this activates PFK1 which continues its usual enzymatic role. Insufficient pyruvate in the cytosol will not allow Krebs to do its cycle. Indirectly, these enzyme-mediated metabolic processes regulate each other at the coarse-grained level. Like glycolysis, the citric acid cycle is regulated at several steps to match its rate to the cell's requirements for ATP [46, 47]. Too much ATP also decreases the activity of citrate synthase for which Krebs also halts its oxidative activity by freezing the conversion of incoming Acetyl-CoA to citrate. Oxidative phosphorylation also is not spared from these regulatory mechanics since it depends heavily on electrons supplied by Krebs. This homeostatic rigidness of cells are due to oscillatory mechanisms that occurs to maintain equilibrium in the concentration of all metabolites. We discuss these oscillations in the next section.

2.4.1 Metabolic oscillations

Cellular metabolism is an open system which continuously allows the exchange of materials with its environment. The up-regulation and down-regulation of the quantity of biological components are responsible for oscillatory mechanisms manifested in many biological systems. These oscillations maintain the dynamic stability of cells as they shift between catabolism and anabolism. We take the example of glycolytic-fermentative loop in Figure 2.6 where we have NADH produced (NAD+ consumed) by glycolysis and consumed (NAD+ produced) by fermentation. Similar events occur for cofactors like O_2 as well as ATP (v/s ADP) which represent biomarkers for metabolic oscillations. An example is shown in Figure 2.11. These oscillations can effectively monitor and cause perturbations in the cell cycle. At the same time, this represents homeostatic response of the cells to maintain the correct balance of cofactors for proper functioning of the cells. Gaining empirical knowledge on the role of these oscillations can open avenues for investigation on highly proliferative cells. These oscillations of metabolites are permissible as they are capable of transiting between different cell compartments via shuttles. We end this section with a reminder that these are types of phenotypic observations to be captured during model simulations and the shuttles that allow these are discussed in the next section.

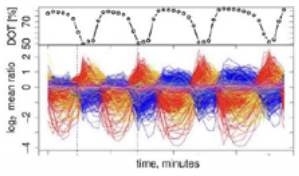


Figure 2.11: Alternating expression of anabolic and catabolic genes. The top panel shows the time courses of the dissolved O2 trace (DOT) in the culture medium in percent of the saturated concentration. (Figure taken from [13]). Catabolic and anabolic activities are mutually exclusive as we have shown in Figure 2.1; when one is low, the other activity is high.

2.4.2 Metabolic shuttles

The translocation of electrons, protons and other biochemical species are useful for regulating metabolic processes. Transporters and shuttles, mostly in the form of enzymes, are put in place across membranes

to facilitate these movements between specific compartments. This occurs because membranes of some organelles as well as cells display impermeability properties. We give two important examples of shuttling systems as follows:

i. Malate-aspartate shuttle
The electrons stored in NADH from glycolysis use the malate-aspartate shuttle to reach the long-chain of electron carriers inside the mitochondria for oxidative phosphorylation (Figure 2.12). This is because NADH cannot cross the mitochondrial membrane. The oxidised form NAD+ can flow in the reverse direction from mitochondria to the cytosol using the same shuttle. This is a systematic way of modulating the level of NADH:NAD+ inside cells by oxidative phosphorylation. The malate-aspartate shuttle is reversible. In period of fasting, gluconeogenesis occurs where some oxaloacetate molecules diffuse into the cytosol where it undergoes a conversion to oxaloacetate and then to malate. This generates NAD+ to replenish the NADH pool.

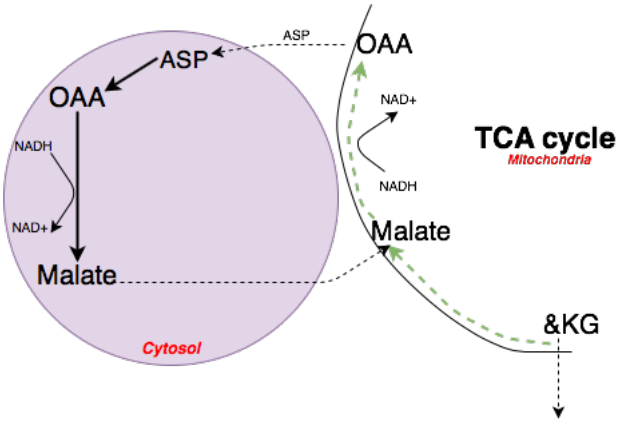


Figure 2.12: Malate-Aspartate shuttle: Malate is converted into oxaloacetate in the oxidative phase of TCA cycle. Some oxaloacetate molecules escape through the mitochondrial membrane into the cytosol in the form aspartate.

ii. Citrate-pyruvate shuttle
A similar shuttle is available in reductive Krebs, citrate are exported from the mitochondria across the citrate-pyruvate shuttle into the cytosol (Figure 2.13). It is then converted into Acetyl-CoA before undertaking the lipid synthesis pathway. Excess flow of citrate can also inhibit PFK1 which in turn slow down the rate of glycolysis. Therefore, Krebs acts indirectly as an inhibitor on glycolysis. PFK1 reverses the citrate synthase reaction and produces also oxaloacetate as a by-product. These oxaloacetate molecules can be converted to pyruvate which eventually can return to the mitochondria.

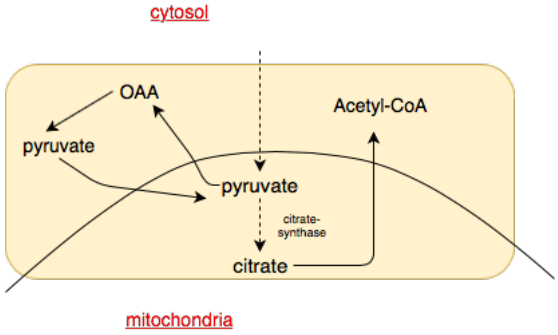


Figure 2.13: Citrate-pyruvate shuttle: Pyruvate can be converted to Acetyl-CoA which moves out of the mitochondria and enters the cytoplasm as citrate

2.5 Conclusion of the chapter

In this chapter, we find it intuitive to give some granular explanations of the general metabolic network without going into the detail of enzyme and gene regulations. Metabolic regulations form an integral part

of cells and allow them to support the environmental pressure. We covered the individual units of the energy metabolic pathway and illustrated how each unit are interlinked and regulates each other. Important points of each metabolic process have been described and we have shown the importance of certain metabolites / cofactors in driving these regulations. Perturbations at the molecular level (metabolites, enzymes) generate global effects on the stability of the whole metabolic network.

We gave a panoramic view of the different catabolic and anabolic activities which govern the metabolism of different nutrients important to sustain growth and proliferation of cells. We can say that cells have the necessary built-in mechanisms to deal with low-level and high level concentrations of metabolites. There exists a kind of synergy between all the metabolic processes which results in high degree of self-organisation of living cells.

Finally, we summarise all these interactions in a regulatory graph to have a global picture of the energy metabolism in Figure 2.14. In the next chapter, we will have profound look at the diverse modelling techniques available in the context of biological networks. Along the way, we will show their specificities and how the context to which they are applied varies and how it depends highly on the biological question.

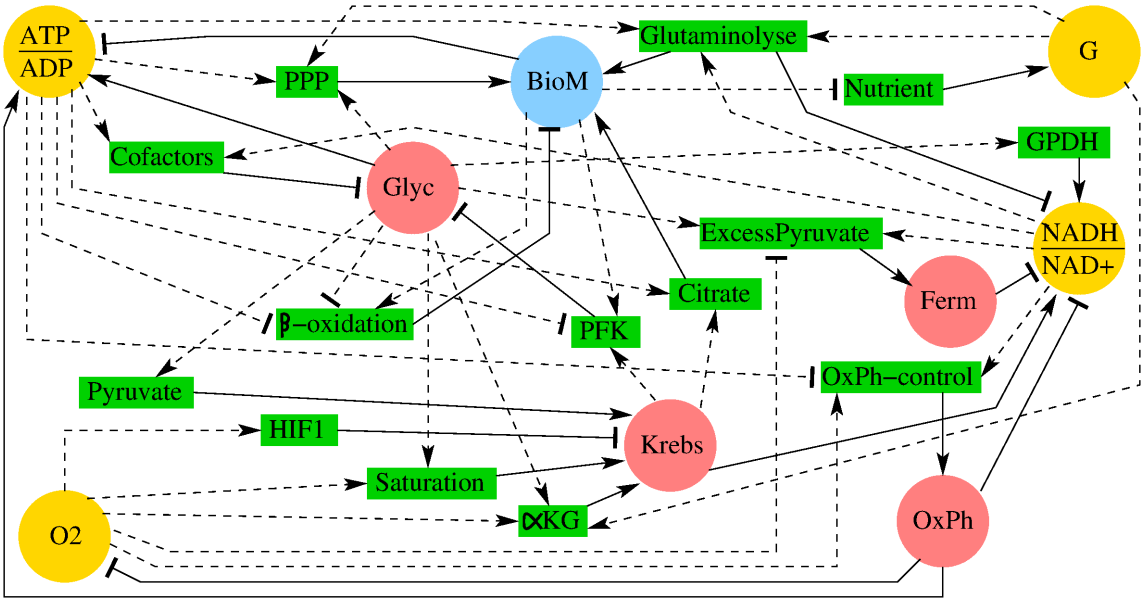


Figure 2.14: Our proposed regulation graph. It summarises, at a coarse grained level, the diverse regulations mentioned in this chapter.

CHAPTER 3

FRAMEWORKS FOR THE DYNAMICS OF BIOLOGICAL NETWORKS

3.1 Introduction

An array of formalisms is available to model the dynamics of biological phenomena and they can be broadly categorised either as quantitative or qualitative. Quantitative methods (section 3.2) rely more on real values based on precise experimental data and make use of either linear algebra (e.g FBA) or differential equations (mostly ODE). On the other hand, qualitative methods rely more on abstract values based on biological knowledge of the underlying biological network (but can also use experimental data). Qualitative methods focus more on a combination of logical approaches (section 3.5 and following of this chapter) and discrete mathematics. The choice of formalism depends on the biological questions to be addressed, and on the nature of the mechanisms and the level of details (molecular, genetic, reactions or processes) we intend to capture in the mathematical model.

Formalisms dedicated to metabolism are mostly of quantitative nature. They are based on reactions catalysed by enzymes of the general form: substrate $\xrightarrow{enzyme}$ product, (with possible reverse reaction).

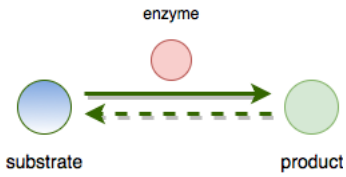


Figure 3.1: Enzymatic reaction : A dotted arrow meaning a reversible reaction.

Reactions can be reversible (bidirectional arrows) or irreversible (single arrow). A metabolite in a given reaction can be a substrate (on the left of the arrow) or a product (on the right of the arrow), as shown in Figure 3.1. Quantitative frameworks often have constraints such as bounded flux speed, conservation of mass or energy and concurrency between reactions to consume substrates. Most of these frameworks have been conceived to incorporate large numbers of basic metabolic reactions and consequently large numbers of possible pathways. For differential equations, the pathways are implicit and we analyse only the evolutions of the respective concentrations of substrates / products over time. Currently, both quantitative and qualitative modelling are hindered by lack of well established values *in vivo* with regards to kinetic parameters which are difficult to identify experimentally.

For formalisms targeted at metabolic modelling, the identification of kinetic parameters follows some hypotheses which are generally based on optimisation of certain productions by the cells : for example, the production of biomass. In the context of our study, these hypotheses are sometimes questionable because the cells do not optimise systematically one criteria or a given set of criteria, and certain decisions are not quantitative in nature.

Differential equations mostly use curve fitting (often based on least-square method), in which we try to minimise the distance between some experimentally established biological curves and the differential equation curves (obtained under constraints that are supposed to mimic experimental contexts). Such mathematical models of regulatory frameworks do not focus on possible hypothesis on cells optimisation. Models can be validated using temporal knowledge or error-free experimental data.

In all cases, the main true problem lies with the identification of parameters. These parameter values are generally unattainable in experiments : only their impacts on the system trajectories are observable, leading to *inverse solving problems*, similar to *reverse engineering*. More often, even if we get the kinetic parameters from *in vitro* knowledge, they often show noticeable differences between simulated and *in vivo* experimental data. The conditions in which *in vitro* experiments are carried out are in fact different from the conditions inside the cell [52].

In the light of choosing a well-suited framework with respect to our coarse-grained vision of metabolism, some of the criteria we would be looking forward to for our choice of formalism are as follows :

- **the level of abstraction**
  A formal mechanism with a certain degree of proof mechanism and the capacity to group possible metabolic processes. For example, the set of chemical reactions occurring in the Krebs cycle are grouped in the Krebs variable.
- **selection of important objects**
  A framework that offers a certain degree of clarity in the selection of important variables of the model. This can allow abstraction and thereby reducing the number of variables.
- **possibility to implicitly represent the notion of resources**
  A coarse-grained framework which will allow significant reduction of concurrent variables. For example, if Krebs is consuming a certain cofactors, this should not reduce the activity of glycolysis. In other words, concurrency will have to be explicit and easily tunable in our model.
- **logical reasoning**
  The ability to allow systematic reasoning of causalities using computer-aided tools.

This chapter will give a panoramic view of the two main categories of modelling frameworks dedicated to the study of the dynamics of biological networks. In the first part, we discuss classical frameworks which are more of quantitative nature focusing on metabolic reactions and using linear algebra. The second part deals with the application of differential equations as a quantitative method to the modelling of regulatory networks in general. Finally, the last part is dedicated to formal frameworks which are more oriented towards qualitative analysis of biological networks using discrete maths and formal logic. The formalism of René Thomas for biological regulatory networks, which we will finally choose, is detailed in the next chapter.

3.2 Classical frameworks for metabolism

Quantitative modelling has gained momentum in the past decades with the explosion of omics data. Following this, there has been tremendous attention to the study of structure, function, and evolution of metabolic networks. Experimental analysis including measurements of enzyme concentrations, enzyme activities, reaction rate constants and metabolic fluxes have become standards. Many formalisms have been proposed in the literature but we give a special attention to the two most representative quantitative methods based on algebra, namely: Flux Balance Analysis (FBA) and Elementary Flux Modes (EFM). These methods work at the molecular level (that is the basic elements of a model are molecule names). In the context of these formalisms, a metabolic network will therefore refer to a series of intricate and interconnected biochemical reactions. They are developed from the same common core principles discussed in detail in section 3.2.1.

3.2.1 General concepts

Enzymes, substrates and metabolites
Plants and animals are able to sustain life via a few hundreds of chemical reactions occurring in cells. They are usually classified in three fundamental network modelling types: metabolism, regulation of genes and transmission of signals. Metabolic networks are large systems of chemical reactions allowing, among others, generation of energy from food sources and the synthesis of small molecules. These processes are catalyzed by small proteins called enzymes which are themselves product of genetic networks. Enzymes are supposed to remain unchanged in the metabolic network formalisms under consideration. This is because the speed of metabolic reactions are, by far, quicker than genetic changes of configuration. Enzymatic reactions take the general form shown in Figure 3.2.

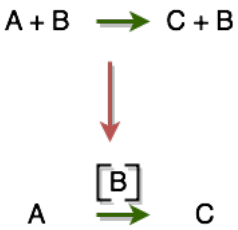


Figure 3.2: Enzymatic reaction: B remains unchanged after the conversion of A to C.

Several useful techniques exist to analyse combined biochemical reactions via substrate and product metabolites. Network theory, stoichiometric analysis, information on protein structure/function and metabolite properties are some examples. Here, we introduce the general concepts which are used as a blueprint for studying the two most popular quantitative methods: FBA and EFM.

Metabolic reactions

A metabolic network is incredibly complex and is governed by a lot of molecules interacting among themselves through chemical reactions. In the analysis of these metabolic reactions, two important notions that are common to quantitative methods are *stoichiometry* and *flux speeds*. The quantitative relationship among reactants and products is called stoichiometry. The stoichiometric coefficient is the number that appears in front of each reactant showing the chemical relationships. Normally, it is an integer but it can also take the form of fractions. It is also important to pinpoint that in any chemical reaction, the law of conservation of mass applies. Once we have the set of reactions and stoichiometric coefficients (the first step), the next interesting step is to determine the flux speed of each reaction; that is, the speed at which they occur. Here, the stoichiometric coefficients are handy for use as constraints to control the flux of reactions and to achieve this we will use a matrix representation described later in this section.

We focus on the example in Figure 3.3 to illustrate the use of stoichiometry and flux speed as a prerequisite in Flux Balance Analysis, Elementary Flux Modes and sometimes in differential equations. Stoichiometric coefficients can be easily deduced from chemical experiments. In reaction $r1$, the stoichiometric coefficient of both u and b are 1, whereas in reaction $r4$, the stoichiometric coefficients of b, c and e are respectively 2, 1 and 1. Furthermore, the flux speed for these reactions will be the speed at which u is converted to b in reaction $r1$ and for $r4$, it would be the rate at which b and c are converted to e. The set of four biochemical reactions $r1$ to $r4$ are captured in Figure 3.3, where we consider u and v as inputs of the system and, d and e as outputs.

r1 : $u \rightarrow b$

r2 : $v \rightarrow 2c$

r3 : $b \rightarrow d$

r4 : $2b + c \rightarrow e$

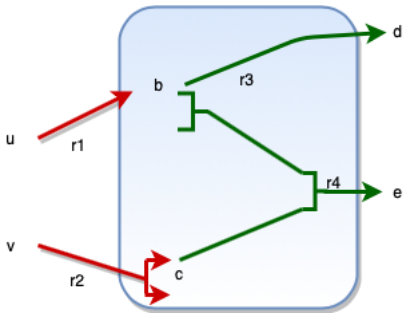


Figure 3.3: Toy example of a metabolic network : u and v are substrates; d and e are products; b and c are internal metabolites

Note here that a metabolite can act as a product (b is produced from u) and as a substrate (b is a substrate for d and e).

Fluxes ($v1$ to $v4$ corresponding to reactions $r1$ to $r4$ respectively) are important measurements that can be rigorously controlled to optimise certain productions (for example optimisation of either d or e or both $d + e$) and that is mostly the goal of FBA and EFM.

Stoichiometric matrix and steady state
As already mentioned, kinetic information are difficult to identify as they are not directly measurable in biological experiments. Contrarily, the stoichiometric structure and thermodynamic constraints are readily available due to knowledge of the underlying networks through chemical experiments. Similarly, it may be feasible to measure the rate of external fluxes but internal fluxes are difficult to measure. In the toy example of Figure 3.3, fluxes for reactions $r1$ and $r2$, and fluxes for d and e are external fluxes. It is also possible to have internal fluxes, for example one may imagine a reaction of the form $b \rightarrow c$.

The whole system can be partly represented in a matrix called the stoichiometric matrix, S, of dimension m x n, where m is the number of different metabolites and n is the number of reactions. The metabolites form the rows and the reactions form the columns. A value "0" in the matrix means the reaction r_n does not include the given metabolite (that is neither product nor substrate). A positive value means the metabolite is a product of the corresponding reaction and a negative value means it is consumed. The stoichiometric matrix serves as an initial step for each of FBA, EFM and sometimes differential equation. For our toy example, we obtain S as follows:

$$\mathbf{S} = \begin{array}{c} \begin{matrix} r1 & r2 & r3 & r4 \end{matrix} \\ \begin{bmatrix} -1 & 0 & 0 & 0 \\ 0 & -1 & 0 & 0 \\ 1 & 0 & -1 & -2 \\ 0 & 2 & 0 & -1 \\ 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 1 \end{bmatrix} \end{array} \begin{matrix} u \\ v \\ b \\ c \\ d \\ e \end{matrix} \tag{3.1}$$

We use the notation $[x,r_i]$ to refer to the intersection of a row x and a column r_i in the matrix. For reaction $r1$, u is consumed(-1 in $[u,r1]$) to produce b(1 in $[b,r1]$) whereas for reaction $r4$, we have both 2 molecules of b and 1 molecule of c that are consumed (-2 in $[b,r4]$ and -1 in $[c,r4]$ respectively) to produce 1 molecule of e (the value 1 in $[e,r4]$).
A vector $\vec{v}$ is designed for reaction speeds. v is a priori unknown, so, it is made of variables. Let us say that reaction $r1$ occurs at speed $v1$, reaction $r2$ to reaction $r4$ at speeds $v2$ to $v4$ respectively, then vector $\vec{v}$ is represented as:

$$\vec{v} = \begin{pmatrix} \text{v1} \\ \text{v2} \\ \text{v3} \\ \text{v4} \end{pmatrix} \tag{3.2}$$

So, the mass balance can be defined as the product of S and $\vec{v}$. The general form of mass balance for a given metabolite is given as shown in Equation 3.3.

$$\mathbf{S}.\vec{v} = \begin{bmatrix} -1 & 0 & 0 & 0 \\ 0 & -1 & 0 & 0 \\ 1 & 0 & -1 & -2 \\ 0 & 2 & 0 & -1 \\ 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 1 \end{bmatrix} \begin{pmatrix} \text{v1} \\ \text{v2} \\ \text{v3} \\ \text{v4} \end{pmatrix} = \begin{bmatrix} -v1 \\ -v2 \\ v1 - v3 - 2v4 \\ 2v2 - v4 \\ v3 \\ v4 \end{bmatrix} \tag{3.3}$$

From 3.3, the flux of u is $-v1$, the flux of v is $-v2$, the flux of b is $2v2 - v4$,the flux of c is $2v2 - v4$, the flux of d is $v3$ and the flux of e is $v4$.

At steady state, the change in the amount of a given *internal* metabolite x over time t across the network is zero. This means that the number of internal metabolites is supposed to be constant, otherwise the number of metabolites would tend to infinity (if the speed is positive) or disappear (if the speed is negative). Therefore, from Figure 3.1, external metabolites u,v,d and e will not be considered and only speeds relative to b and c will be equal to 0:

$$\begin{bmatrix} v1 - v3 - 2v4 \\ 2v2 - v4 \end{bmatrix} = \begin{bmatrix} 0 \\ 0 \end{bmatrix} \tag{3.4}$$

From the stoichiometric matrix, by using $S.\vec{v}$=0 on internal metabolites, we impose certain constraints on $\vec{v}$ and at the same time, the fluxes are also interdependent. The "rest" of this vector of fluxes $\vec{v}$ can be tuned for the optimisation of certain products.
In our example, the list of internal metabolites are only b and c, from 3.4, this gives $v1 - v3 - 2v4$=0 and $2v2 - v4 = 0$. And finally, this will end with $v1 = v3 + 2v4$ and $v2 = 0.5v4$, with only $v3$ and $v4$ left as unknowns. Thus, $v3$ and $v4$ "drive" the behaviour of external metabolites via the vector shown in 3.5, generating equations shown in 3.6:

$$\begin{bmatrix} -v3 - 2v4 \\ -0.5v4 \\ v3 \\ v4 \end{bmatrix} \begin{matrix} \text{u} \\ \text{v} \\ \text{d} \\ \text{e} \end{matrix} \tag{3.5}$$

$$\begin{aligned} v4 &= 2v2 \\ v3 &= v1 - 4v2 \end{aligned} \tag{3.6}$$

While these two basic steps are shared in quantitative metabolic analysis, the other remaining steps will be different for FBA and EFM.

The next section continues with FBA to demonstrate how constraints are incorporated on these reactions based on biological knowledge of the studied metabolic network.

3.2.2 Flux Balance Analysis

Introduction
Flux balance analysis (FBA) has emerged as an effective modelling framework to analyse metabolic networks in a quantitative manner [54] with a large repertoire of rigorous applications [55, 56, 57].

Five steps are involved in FBA metabolic reconstructions and analysis:

1. construction of the biochemical reactions for the system in question
2. linking the sets of enzymatic reactions with metabolites in a stoichiometry matrix and using it on internal metabolites which are neither produced nor consumed (steady state condition)
3. using biological knowledge to extract minimum and maximum flux speeds of reactions
4. determining the objective function that is supposed to be optimized by the cell
5. identify the parameters in order to optimise the objective function

The first two preliminary steps of FBA have been elaborated in the previous section. We continue with the same toy example to illustrate the next two steps of FBA. More often, there are normally more reactions than the number of internal metabolites in a given model. Consequently, there remain unknown reactions speeds after the second step which results in a large number of solutions. When mass balance constraints imposed by the stoichiometric matrix S, and capacity constraints imposed by the lower and upper bounds are applied to a network, it defines an allowable solution space. With no additional constraints, the flux distribution of a biological network may lie at any point in a solution space. To get a more accurate set of solutions, FBA introduces certain constraints. There exists two types of capacity constraints : non-negativity constraints which reflect non-reversibility of reactions and main constraints which reflect maximal possible reactions speeds.

In our toy example, the constraints, $v1 \geq 0$, $v2 \geq 0$, $v3 \geq 0$ and $v4 \geq 0$ are non-negativity constraints. If a reaction is non-reversible, then the minimum and maximum flux speeds for that reaction is always positive. In our toy network, we can, for example, have the following inequalities for the four fluxes: $0 \leq v1 \leq 20$, $0 \leq v2 \leq 7$, $0 \leq v3 \leq 8$ and $0 \leq v4 \leq 7$.

Within the solution space, many possibilities are available. However, depending on the cellular pressures (cofactors, metabolites, gene regulations, environment, etc), the network will usually manifest itself on a given phenotype (or a set of phenotypes). This means that the network (and the cell) will try to go towards an "optimal solution" to achieve this phenotypic transition. For many metabolic networks, we

can imagine, for example, optimal production of biomass or energy production. In mathematical terms, the modeller will create an objective function which is biologically relevant and optimise this function (maximise or minimise).

With respect to unknown variables, the set of linear inequalities and their constraints give rise to a polyhedral. FBA tries to identify the optimal points within this constrained space. Normally, the optimal solution is located in one of the corner of the polyhedral. To achieve this, FBA expresses the objective function as $Z = c^T.v$, where c is a vector of weights, indicating how much each reaction contributes to the objective function. In practice, when only one reaction is desired for maximization or minimization, c is a vector of zeros with a one at the position of the reaction of interest. For example, if we want to optimise the production of e, then we put a weight of 1 on reaction $r4$ and a weight of 0 on reactions $r1$, $r2$ and $r3$. In this case, the vector will be $\vec{c} = (0001)$. Suppose we want to optimise the production of e twice to that of d, then the vector $\vec{c}$ will be (0 0 1 2), and so on.

To solve this optimisation problem and find the right flux distribution, FBA uses linear programming. Since our toy example is a small network, it is easy to solve the two optimisation functions as follows:

- if $\vec{c}$= (0 0 0 1), then we want to maximise v4 = 2v2; that is maximise v2 as well. Nevertheless, v2 cannot attain its maximum of 7 as we also need to have v4=2v2 $\leq$ 7. So, v2=3.5 and v1=7 is the maximum. Moreover, v3 $\geq$ 0 and therefore v1 $\geq$ 4v2 = 14 which means 14 $\leq$ v1 $\leq$ 20. Most probably, one would choose the strict minimum value of v1=14, as a greater value of v1 would consume u and produce d uselessly.
- if $\vec{c}$= (0 0 1 2), then we want to maximise v3 + 2v4 = v1-4v2 +4v2; that is, we have to maximise v1. So, we choose the maximal value of v1=20. Moreover, we must choose v2 in such a way that v3 $\leq$ 8 because v3 = v1 - 4v2. Lastly, v4 must be less than or equal to 7, thus 3 $\leq$ v2 $\leq$ 3.5 because v4 = 2v2. Most probably, one would choose the strict minimum value of v2=3, as a greater value of v2 would consume v uselessly.

Tools
Constraint-based models, particularly those using FBA, have enabled the analysis of several large systems, including entire (GSMNs) for prokaryotes [58], eukaryotes and human [40, 43], with a wide range of applications from metabolic engineering [56, 57] to drug discovery. Many simulation and visualisation tools are used in the research community for in-silico FBA analysis. These include algorithms from MOMA (minimisation of metabolic adjustment) and ROOM (regulatory on-off minimisation), and more powerful network design tools like CellNetAnalyser (CNA) and COBRA.

Discussion
A wide spectrum of objective functions for analysis, with increasing biological relevance, are used to enable various types of predictions on the capabilities of metabolic networks. There have been interesting advances in the area of FBA, with the integration of regulatory information as well as signalling networks into the metabolic models. FBA has some remarkable advantages which are as follows:

- It is a fast metabolic framework as it relies only on three information on the network: stoichiometry of metabolites, metabolic demands and some few flux parameters. Since, there is no need for kinetic parameters (except for the determination of maximal speeds), FBA is computationally cheap even in large networks. This stimulates model-driven discoveries by introducing a large number of perturbations.
- Interestingly, reverse engineering can also be performed using FBA by extracting information from large genome-scale experiments. Predicting, for example, gene knockouts to produce a biotechnological viable product.
- Additionally, FBA can help identify the knowledge gap presents in genomic data; that is reactions that are missing can be systematically identified by comparing in silico growth simulations to experimental results [59].

However, the simplicity of FBA has the following drawbacks:

- The lack of kinetic parameters means FBA cannot predict metabolite concentrations and their variations in time, oscillations and so on. Furthermore, this also means that FBA cannot take into account the dynamic behaviour of the model over time.

- It is also only capable of determining fluxes at steady state for a particular objective function. But, the temporal evolution of the cell forces it not to optimise a certain metabolite infinitely. For example, the cell will not always optimise the growth of cells as it has other objectives like production of energy to sustain other activities. These successive cell choices are of course regulated by higher level regulatory networks.
- A major challenge is often to find the definition of a biologically relevant objective function in networks where there is a regulatory loop. Finally, FBA does not account for regulatory effects such as activation of enzymes by protein kinases or regulation of gene expression, so it lacks a degree of accuracy in its predictions.

As a small reminder, our objective is to formulate the regulation of the metabolic network at the coarse-grained level to understand causalities of metabolic processes. A shift from this coarse-grained level to go down at metabolic levels would mean a shift from our objective. The dependency of FBA on metabolites would mean the analysis of too many biochemical reactions. The metabolic network is highly regulated and is driven by a large number of regulatory loops with sophisticated feedbacks, which cannot be handled by FBA tools. Our research focuses on the successive changes of the metabolic state of the cell with a particular attention to biomass and energy. Using FBA will explicitly demand the list of all enzymatic reactions which amounts to hundreds and this is righteously not our main objective. Reasonably, to achieve this vision, we will therefore require a higher granular modelling. In conclusion, we will put aside FBA as a metabolic engineering tool, not suitable for the question we want to address in this thesis.

Next, we will see a variant and a complement of FBA called Elementary Flux Modes (EFM), which is a promising tool for pathway analysis and metabolic engineering.

3.2.3 Elementary Flux Modes

Introduction
While FBA proposes only an optimal solution (single flux distribution) for a given metabolic network, a variant has been proposed by Schuster et al. [84, 85] called Elementary Flux Modes (EFM). This metabolic framework shares the same mathematical principle (first steps) with FBA; that is, firstly, constructing the network with an initial set of reactions (both reversible and irreversible) for both internal and external metabolites, and secondly building the stoichiometric matrix by aligning the reactions with the metabolites. However, EFM introduces a systematic way of extracting biologically meaningful pathways from an intricate metabolic network. These pathways (called elementary modes abbreviated as EM) consist of a minimal set of input metabolites and a minimal set of outputs. Compared to FBA, EFM does not require knowledge of any fixed flux rate and does not use any objective function. In general, a set of properties must be verified to generate EMs. They are as follows :

1. $S.\ \vec{v} = \vec{0}$ for internal metabolites (for steady state).
2. all reactions must be directed (proceeding in one direction will ensure positive flux values). When a reaction is reversible, EFM simply asks to decompose it into two directed reactions for each direction.
3. the first two properties admit an infinite set of solutions. This additional constraint allows EFM to generate finite sets of solutions.
4. in the attempt of optimising the outputs, we must have sufficiently EMs so that we can generate all the linear combinations of EMs with coeff ≥ 0.

The decomposition into elementary flux modes of a given metabolic network is not unique. It depends, for example, on the order of removal of non-linearly independent flux modes. To illustrate the procedures involved in EFM, let us use the following set of reactions (captured in Figure 3.4 left), where $r4/r5$ and $r6/r7$ denote in fact two reversible reactions.

r1 : $a \rightarrow b$

r2 : $b \rightarrow c$

r3 : $b \rightarrow d$

r4 : $d \rightarrow e + f$

r5 : $d \leftarrow e + f$

r6 : $e \rightarrow f$

r7 : $e \leftarrow f$

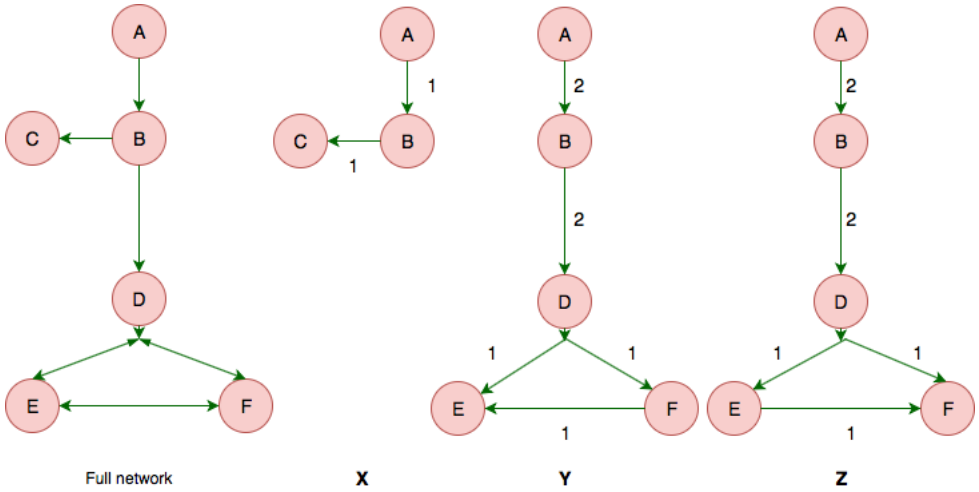


Figure 3.4: An example of a toy network (left) with four external metabolites: A as input, and C, E and F as outputs. Three elementary modes (X, Y and Z) are extracted from the network, with stoichiometric coefficients on the arrows. This figure will be used to illustrate how the three EMs participate to produce a certain flux of interest.

Using the reactions r1 to r7, we construct the stoichiometric matrix S.

$$\mathbf{S} = \begin{array}{c} \begin{matrix} \text{r1} & \text{r2} & \text{r3} & \text{r4} & \text{r5} & \text{r6} & \text{r7} \end{matrix} \\ \begin{bmatrix} -1 & 0 & 0 & 0 & 0 & 0 & 0 \\ 1 & -1 & -1 & 0 & 0 & 0 & 0 \\ 0 & 1 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 1 & -1 & 1 & 0 & 0 \\ 1 & 0 & 0 & 1 & -1 & -1 & 1 \\ 1 & 0 & 0 & 1 & -1 & 1 & -1 \end{bmatrix} \end{array} \begin{matrix} \text{a} \\ \text{b} \\ \text{c} \\ \text{d} \\ \text{e} \\ \text{f} \end{matrix} \tag{3.7}$$

We then calculate the mass action by taking the product of the stoichiometric matrix S and the vector $\vec{v}$.

$$\mathbf{S}.\vec{v} = \begin{bmatrix} -1 & 0 & 0 & 0 & 0 & 0 & 0 \\ 1 & -1 & -1 & 0 & 0 & 0 & 0 \\ 0 & 1 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 1 & -1 & 1 & 0 & 0 \\ 1 & 0 & 0 & 1 & -1 & -1 & 1 \\ 1 & 0 & 0 & 1 & -1 & 1 & -1 \end{bmatrix} \begin{pmatrix} \text{v1} \\ \text{v2} \\ \text{v3} \\ \text{v4} \\ \text{v5} \\ \text{v6} \\ \text{v7} \end{pmatrix} = \begin{bmatrix} -v1 \\ v1 - v2 - v3 \\ v3 \\ v3 - v4 - v5 \\ v1 + v4 - v5 - v6 - v7 \\ v1 + v4 - v5 + v6 - v7 \end{bmatrix} \tag{3.8}$$

Using 3.8 with the only internal metabolites b and d, we will have the following two equations set to 0 leaving us with: $v1 = v2 + v3$ and $v3 = v4 + v5$

$$\begin{bmatrix} v1 - v2 - v3 \\ v3 - v4 - v5 \end{bmatrix} = \begin{bmatrix} 0 \\ 0 \end{bmatrix} \tag{3.9}$$

Since all elementary modes are unique up to scalar multiples, the fluxes in each mode represent only relative values. The most meaningful values are fluxes of an entire pathway that are normalised with respect to a flux of interest in a reaction such as a substrate flux or a product flux. This pathway definition allows a systematic approach to accurately compare molar yields of a metabolite with respect to another in multiple pathways [84, 85].

Let us illustrate how we can use the linear combinations of these three EMs (X,Y and Z) to produce three different examples of fluxes (M1, M2 and M3) of interest (among others) :

1. **M1** : let us assume that one wants to produce the following molecular combinations : 2 for C, 0 for E and 2 for F. To achieve this yield, we can use only the pathways X and Z with the computation 2X + Z as shown in Figure 3.5

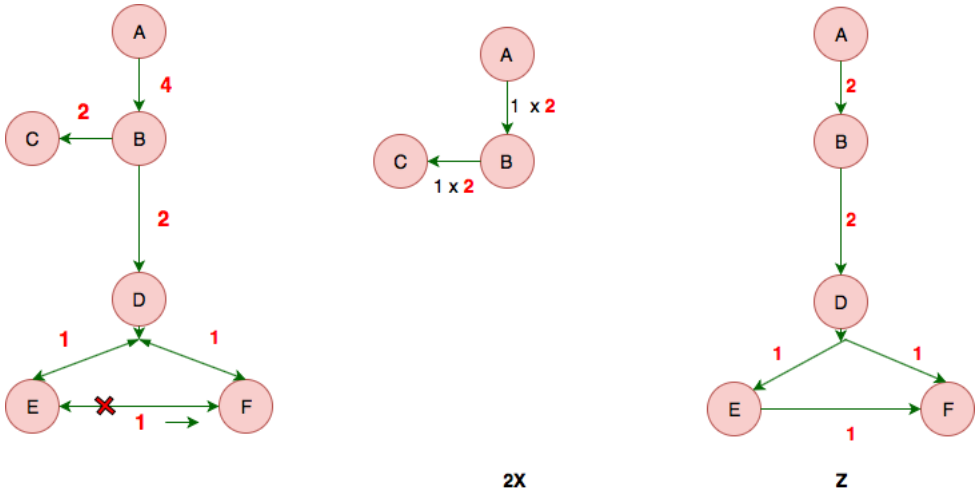


Figure 3.5: Elementary mode 1 (EM1): 2X + Z; there is no need to have Y in the computational paths as we target only the flux distribution of C and F.

2. **M2** : 2X + 1/2 Y + 1/2 Z
In the second (M2), the optimal solution is to find the following molar yields : 2 molar yield for C, 1 for E and 1 for F. We can formulate this computation as : 2X + 1/2 Y + 1/2 Z. We still use 4 as coefficient for $r1$, 2 for $r3$ and 1 for $r4$, except that 1/2 mole of E flows to F and vice-versa. In this way, we expect an equivalent 1 mole for E and F are produced. The repartitions of the coefficients for the production of E and F are shown in Figure 3.6.

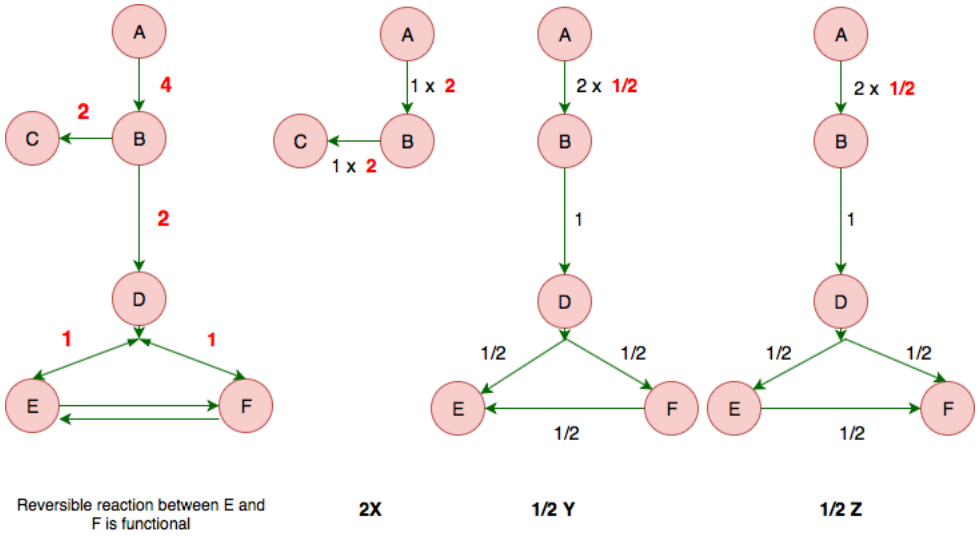


Figure 3.6: Elementary mode 2 (M2): 2X + 1/2 Y + 1/2 Z; an equal distribution of weight between Y and Z allows us to produce the required metabolic phenotypes shown in (a)

3. **M3** : 2X + 1/8 Y+ 3/8 Z
In the final example, we still maintain the output of 2 moles of C but this time, we assume that 1/4 part of E is distributed to F only. The computation to attain these fluxes for M3 can be calculated as 2X + 1/8Y and 3/8 of Z as shown in Figure 3.7.

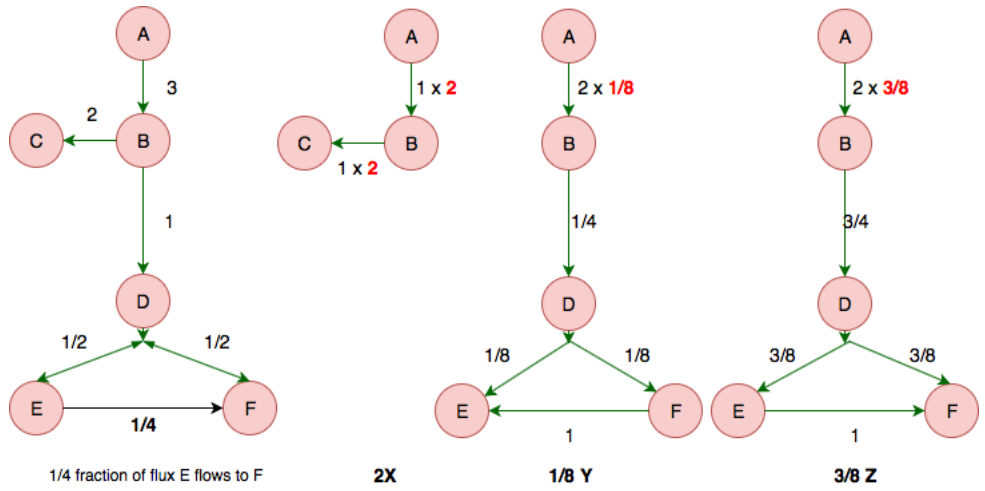


Figure 3.7: Elementary mode 3 (EM3): 2X + 1/8 Y + 3/8 Z; we need to put a greater weight of F in Z to be able to achieve the output shown in (a).

Discussion
EFMs have proved their effectiveness as a mathematical tool in metabolic engineering research. Many metabolic tools dedicated to EFM have been used in the past decade [87, 88, 89]. Moreover, there are growing attempts to develop more effective computational algorithms to reduce significantly the number of pathways and use EFMs for genome-scale networks. One possibility is to use a clustering approach to group pathways having the same topology [63]. In our example, EMs Y and Z can be grouped as they shared similar topologies. The following are other advantages of EFMs among others:

- EFMs is fast (although relatively slow compared to FBA) as it depends only on stoichiometric coefficients; there is no need for kinetic parameters.
- Once a set of EFM is identified, any non-negative set of linear combination of those pathways is possible; this is a useful feature which means a higher degree of flexibility and open doors to time-dependent solutions.
- An improvement in EFM metabolic engineering is to introduce kinetic parameters for dynamic interaction between metabolites.

Unfortunately, despite its powerful methodology and effectiveness, we will have to ignore EFMs in our approach as they share the following disadvantages:

- high dependency on precise experimental data which are also real values
- it remains impractical for the study of large metabolic networks; the computation is expensive for generating all EFMs which can rapidly go to thousands even millions of pathways [86].

3.2.4 Conclusion on metabolic frameworks

Classical quantitative formalisms like FBA and EFM which are dedicated to the analysis of metabolic networks have been discussed in this section.

We have seen that classical metabolic frameworks work on the same mathematical principle of quasi-steady states; that is flux speeds of substrates are constant. But, in our case, we are interested in the regulatory networks of metabolism; that is, evolution of flux speeds among metabolic processes with respect to time. Even if some variations exist for FBA and EFM, they still do not put the focus on causalities that drive the overall dynamics.

Despite their usefulness in metabolic engineering and their integration in diverse applications [64, 65], our coarse-grained vision of energy metabolism cannot be achieved with FBA nor EFMs. We insisted on coarse-grained to avoid all sorts of enzymatic reactions and metabolites transformations. In our model, for example, ATP and ADP are treated as only one variable (we refer to their quotients only and we do not differentiate between their cytoplasmic and mitochondrial presence) and we do not involve at any time the transformation of ADP to ATP by the addition of one phosphate group. Our focus is more on

the regulation between metabolic *processes* for the production of energy and biomass.

The next section will detail a last quantitative method called differential equations, which diverge from both FBA and EFM.

3.3 Differential Equations

Traditionally, the set of biological reactions (metabolites, chemicals or genes, which we refer to as entities) can be described by linear or non-linear, ordinary or partial differential equations, solutions of which provide insights into the dynamics of the studied processes [60]. Classical kinetic modelling approaches describe the rate of change in the concentration of the considered entities based on the enzyme kinetics (e.g., mass action or its derivative, Michaelis-Menten) with the corresponding parameters (e.g., rate constants) [61]. For example, the classical Michaelis-Menten for an enzymatic reaction takes the general form as in Figure 3.8.

The power of ODE is its capability to embrace rather heterogeneous phenomena (metabolic reactions,

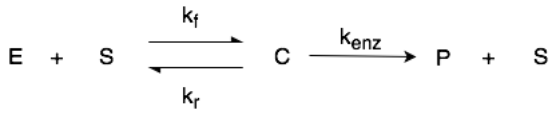


Figure 3.8: A simple enzymatic reaction of the conversion of substrate(S) to product(P) catalysed by an enzyme(E). k_f, k_r and k_{enz} are rate constants. C denotes an intermediate reaction.

gene regulations, signalling pathways, etc) within a single homogeneous framework. The coupling of gene regulations as well as enzymatic reactions can be encoded into differential equations, the system of which models the functioning of the whole metabolic network. Finally, differential equations give the possibility to the modeller to regroup all these reactions or interactions (genes, metabolites, etc) in the same system.

Due to its universality property, ODEs can represent any kind of reactions (genes, metabolic, enzymes) in the same system with a global view of behaviours. The popularity of Ordinary Differential Equations (ODEs) is due to their capacities to describe temporal evolution of the components of a system as well as the possibility to provide analytic solutions. It has been used in diverse applications in engineering and natural science. Let us see how ODE can be used to model the toy example in Figure 3.3.

After obtaining the stoichiometry matrix and the flux vector (denoted by S and $\vec{v}$ respectively), at steady state, the system (x_i represents the metabolites) can be simply expressed as :

$$\left(\frac{\mathrm{d}\vec{x_i}}{\mathrm{d}t} \right) = S.\vec{v} = 0$$

The rate of change for a given metabolite is, therefore, a linear transformation of the fluxes affecting the metabolite (a flux is negative if the metabolite is consumed). At steady state, internal metabolites are not considered as in FBA and EFM. Therefore, the ODEs of metabolites b and c, which are internal metabolites (from Figure 3.3), can be expressed as:

$$\frac{\mathrm{d}b}{\mathrm{d}t} = -v_3 - 2v_4 + v_1 (= 0)$$

$$\frac{\mathrm{d}c}{\mathrm{d}t} = -v_4 + 2v_2 (= 0)$$

$$\frac{\mathrm{d}d}{\mathrm{d}t} = v_3 (= 0)$$

$$\frac{\mathrm{d}e}{\mathrm{d}t} = -v_4 (= 0)$$

After obtaining a mathematical representation of the model in terms of ODE, many numerical methods are available to explore the system. For each metabolite, we can graphically plot its evolution over time. Similar plots can be obtained from experimental data which are real values and which may contain noisy data as well. We need rigorous methods to bring simulated data closely enough to experimental analysis.

Let us see the use of differential equation to model gene regulations in which the expression of genes changes over time (see Figure 3.9).

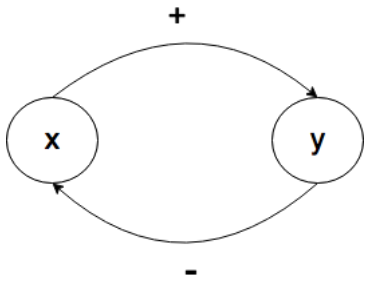


Figure 3.9: Interaction graph between gene x and gene y. x activates y and y inhibits x

Here, we have two regulations :x activates y (therefore, it would be interesting to model the rate of change of y which can be expressed as in dy/dt) and contrarily y inhibits x (expressed as dx/dt). Both rate of changes can be shaped as in the Figures 3.10 and 3.11 respectively. f_y^x and f_x^y are sigmoidal functions.

In ODE, the rate of change of gene X can be expressed as :

$$\frac{\mathrm{d}x}{\mathrm{d}t} = k_0^x + f_y^x(y) - \gamma^x xx$$

where :

- f_x^y : This is a function representing the action of x over y.
- γ^x represents the degradation of gene x
- k_0^x represents the minimum production speed when x is inhibited

Similarly, the rate of change of gene Y would be expressed as :

$$\frac{\mathrm{d}y}{\mathrm{d}t} = k_0^y + f_x^y(x) - \gamma^y xy$$

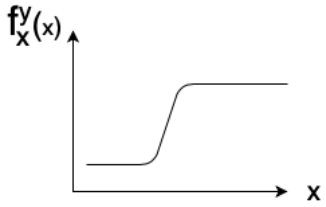


Figure 3.10: A sigmoid representing the action of x on y. Here, we have a positive sigmoid demonstrating activation. x will gradually increase the derivation of the expression level of y over time.

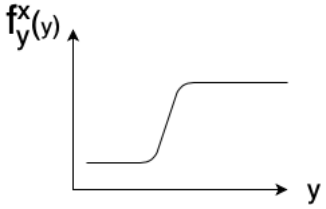


Figure 3.11: A sigmoid representing the action of y on x. A negative sigmoid demonstrating inhibition where y decreases the derivation of the expression level of x over time.

Given a set of experimental data and a model structure, the aim of parameter estimation is to calibrate the model by solving an optimisation problem where the objective function represents the distance

between the model and experimental data. Many parameter estimation techniques have been developed [91]. We illustrate one technique here : curve-fitting. For this, we need to construct an error function and minimise it (several minimisation algorithms are available [90]). This can clearly be depicted in Figure 3.12.

Discussion

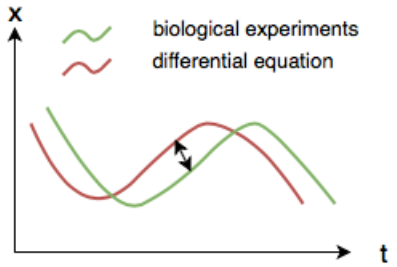


Figure 3.12: Curve-fitting in differential equation by calculating distance between model and experimental data.

ODEs can do well to describe metabolic network dynamics in terms of changes in metabolite concentrations over time but require some preliminary information. This information which can be obtained by carrying a large number of experiments include initial concentration of metabolites, kinetic parameters and kinetic rate laws of enzymatic reactions. Kinetic parameters are among the most difficult to find, especially in large complex networks. However, some advantages of ODEs are :

- Compared to FBA and EFMs, ODEs have the ability to adapt to any abstraction level : the variables can be "coarse-grained".
- The availability of regulatory mechanisms (information on activators and inhibitors) make it possible to infer general knowledge and easily write down the form of the differential equations.
- The causalities are visible on the arbitrary functions $f_i(x)$ in which the graph structure is encoded by parameters to functions $f_i(x)$.

Despite a large range of ODEs modelling tools (like Copasi [71]), there has been a poor penetration of ODEs in the modelling of very large metabolic networks and their regulations. Difficulties lie in the following:

- The identification of kinetic parameters is scarce and difficult to establish from poorly precise experimental data.
- The true mechanistic kinetic rate law for a specific reaction is frequently not known for most of the enzymes [52]. However, there are approximations like Generalized Mass Action, Lin-Log and Power Law [52] that can be applied to represent these kinetics.
- The very different nature of metabolic reactions on one hand, and regulatory phenomenon on another hand, is badly captured by ODEs, where the specificity of transformations is not helpful to simplify predictions.

However, the Michaelis-Menten kinetics assume that the rate at which an enzyme binds to its substrate is much faster than the rate of the product formation, and that the intermediate reaction is therefore at steady-state. But, similar techniques do not easily apply to our problem, as this would necessitate the formulation of a mechanistic equation for each enzymatic reaction. Our view is more abstract, leaving behind the details of molecules and their enzymes counterparts. Moreover, ODEs can also be successfully applied to non-linear interactions and this captures gene regulatory networks which are non-linear systems, but even genes would be too numerous to study the whole regulation of the metabolism.

Using ODEs, it would be practically impossible to consider all the molecules and all the genes in the metabolic network. This is too complicated for this large network and working with more abstract entities would make it impossible to measure their speeds as real values.

More precisely, we can choose to fairly group many processes and enzymatic reactions for abstract representation of main processes like Krebs, and important metabolic cofactors like ATP/ADP. This will result in less variables and thereby reduce the complexity of the model. In fact, this is exactly what we are

going to carry out further in our study. Unfortunately, when making such abstractions, the quantitative measurements become unfeasible. For example, the speed of Krebs does not have any sense in terms of quantitative method as it is difficult to measure the flux speed of Krebs as a whole. So, the *validation* of the model would be impossible.

Following this point, we are going to abstract the speed as they have thresholds. For example, the level of Krebs is sufficient enough to inhibit NADH/NAD+. This makes good metabolic sense and imply that we can do some discretisations (the application of formal methods) on these types of information.

3.4 Conclusion on quantitative methods

In the previous sections, we have shown the most commonly used quantitative formalisms in metabolic engineering, their applications and why they are not suitable for our modelling questions. Biochemical networks are incredibly complex. Quantitative models work more at the molecular level. A panoply of software tools is available both for simulation and partly for the identification of parameters. The sole default of these formalisms is precisely the occurence at the molecular level (concurrency in terms of speed and resources). The identification of parameters is difficult with real numbers as this gives infinite number of possibilities. For example, it would be very difficult to estimate kinetic rate constants for all the enzymes in respiration. It would also be very difficult to measure all metabolic fluxes in a large metabolic network.

We are conscious that quantitative models describing metabolic network dynamics are powerful tools to explain properties of complex biological systems and to guide experimentation [75]. Interestingly, we have seen the use of non-linear ordinary differential equations to model dynamic changes in metabolite concentration over time [76, 58]. But, it also represents some inconveniences: it requires a previous knowledge on the fine-grained network structure and a large amount of experimental information, such as initial concentrations of metabolites, kinetic parameters and detailed kinetic rate laws.

Our research is mainly targeted at understanding the *global* metabolic network in terms of energy and biomass production. At the molecular level, this means too many metabolic reactions for which it would be practically impossible to perform precise measurements and parameter identifications. The system itself is far too large to cater for all the enzymatic reactions and modelling at this level. These are the prime reasons we avoid using these quantitative formalisms. In our case, the goal is to understand the main causalities that drive the dynamics of the energy metabolism network at a "coarse-grained" level. The identification of quantitative parameter values is of interest to us only to elucidate the functioning of well-known biological phenomena in terms of phenotypes.

Qualitative modelling compared to quantitative methods is less data-dependent but requires sufficient knowledge to model the system. In some research experiments, data is of little interest because, by using formal logic, we can draw some meaningful conclusions with less cost and time. From this perspective, qualitative modelling can be viewed as an abstraction of quantitative modelling where reasoning capabilities are increased, and which helps the prediction of "interesting" new experiments as well as avoiding logically "redundant" experiments. Qualitative approaches ignore some numerical details which quantitative methods would require. The power of qualitative frameworks lies in their simplicity and abstraction of biological facts that are transparent with respect to the studied question. In this frame of reasoning, qualitative modelling techniques sacrifice the quantitative knowledge and focus more on the qualitative relations between model variables in order to explore and establish general properties.

For all these reasons, we focus our attention to formal methods. Formal frameworks in general are based on logic, and we give in Annexe 11.1 a general introduction to the notion of general logics and its properties, with the examples of propositional logic and first order logic. Modelling biological networks asks for more powerful logics than first order logic because one needs to state formulas about time. Temporal logics are well suited and we give here a description of CTL (Computation Tree Logic) that we will intensively use because its corresponding model checking is particularly efficient.

3.5 Computation Tree Logic(CTL)

CTL is a temporal logic in which time has a tree structure. Starting from an initial *state* that can be seen as a given model in propositional logic, there are possible *transitions*, each of them defining a possible

next state model. Then, the process is iterated starting from each of these next models, giving rise to an infinite tree (see Figure 3.13). CTL formulas are constructed from propositional logic formulas and time is taken into consideration in terms of path along the tree structure starting from the root of the tree. More often, the system is modelled as a state transition diagram in which there are states, and transition between states, which dictate the system dynamics : each state can be considered as an initial state, and, for each of them, the transition system can be 'unwinded' to produce a computational tree as shown in Figure 3.13 (right). In this example, we assume a is the initial state. From this example, we can conclude that if we go from state a to state b, we will always stay in state b. In this context, CTL provides an ideal method to express formal properties on state transition diagrams.

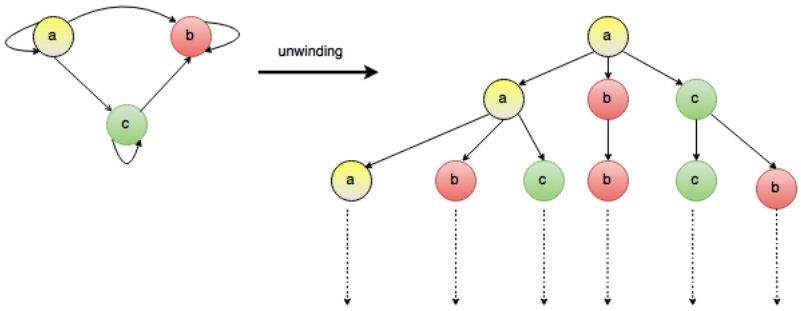


Figure 3.13: Computation Tree: To explore the whole structure, we can intuitively see CTL as unwinding the state transition graph so that it works down the tree when validating a particular formula.

CTL is a well-suited approach for analysing biological events (for example, discrete models of gene interactions [113]) in which the succession of events is important. This type of formalisation is convenient especially when we are dealing with discrete biological events which are mostly time-dependent. Temporal logic has some forms of tractability which justifies its importance in our research. However, it is worth mentioning that CTL does not handle the duration in terms of real time. It handles the order of events as they occur. In other words, by using CTL, we are able to verify the sequence of states where certain formulas hold or not. The *syntax* and *semantic* rules of CTL are explained in the next subsections.

3.5.1 Syntax

This subsection describes the basic syntactic rules of CTL. The building blocks for a temporal logic formula includes atomic propositions, logical connectives ($\neg, \vee, \wedge$), quantifiers (A for 'all paths', E for 'there exists some paths') and temporal modalities (neXt, Future, Globally and Until which are abbreviated as X,F,G and U respectively).
A CTL temporal modality must be written as a couple quantifier-temporal modality resulting in eight combinations as listed in Table 3.1. More details are provided in Annex (11.4).

Quantifier	Temporal	Combinations
A	F,G,X,U	AF, AG, AX, AU
E	F,G,X,U	EF, EG, EX, EU

Table 3.1: Syntactic temporal formulas: Temporal identifiers are always preceded by quantifiers.

For example: $EF\,(GLYC \geq 2 \wedge O_2 \geq 1)$ means there exists a path (E) such that in the future (F), glycolysis will reach the level 2 in the presence of oxygen. This example is truly propositional because each variable of a Thomas model has a bound, so the number of possible comparisons is finite.

Now, given a signature of propositional logic $P = p_1 ... p_n$, the set of well-formed CTL formulas is defined inductively by:

1. all propositions p_i are well-formed
2. if ϕ and ψ are well formed, then: ψ, $\psi \wedge \phi$, $\psi \vee \phi$ and $\psi \Rightarrow \phi$ are well-formed

3. if ϕ and ψ are well formed, then: $EX\psi$, $EF\psi$, $EG\psi$, $E(\psi U\phi)$, $AX\psi$, $AF\psi$, $AG\psi$, $A(\psi U\phi)$, are well-formed

Remarks :
Each of the eight formulas can be expressed in terms of the following existential normal forms : EX, EG and EU.

- $AX\phi \Leftrightarrow \neg EX(\neg\phi)$
- $AG\phi \Leftrightarrow \neg EF(\neg\phi)$
- $AF\phi \Leftrightarrow \neg EG(\neg\phi)$
- $EF\phi \Leftrightarrow E[True\ \mathrm{U}\ \phi]$
- $A[\phi U\psi] \Leftrightarrow \neg(E[\neg\psi U(\neg\phi \wedge \neg\psi)] \vee EG(\neg\psi))$

3.5.2 Semantics

This subsection is devoted to the meaning of CTL formulas. For efficacy reasons in model checking, "The future includes the present" in CTL, as shown in the following definitions:

Let us assume there is a rooted tree $\mathcal{A}$ where all the nodes are propositional models. $\mathcal{A} \models \phi$, where ϕ is a CTL formula, is defined inductively by:

- $\mathcal{A} \models p_i$ (where $p_i \in P$) iff the model at the root of $\mathcal{A}$ satisfies p_i
- the logical connectives $\neg$, $\wedge$, $\vee$ and $\Rightarrow$ follow the same definition as propositional logic
- $\mathcal{A} \models \mathrm{EX}(\phi)$ iff there exists a subtree of $\mathcal{A}$ ($\mathcal{A}'$) which is a direct child of the root of $\mathcal{A}$ such that $\mathcal{A}' \models \phi$
- $\mathcal{A} \models \mathrm{EG}(\phi)$ iff there exists an infinite path from the root of $\mathcal{A}$ such that all the subtrees on this path satisfy ϕ (including A itself)
- $\mathcal{A} \models \mathrm{E}(\phi\ \mathrm{U}\ \psi)$ iff there exists a subtree $\mathcal{A}'$ of $\mathcal{A}$ such that:
  1. $\mathcal{A}' \models \psi$
  2. for all subtrees $\mathcal{A}''$ for which the root is in the path from A to $\mathcal{A}'$, $\mathcal{A}'' \models \psi$
  3. $\mathcal{A} \models \mathrm{AX}(\phi)$ iff $\mathcal{A} \models \neg\ \mathrm{EX}(\neg\ \phi)$
  4. $\mathcal{A} \models \mathrm{AG}(\phi)$ iff $\mathcal{A} \models \neg\ \mathrm{EF}(\neg\ \phi)$
  5. $\mathcal{A} \models \mathrm{AF}(\phi)$ iff $\mathcal{A} \models \neg\ \mathrm{EG}(\neg\ \phi)$
  6. $\mathcal{A} \models \mathrm{EF}(\phi)$ iff $\mathcal{A} \models \neg\ \mathrm{E}(\mathrm{True}\ \mathrm{U}\ \phi)$
  7. $\mathcal{A} \models \mathrm{A}[\phi\ \mathrm{U}\ \psi]$ iff $\mathcal{A} \models \neg(E[\neg\psi U(\neg\phi \wedge \neg\psi)] \vee EG(\neg\psi))$

  Notice that $\mathcal{A}' = \mathcal{A}$ has also to be considered.

Let us consider the state transition graph of Figure 3.14.

1. $(x = 2 \wedge y = 0) \Rightarrow AG(x = 2 \wedge y = 0)$; means that (x=2, y=0) is stable state (once we are in, we stay for an infinite time). This formula is satisfied by our state transition graph.
2. Then, $AF(x = 2 \wedge y = 0)$ means that the attraction basin leading to steady state is the whole state graph. Beware that this formula is not satisfied here, because there are paths that infinitely loop between states (x=0,y=1) and (x=1,y=1). So, this formula is only satisfied for initial states where $y = 0$. Nevertheless, $EF(x = 2 \wedge y = 0)$ is satisfied for all states. An attraction basin is a set of states in which if we form part, we cannot escape. In this case, we are guided towards the stable state from which we get trapped.
3. $(y = 1) \wedge EF(x = 0)$ is obviously satisfied only for the 3 states where $y = 1$.
4. We must be careful on the manipulation of temporal modalities. Consider for example $AG(y = 1) \Rightarrow AFAG(x < 2)$. It does not mean that all paths in the line $y = 1$ will end in the two states where $x < 2$. In fact, $AG(y = 1)$ is false as y can get down to 0. Therefore, because $false \Rightarrow anything.$, the formula is satisfied but it has nothing to do with the values of x along any path. $AG(y = 1) \Rightarrow AG(y = 0)$ is also satisfied.

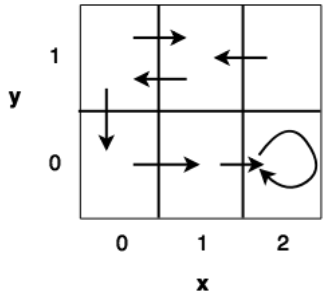


Figure 3.14: Example of a state transition graph: stable state (x=2,y=0), its attraction basin (covering states starting from x=0,y=0) and infinite loop (transitions between states 1,1 and 0,1)

3.5.3 Model checking

Model checking is an exhaustive technique for system verification that, given a CTL formula and a state transition graph, returns all states in the system where the formula does not hold or it simply returns success (Figure 3.15). In this verification procedure, it appears logical to start with an initial state and traverse all the paths from this same initial state, and to verify if a given CTL formula holds. But, this path traversal mostly leads to infinite number of infinite paths. To be computationally efficient, an alternative is to go through CTL formulas instead of the infinite paths. Model checking adopts this method.

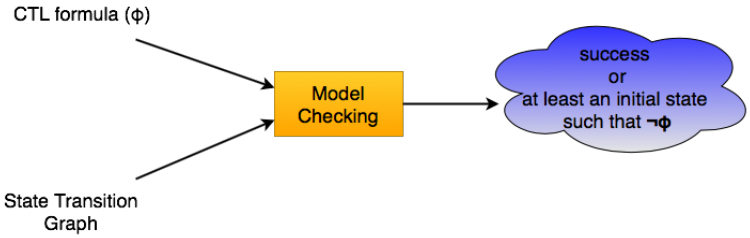


Figure 3.15: Model Checking as a black box. It takes two inputs: a state transition graph and a CTL formula, and outputs whether the CTL formula is valid or at least a state which does not satisfy the CTL formula.

The simplest version of model checking algorithms on CTL formulas can be inductively defined on the structure of the formula to model-check as follows:

1. if $\phi = p_i$, we label all the states such that $\eta \models p_i$ by p_i. This is computationally cheap as we evaluate all CTL formulas on the nodes of the state graph.
2. if the top operator of ϕ is a logical connective, then, with the use of truth tables, we can easily cross logical connectives $\wedge$,$\vee$ and $\neg$ to label states accordingly. Let us assume for example that states in the state transition graph have already been labelled for satisfying CTL formulas ϕ and ψ. All states not satisfying ϕ can be labelled with $\neg\phi$ ($S \not\models \phi$ is the same as $S \models \neg\phi$), states where both ϕ and ψ hold can be labelled $\phi \wedge \psi$ and so on.
3. lastly, if the top operator of ϕ is a CTL modality, following the CTL semantics discussed in 3.5.2, model checking can be applied with a minimum set of existential quantifiers (the set for quantifier A can be derived from E). We elaborate on the existential quantifiers as follows:
   - $EX\phi$: if a state in a transition graph is labelled with ϕ, then by induction, we can label all the states that are predecessors of that state with $EX\phi$ as in Figure 3.16.
   - $EG\phi$: First, we must label the sets of all states where ϕ holds. Next, if there exists a cycle in this set where all states satisfy ϕ, then the cycle is labelled as $EG\phi$ (see 3.17(b)). This is computationally expensive as for each state, we need to check for cycles. Then if a given state is labelled with $EG\phi$, then all its predecessors that are already labelled with ϕ are labelled with $EG\phi$ (there are improved algorithmic computation by substituting E with A which is not discussed in this text).

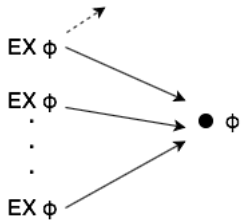


Figure 3.16: Model checking using EX : All predecessors of a state labelled ϕ are labelled with $EX\phi$ even if they have other successors (dotted arrow).

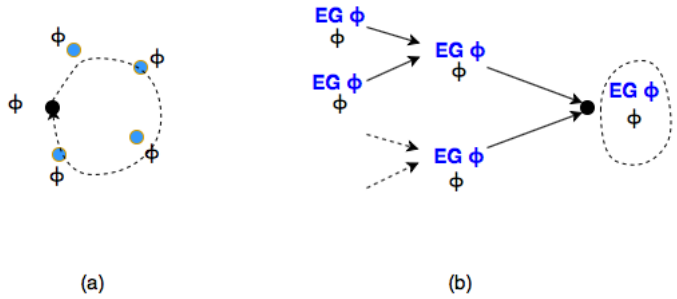


Figure 3.17: Model checking using EG : All states labelled with ϕ which are also the predecessors of state satisfying ϕ, are labelled with $EG\phi$

.

- $E[\phi$ U $\psi]$: For EU, let us assume that states have been labelled according to whether they satisfy ψ or ϕ. Then, all states that have been labelled with ψ are also labelled as $E(\phi$ U $\psi)$, because the future includes the present. All predecessors where ϕ holds and at the same time ψ holds are equally labelled with $E(\phi$ U $\psi)$ (Figure 3.18). All states of the transition graph are checked in the same way until we reach a state already labelled with $E(\phi$ U $\psi)$.

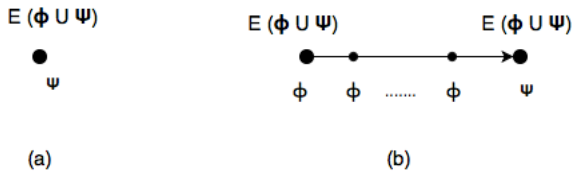


Figure 3.18: Model checking using $E(\phi$ U $\psi)$. (a) A state where ψ holds can be labelled as $E(\phi$ U $\psi)$ (b) If the predecessor of this state satisfies ϕ, then the predecessor can also be labelled $E(\phi$ U $\psi)$.

To give a flavour of what model checkers bring to improve performances, one can mention among others that, in situations where many states satisfy the same set of CTL formulas, model checking algorithms regroup them and consider this group as only one state. Depending on model checkers, this regrouping of states are implemented behind the scenes on top of automata networks using complex algorithms with structures extending binary decision diagrams. This goes beyond the discussions here and are therefore, not further elaborated.

3.6 Automata networks

Biological networks are controlled by inputs from their respective environments and they are, at a given time, in a particular state. In this way, automata networks mimic biological regulatory networks.

The concept of automata network was conceptualised by Alan Turing in the early 1930's and finds its applications in many areas from linguistics to biology [95]. In this concept, an automaton is an abstract model of a given machine which shows how it evolves over time based on inputs. At any time, the machine is in a given state. In this chapter, we discuss some of these formalisms having the capacity to model cellular activities in terms of automaton.

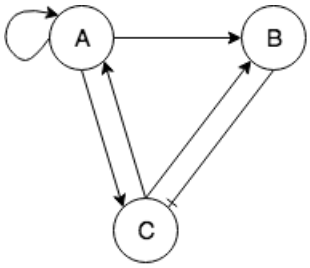
3.6.1 Boolean network (BN)

It is perhaps the simplest yet powerful formalism to describe biological processes. Initially meant for inferring gene networks and modelling gene regulations [100, 101], boolean networks consist of nodes (called *vertices*) and boolean functions that can link the nodes, and at the same time model transition between states. Nodes can take only two values: 0 or 1. In biological terms, this would mean, for example, that a gene is expressed or not expressed, a transcription factor is active or inactive, and a molecule's concentration is above or below a certain threshold [94]. This discrete dynamic model can be also scaled to model large-scale biological networks [92, 93]. For a given network with n nodes, we will have 2^n possible states, each state being modelled as a vector storing the boolean value of each node.

Four steps are required to model BNs. They are as follows:

1. First, we construct the network based on biological knowledge with interactions between the network variables. This also includes the identification of activators and inhibitors.
2. In the second step, we derive the boolean functions (F) applicable to each node (using logical connectives). Here, we note the actions each node has on its successors either alone or in combination with other nodes.
3. Third, we build the state transition table (truth table based on F) which shows the effect of the source nodes for each individual node. We use the logical relations described from the previous step.
4. Finally, we infer the dynamic of the network based on the transition type obtained from the truth table.

In a boolean network, transitions between states can be viewed in at least three ways: *synchronous*, *asynchronous* and *block synchronous*. We consider a simple network of three nodes in Figure 3.19 to illustrate these distinct transitions.



(i)

F(A) = A or C

F(B) = A and C

F(C) = A and !B

(ii)

A	C	F(A)
0	0	0
0	1	1
1	0	1
1	1	1

A	C	F(B)
0	0	0
0	1	0
1	0	0
1	1	1

A	B	F(C)
0	0	0
0	1	0
1	0	1
1	1	0

(iii)

Figure 3.19: (i)Boolean network showing the interaction between three variables A, B and C. (ii) Boolean functions for expressing the type of cooperations between the variables. (iii) Truth tables for nodes A, B and C

1. **Synchronous boolean network**
   In a synchronous boolean network, all the boolean functions are updated simultaneously between transitions in consecutive time points. As a result, there is only one outgoing edge for each node in the state transition graph. From Figure 3.19, we get the state transition graph of Figure 3.20.
2. **Asynchronous boolean network**
   Asynchronous models are closer to biological phenomena. In the asynchronous model, only one node can be updated at a time. So for each state, there are at most three possibilities: either one of A, B or C changes in the next state which means at least one outgoing edge for each node (if we consider that a stable state has always a transition on itself). From Figure 3.19, we got the state transition graph of Figure 3.21.
3. **Block synchronous network**
   In block synchronous models, subsets of the variables are chosen to update their values simultaneously in a predefined order. We continue with the same example of Figure 3.19 and assume that the subsets {A,B} and {C} are allowed to change states in this order. The state transition graph for this block synchronous model is illustrated in Figure 3.22.

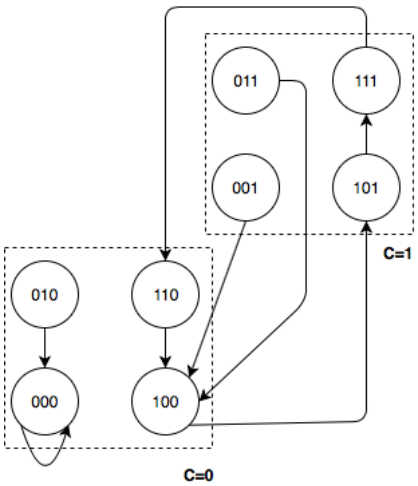


Figure 3.20: Boolean network - Sample synchronous transition : Only one transition for each state is possible.

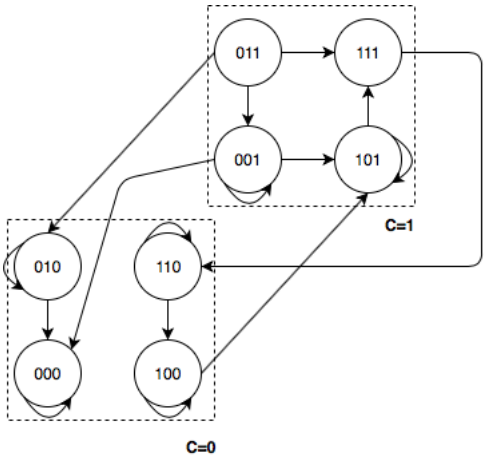


Figure 3.21: Boolean network - An example of an asynchronous transition : Many transitions possible for each state.

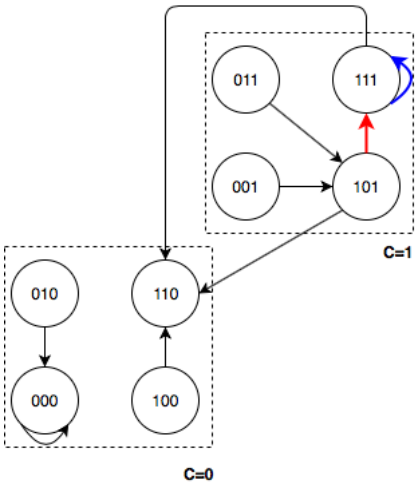


Figure 3.22: Boolean network - An example of block synchronous transition : For instance, by allowing changes to {A,B} followed by {C}, state 101 changes to 111 (red arrow) then stay in 111 for changes in {C} as shown with blue arrow

.

The boolean approach has a too poor expressivity for us as in our system we will be confronted with

variables having more than 2 values. For example to express biological properties like low, medium and high, we need more than 2 values. This overrides the boolean approach and for this reason, it does not directly apply for our network analysis (see next chapter on the Thomas framework).

3.7 Petri Nets and extensions

Biochemical reaction systems have by their very nature two distinctive characteristics:

1. They are inherently bipartite; that is, they consist of two types of game players, the species and their interactions.
2. They are inherently concurrent, that is, several interactions can usually happen and compete for the same resources.

3.7.1 Normal Petri Nets

Petri nets (PNs) are devoted to the study of concurrency and they have recently emerged as a useful tool among the various methods employed for the modelling and analysis of molecular networks. Petri nets were introduced in 1962 by Dr. Carl Adam Petri as a formalism for modelling complex networks. The theory of Petri nets provides a graphical notation with a formal mathematical semantics for modelling and reasoning about concurrent, distributed systems. Metabolic models using Petri nets are numerous ([102], [103], [104]), as the set of metabolic pathways can be considered as a concurrent system.

Petri nets are mathematical structures having three types of components namely: *places* (denoted by P and represented as circles), *transitions* (denoted by t represented by rectangles) and *arrows* (represented as arcs).The displacement of tokens between places describes state transitions which allow the modeller to study the dynamic behavior of the system. When a transition occurs, the source will pass some token to the destination place, resulting at a given moment to either zero or positive number of tokens. Formally, the Petri net can be defined as follows:

Definition 1. A Petri net is a 5-tuple structure denoted by $N = (P, T, I, O, M_0)$

1. P is the set of finite places, $P = \{p_0, p_1, .., p_i\}$
2. T is the set of transitions, $T = \{t_0, t_1, ..., t_j\}$
3. I is the set of directed arcs from P to T labelled with an integer, I : P x T $\rightarrow N_0$, where N_0 is the set of non-negative integers.
4. O is the set of directed arcs from T to P labelled with an integer, O : T x P $\rightarrow N_0$
5. M_0 is the initial state of the network (M_i is referred to as *marking*), that is, the initial number of tokens in each place of P is often represented by a transpose matrix.

In Figure 3.23, we illustrate a simple Petri net and its corresponding 5-tuple information.

The dynamics of the Petri nets can be observed by a series of transitions that are fired over time. This firing procedure which will distribute tokens over the net, is defined by two rules as follows:

- Enabling rule
  A transition t_j is enabled if there exists (t_j, p_i) such that p_i contains at least the same number of tokens as the weight I(t_j, p_i), that is $M(p_i) \geq I(t_j, p_i)$.
- Firing rule
  One transition among the enabled ones is chosen and the firing will remove the number of tokens $I(t_j, p_i)$ from each p_i connected to t_j via I, then add $O(t_j, p_i)$ tokens to each p_i connected to t_j via O.

Successive states can then be computed using an *incidence matrix* that contains the difference between the number of tokens produced and the number of tokens consumed for each firing transition (see Figure 3.24). For our toy example, the incidence matrix, C is given by : C=O - I, and successive states can be calculated as follows : $M'(p_i) = M(p_i) + C$.

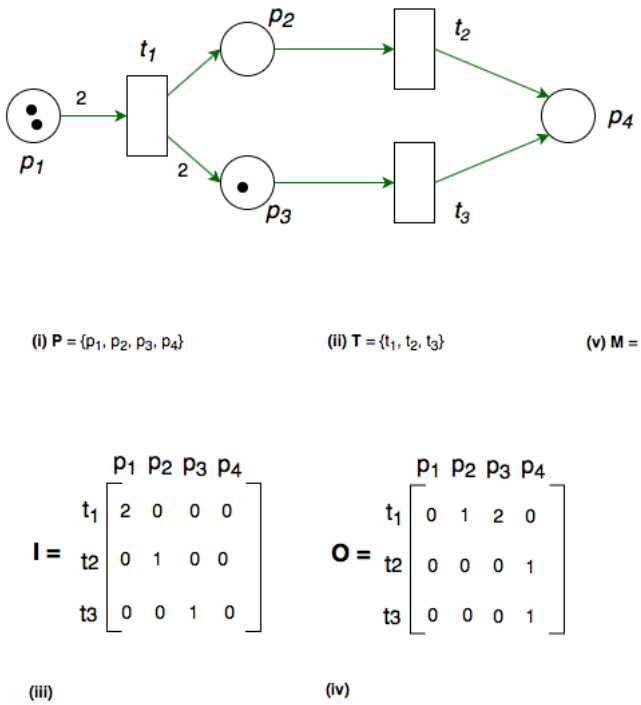


Figure 3.23: A Petri net with (i) 4 places (ii) 3 transitions ($t1$,$t2$,$t3$) (iii) 2 tokens in place P_1 ,(iv) and (v) show the corresponding input and output matrices respectively.

$$C = I - O = \begin{array}{c} \\ t_1 \\ t_2 \\ t_3 \end{array} \begin{array}{c} \begin{array}{cccc} p_1 & p_2 & p_3 & p_4 \end{array} \\ \begin{bmatrix} -2 & 1 & 2 & 0 \\ 0 & -1 & 0 & 1 \\ 0 & 0 & -1 & 1 \end{bmatrix} \end{array}$$

Figure 3.24: Incidence matrix, C, calculated using $C = O - I$. The incidence matrix can be read as follows : for example if t_1 fires, then 2 tokens have to be removed from p_1, 1 and 2 tokens added to p_2 and p_3 respectively while a "0" value means no direct transitions exist between t_1 and p_4.

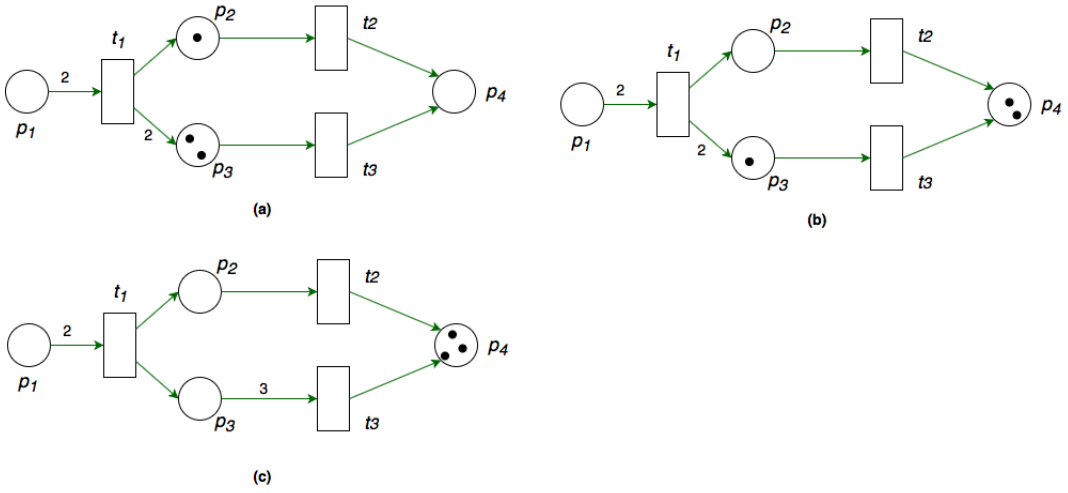


Figure 3.25: (a) Starting from Figure 3.23, only transition t_1 will fire as $I(t_1, p_1)$ contains sufficient number of tokens from p_1 to pass on to p_2 (1 token) and p_3 (2 tokens). (b) Only transitions t_2 and t_3 will be enabled and we assume t_2 will fire first followed by t_3 (c) Only t_3 will be enabled and is the only one to fire.

Using Figure 3.23 and M_0=(2 0 0 0) as initial state, we can fire transitions as shown in Figure 3.25.

Advantages of Petri Nets:

1. We can do algebraic manipulations easily with the incidence matrix that allows us to view the "transfer" of tokens as well as the state transitions.
2. Petri nets are formalisms that are concurrent to FBA and EFM. In contrast to FBA and EFM, Petri nets are abstract to us since, instead of fluxes which are real values, tokens which are positive integers, are used.
3. The ability to use of CTL to view the evolution of Petri nets [105].

Disadvantages of Petri Nets:

1. Petri nets represent the fluxes in networks like FBA and EFM rather than regulations.
2. The inability to distinguish tokens in a Petri net.

For the entire metabolic network, the use of Petri nets would require too many information on the biochemical reactions to choose the required tokens. To conclude, our focus is more on the regulations rather than fluxes, therefore, we will not choose Petri nets as a formalism to address our question.

3.8 Rule-based frameworks

Biochemical processes inside cells are regulated by intricate chemical reactions involving diverse molecules which are catalysed by enzymes. The latter are in turn mediated by genes. In rule-based frameworks, these chemical reactions can be rewritten as *rules* with a well-defined syntax. Two commonly used rule-based frameworks, namely BIOCHAM and Kappa, will be briefly discussed in this section.

3.8.1 BIOCHAM

BIOCHAM (BIOCHemical Abstract Machine) is a rule-based language together with a software environment for modelling biochemical signalling systems [106]. It also provides parameter identification and validation tools (uses temporal logic). BIOCHAM is designed using a logic programming setting and a language of reaction rules.

To specify a signalling system, BIOCHAM asks first to declare a "signature" which is an initial starting set of abstract molecules (multisets of them are called a *solution*) and it also asks for evolution rules(reactions) from which dynamical properties of the system can be formally established. BIOCHAM also offers the possibility to choose within a set of semantics for models using one of the following frameworks :

1. asynchronous boolean networks (focuses on the presence/absence of molecules)
2. continuous-time Markov processes (focuses on numbers of molecules as a Markov chain)
3. ordinary differential equations (relies on molecular concentrations)

The syntax of BIOCHAM follows two forms namely : reaction networks (like biochemical reactions) and influence networks (like genetic and metabolic networks). In this text, we focus solely on the influence network, as our metabolic network is regulated by many influencers (glucose, glutamine, etc).

Syntax
The syntax for BIOCHAM is divided into four parts:

1. Objects
   An object in BIOCHAM simply represents a biochemical species which can have one of the following notations :
   - An object containing only letters and numbers represents a "simple" molecule.
   - An object with a "-" represents a complex and the "-" is commutative as well as associative. This means that *a*-*b* is the same as *b*-*a*.
   - "Sites" can be integrated using the "$\backsim$" operator. For example the two sites of phosphorylation $s1$ and $s2$ for a given molecule m is given by $m \backsim (s1, s2)$.

   Abstract objects, which are not molecules, can be prefixed with the @ character.

2. Solutions
   A solution in BIOCHAM is a set of molecules which are combined together by the "+" operator. Therefore, "o1 + o2" represents a solution where we have two objects o1 and o2. An empty solution is represented by "_".

3. Reactions
   Reactions involve solutions of the form $soln1 \Rightarrow soln2$ ($soln1$ and $soln2$ are the two solutions in this reaction) which can be further extended as follows:

   - $(soln1 = [C] \Rightarrow soln2)$ is an abbreviation for $(soln1 + C \Rightarrow C + soln2)$ where C is a catalyst
   - A catalyst can also represent a reaction rather than a molecule alone. For example $(soln1 = [soln3 \Rightarrow soln4] \Rightarrow soln2)$ is a shortcut for $(soln3 + soln1 \Rightarrow soln4 + soln2)$
   - At last, reversible reactions can be written as either $(soln1 \Leftrightarrow soln2)$ or in the catalysed form $(soln1 \leftarrow [C] \rightarrow soln2)$, in both cases there are two reactions in which $soln1$ produces $soln2$ and $soln2$ produces $soln1$.

4. Patterns
   Patterns allow one to write many reactions in a simple equation for declaring objects using variables prefixed by the $ sign. For example : if we have "declare COM parts-of ({p1,p2})", then COM$p can take 4 possibilities as the $p can take the values : {},{p1},{p2} and {p1,p2}.

There are three main modelling methods in BIOCHAM namely : boolean, stochastic and differential equation.

a) Boolean
   In the boolean configuration, each substrate can be either consumed or not. In the example expressions below, with 3 variables, we have 8 possibilities as shown in Figure 3.26. As such, we elaborate all possibilities of the presence or absence of substrates.
   We use the following expressions to illustrate the Boolean method :

   - $c = [b] \Rightarrow a$ is represented by green arrows (Expression 1)
   - $a + b \Rightarrow b + c$ is represented by blue arrows (Expression 2)

   - We start with expression 1 where we need to have b and c as initial conditions for the expression to be true. This gives us only two valid states : abc and bc, out of the 8 states. But, we do not know if both b and c will be consumed. If we start from state bc and we consume all variables, then this will enable us to transit to state ab (b is a catalyst). If we do not consume c, then we will change to state abc. From state abc, if we consume b and c, then this will change to state ab (b is a catalyst). State abc will change to state bc if only a and b are consumed.
   - For expression 2, we need to have a and b present which gives us only two states : abc and ab. If we start from state ab and we consume all variables, then we will be left with bc only (state transitions from ab to bc). If no variable is consumed, then we will shift to state abc. Similarly for abc, if no variable is consumed, we will stay in the same state. If a is consumed, we will change to state bc, and if c is consumed, we will change to state ab.

   This gives us the state transition of the whole system. From this, we can apply CTL for the validation of biological properties. We illustrate the use of CTL in Figure 3.26 with two simple examples:

   (a) $(b \geq 1) \wedge (\mathrm{c} \geq 1) \Rightarrow \mathrm{EF}\ (\neg b \wedge \neg c \wedge \neg a)$; Is there a situation where we start with b and c, and end up with nothing ?
   (b) $(a \geq 1\ ) \wedge (c \geq 1) \Rightarrow \mathrm{AG}((a \geq 1) \wedge (c \geq 1))$; If a and c are present, do they always remain present ?

   However, in the metabolic regulatory network, there are some variables (like ATP which has 3 possible values) that goes above the boolean semantics. This makes the boolean approach inappropriate for our study firstly, because it is limited to only two possible values and secondly, we do not look at metabolic reactions in our model. Moreover, there is the notion of consumption which is not considered in our metabolic network (for example we do not interpret Krebs producing NADH as a reaction consuming Krebs at the same time). Moreover, in BIOCHAM, boolean networks can

rapidly become large and complex when the number of variables increases.

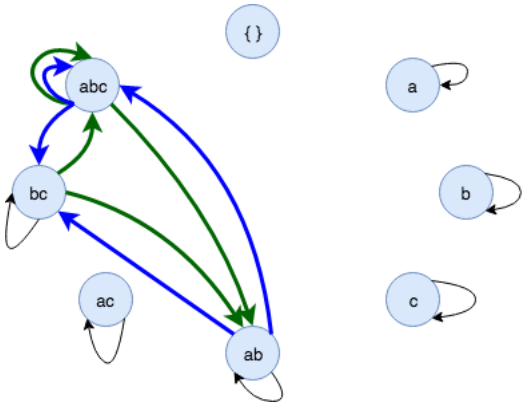


Figure 3.26: BIOCHAM : Boolean method. 3 variables generating 8 possibilities

b) Stochastic
In the stochastic semantics, rules are interpreted as a continuous time Markov chain where transition probabilities are defined by the kinetic expressions of the reaction rules [106]. An integer is assigned to each object, representing the number of molecules in the system. This number is the product of the concentration C of the object, the volume V of the location and the Avogadro's number, K.

$N = C$ x V x K

These transition probabilities are difficult to interpret in the large regulatory network of energy metabolism and as such we will not use this stochastic approach.

c) Differential Equation
Using the differential equation method, reactions are given kinetic expressions. For instance, k*[A]*[B] for A=[B]=>A $\sim$ $\{p\}$ specifies a mass action law kinetics with parameter k for the reaction. Classical kinetic expressions are the mass action law. Using these, BIOCHAM automatically builds, from the syntactic rules, a system of differential equations which are normally difficult to solve but, at least they allow us to simulate the system. In the differential equation semantics, we observe the change in concentration of a given metabolite with respect to time.

But, we have already explained why modelling using differential equations does not answer our biological questions (section 3.3) : difficulty to obtain real parameters and especially we wanted to have a computer-aided tool with logical reasoning capacities.

3.8.2 Kappa

Another rule-based language that has attracted attention in modelling biological networks is *Kappa* [107]. Like BIOCHAM, it works at the molecular level, modelling biochemical reactions as rules. The main difference is that BIOCHAM is based on rules that manipulate *term patterns*, while *Kappa*-rules manipulate *graph patterns*. This induces rather sophisticated soundness constraints that must be followed by any system of *Kappa*-rules. Thus, we shall not explain the syntax of *Kappa* here, as it would be far too long for a language that we will not use in the sequel. A feature of *Kappa*, which probably takes part of its visibility, is the ability to represent rules using abstract pictures of molecules and their affinities. Such a symbolic representation of a system of *Kappa* rules makes it "apparently" intuitive for biologists. Nevertheless, in practice, a deep expertise is necessary to properly write a set of biochemical reactions.

In full *Kappa*, rules can be equipped with rate constants and the graph rewriting process is stochastic. *Kappa* is designed in such a way that many properties can be established formally, and the main advantage of *Kappa* is precisely to facilitate formal reasoning. *Kappa* is resolutely molecule oriented, graph patterns representing the molecule affinities and properties. The power of *Kappa* is the way it

deals with the combinatorial complexity, which is among the main challenges in modelling complex networks. If one does not want to abstract molecular processes in the cell, then there is the possibility to abstract behaviours and make them "emerge" from a huge number of such elementary molecular processes.

So, *Kappa* is probably the most deeply formal framework dedicated to modelling molecular processes in the cell. But paradoxically, it is far too concrete with respect to our motivation to understand the main causality mechanisms in cell metabolism regulation because, it is dedicated to rules at the molecular level.

According to this important consideration, BIOCHAM would be less “tied” to concrete molecular reactions, as the terms used in BIOCHAM can represent arbitrarily abstract concepts. It remains that BIOCHAM is oriented to the modelling of signaling reactions rather that regulation mechanisms.

3.9 Conclusion of the chapter

An array of rigorous techniques for modelling biological networks has been outlined in this chapter with a particular attention to the strengths and weaknesses of each framework. The choice between qualitative and quantitative formalisms resides in the biological question under consideration. These modelling frameworks are representation of the main possible approaches and they are complementary to each other. The main weakness of the frameworks described here is that metabolic *regulations* are neglected and seen as side phenomena.

Interestingly, some of the criteria we would be looking forward to for our choice of formalism include a high degree of abstraction for regulation of metabolism with a selection of important variables supporting this regulation. Our main motive is to understand the underlying causalities in metabolic regulations and we want to be able to use a computer-aided approach to study these causalities. So, formal methods are clearly suitable because logic and formal methods can establish proofs using software tools. The choice of our framework will be explained in the next chapter.

CHAPTER 4

THE THOMAS MODELLING FRAMEWORK

4.1 Introduction

In the late 1990's, formalisms based on differential equation were de-facto the main methods used in the modelling of complex biological systems [109] : often, biological experiments based on concentration level measurements reflect changes of certain molecular species along time (like gene activity via one of its products). Such quantitative dynamics, where several variables interact to produce curves of real numbers in function of time are often captured in systems of ordinary differential equation (ODE) in a rather natural way. The representation of temporal aspects of systems (rate of change of physical quantities) in biology, and as a matter of fact in many other complex systems, is one of the most frequent application of ODEs. The real strong difficulties begin when we try to identify the ODE parameters. Moreover, biological systems are often non-deterministic ones, but ODE only encode deterministic trajectories, due to a hypothesis of "large number of molecules" that justifies the *continuous* setting of ODE. In molecular biology, we cannot ignore a common feature that biological complex systems exhibit : their non-deterministic behaviours.

This chapter enlists some motivations of Réné Thomas in the search of a more simplistic discrete approach to the modelling of gene networks for the study of their dynamics. After a rigorous definition of the formalism, we will see how other researchers have integrated useful features from computer science adding more power to his formalism. Later on in chapters 7 and 8, we will show how the Thomas' modelling framework scales well for our metabolic network with 13 variables and 100 parameters, representing, up to our knowledge, the largest network modelled using this framework.

4.1.1 Motivation

Finding kinetic parameters is a major bottleneck in the study of dynamics of complex systems. ODEs are often too precise, with parameters belonging real numbers, to be used in cellular biology, due to insufficient precise measurement capabilities. Moreover, there are many large intervals of values where any change of parameters in the differential equation does not modify the observable end result.

To gain insight into the interaction and regulation of biological systems, Réné Thomas proposed a boolean framework that is close enough to actual biological regulatory networks, which manifest themselves in a *qualitative* manner. In this context, an object is either activated or inhibited. In genetics, this corresponds to an ON/OFF state of genes which can occur during gene expressions or at the level of transcription. The motive of the next subsection is to detail how Réné Thomas achieved his boolean approach in a manner that can be formally seen as a slight extension of boolean networks described in the previous chapter. Then, the approach was later mathematically defined and extended by Houssine Snoussi [110]. Here, we directly define the Snoussi version with formal extension.

4.1.2 A variant of automata networks

Living cells are in a state of ceaseless activity. The way organisms are constructed, how they function, how they react to external environment and more generally their behaviour, are partly monitored by genes. These controls follow a series of activations and inhibitions, which were previously modelled in ODE as sigmoids. This is nicely captured in Figure 4.1, where an activation is translated into an increasing sigmoid (green curve) and an inhibition into a decreasing sigmoid (red curve). Since we are

interested with the two areas in which, the gene is respectively active or inactive, in discrete modelling, this can suitably be represented as a '< 1 '(to mean inactive state) and a '≥ 1'(to mean active state). This amounts to an approximation of a sigmoid into a step function.

In this chapter, we will see the two major steps involved in the classical R.Thomas framework namely: the static representation of the interactions between the biological entities (eg genes) and their dynamics expressed via kinetic parameters.

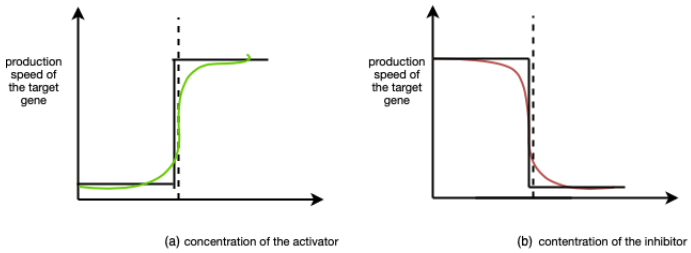


Figure 4.1: Discretisation of sigmoids (a) Activation (b) Inhibition

4.2 Interaction graph

In the Thomas' framework, the interactions between genes (or more generally biological entities) are encoded in a directed graph.

Definition 4.2.1. Directed graph − A directed graph, G, is a couple (V, E), where:

- V represents the set of vertices $\{v_1, v_2, .., v_n\}$
- $E \subset V$ x V is the set of directed edges $\{e_1, e_2, .., e_m\}$ between the vertices.

An interaction graph is a directed graph together with additional annotations: one need to add labels on the edges in order to represent the sets of interactions of each biological entity with others (including itself). For example Figure 4.2 shows the interactions between two boolean variables x and y. We will use this toy example throughout this chapter.

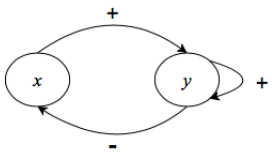


Figure 4.2: Interaction graph between two variables x and y and three edges : (x, y), (y, x) and (y, y) . The labels mean activation $(+)$ or inhibition $(-)$.

In an interaction graph, an edge from $v1$ to $v2$ means that $v1$ (or one of its products) has an influence on $v2$ above a certain concentration. In Figure 4.2, x has an influence on y, y on x and y over itself. The influence can be either an activation ($+$ sign on edge) or an inhibition ($-$ sign on edge).

4.2.1 Thresholds and outgoing edges

In a directed interaction graph, the direction of the edges provides useful information on the influences. For a particular variable v, all its outgoing edges (in graph terminology, the number of outgoing edges is called the *out-degree* of v and we denote it as d_v^+) are synonymous to the influences it has on other variables in the interaction graph. Each influence will occur if the variable v goes above a certain discrete concentration level which is called the *threshold* and is denoted by s in this text. This threshold value is the inflexion point that separates intervals in the discretisation of sigmoids in ODE. If the influence of v is an activation, then the corresponding successors will be more easily expressed when v reaches this threshold otherwise this will possibly reduce the expression of the successors. If the influence of v is an inhibition, then when v is absent (has a too low level of concentration), then this will allow the corresponding successors to be more easily expressed. The threshold for each influence is not necessarily the same, see Figure 4.3. Each inflexion point separates two intervals, which means that the threshold for

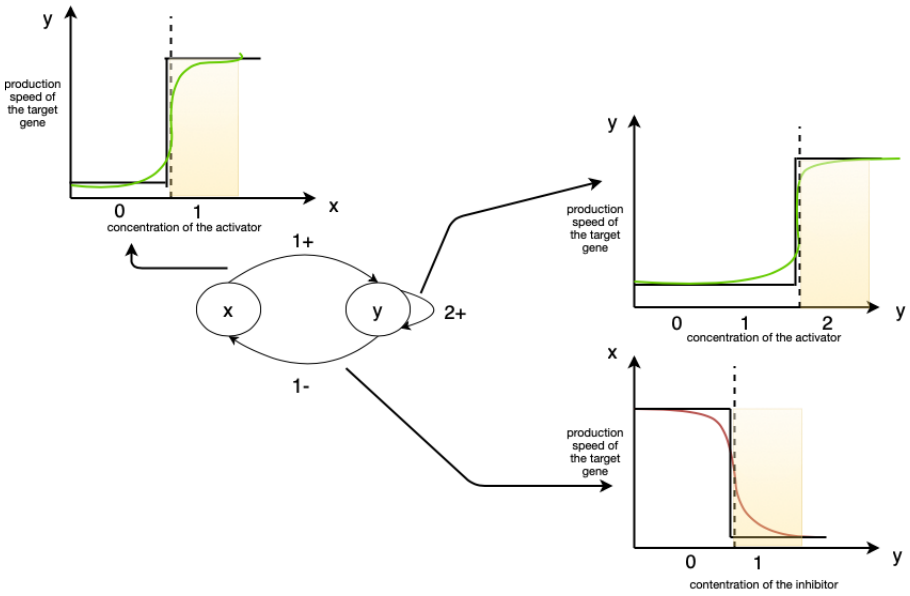


Figure 4.3: The interaction graph of x and y with thresholds, and how their sigmoids are discretised. Here, we assumed that the threshold of action of y on x is lower than the one of the action of y on itself. So, the edge $y \to x$ is labelled by a "1" and the edge $y \to y$ is labelled by a "2'.

the variable v is less than or equal to its out-degree (it is strictly lower when two or more inflexion points are equal). So, if the number of successors for v is equal to 1, then the threshold for v is necessarily 1.

NOTATION 1.1: The domain for a variable v is the discrete domain $[0, b_v]$ where b_v is called the boundary and is the maximum integer threshold value of v over its successors. So each threshold, s, lies in $1 \leq s \leq b_v$. It is important to note that the integer thresholds are *qualitative* representations of the real value of the inflexion point required to allow an action to take place. As such, it is not a quantitative value.

NOTATION 1.2: If the action of a given variable $v1$ over $v2$ is an activation, then the edge $v1 \to v2$ can be labelled with "+" and "s", or equivalently but more formally it can be labelled with $v1 \geq s$, else if it is an inhibition, then it can be labelled with "-" and "s", or equivalently but more formally it can be written as $v1 < s$. To be in conformity with the activation, the inhibition is written as $\neg(v1 \geq s)$ ($\neg$ means inhibition).

Definition 4.2.2. Labelled interaction graph − A labelled interaction graph is defined by $\sum = (V, E)$ where:

- V, is the set of all vertices $(v_1, v_2, .., v_n)$ in the graph and each vertex v_i has a boundary $[0, b_{v_i}]$.
- E, is the set of all edges $(e_1, e_2, .., e_m)$ in the graph and each edge e_i is labelled with a logical formula between two vertices of the form $v \geq s$ for activations and $\neg\ (v \geq s)$ for inhibitions ($s \in [0, b_v]$).

In Figure 4.2, x has an out-degree of 1 and y an out-degree of 2.
Let us assume that following some biological knowledge, we know that y needs a greater concentration level to activate itself than inhibits x. Then, these information is translated into the following: the activation of x over y is transformed into $x \geq 1$ (it has only one outgoing edge), the inhibition of y over x into $\neg\ (y \geq 1)$ and finally the activation of y over itself as $y \geq 2$. All these notions allow us to formalise the Figure 4.2 into Figure 4.4.

4.2.2 Incoming edges and parameters

The number of incoming edges of a variable v (in graph terminology, it is called the *in-degree* and we denote it as d_v^-), represents the number of predecessors influencing it, either individually or collectively.

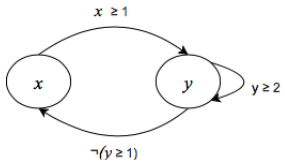


Figure 4.4: Labelled interaction graph with thresholds on the edges. Alternatively, $(x \geq 1)$ can be represented as 1+ or simply +, $(y \geq 2)$ as 2+ and $\neg\ (y \geq 1)$ as $(1-)$

If a variable v has $p_1, p_2, .., p_n$ predecessors, then it can be influenced by any subset of these predecessors. Since each predecessor can be present or absent, this gives rise to a family of $2^{in\_deg_v}$ such possible subsets.

In the Thomas approach, we prefer to keep track of the *positive* influences that a variable v receives a given time. So, we encode these subsets as follows:

- if the expression level of an *activator* p_i is *greater or equal* to its threshold s_i, then p_i belongs to the subset, and if it does not, then p_i does not belong to the subset.
- if the expression level of an *inhibitor* p_i is *strictly lower* to its threshold s_i, then p_i belongs to the subset, and if the expression level of p_i is greater or equal to s_i, then p_i does not belong to the subset.

We note ω this subset at a given time and we call it "the set of resources of v".

These subsets of predecessors define the set of parameters of the models as follows : when v has ω as resources, the expression level of v is *attracted* toward a given value that only depends on ω. We note $K_{v,\omega}$ this value , which will belong to the integer interval [0,b_v].
For our toy example:

- for x, the in-degree is 1, giving rise to 2^1 parameters $\{K_{x,\{\}}, K_{x,\{y\}}\}$
- for y, the in-degree is 2, giving rise to 2^2 parameters $\{K_{y,\{\}}, K_{y,\{x\}}, K_{y,\{y\}}, K_{y,\{x,y\}}\}$

So, for this small regulation network between x and y, we have to find 6 ($2^1 + 2^2$) parameter values. Using our toy example, if we are in state $\eta = (x, y) = (1,0)$, then the parameter for x is $K_{x,\{y\}}$ since the predecessor of x is only y and the edge $y \to x$ is labelled with $\neg\ (y \geq 0)$ which is true in this state. The parameter for y is $K_{y,\{x\}}$ since the edge $x \to y$ is labelled with $x \geq 1$ and this is also true in this state.

4.3 Dynamics in a biological regulatory graph

Once, we have all the information on the variables (in terms of their interactions, thresholds and finally their parameters), the next step is to see how to model the dynamics of the network. In other words, what are the values of the K parameters that will allow the network to change states over time in a way compatible with biological knowledge.

So, one needs firstly to define the notion of *state* of a system. A state of a system simply defines the current value of each variable. For example, according to Figure 4.3, x can have values 0 or 1 and y can have values 0, 1 or 2. Thus, there are 6 possible states: (0,0), (0,1), (0,2), (1,0), (1,1) and (1,2), as represented in Figure 4.5, where each state is a square. Representing states as squares (cube if there are 3 variables, hypercubes if more) is consistent with the fact that our integer values represent *intervals* (between inflexion points).

Definition 4.3.1. (State) $-$: A state η of an interaction graph is a function that associates to every variable v in V a value in $[0, b_v]$. We denote ζ as the set of all possible states of the system and η_v as the value of v in state η.

The state transition graph shows how a system transits from one state to another and which variable changes values.
To analyse the dynamic behaviour of a system, we must first answer two questions:

1. how to characterise a change of state ?

2. how to attribute a new value for a given variable that has changed state ?

The changes of a state must be *asynchronous* in the Thomas framework (see Figure 4.5):

- in synchronous mode, several variables can change values at the same time. But, in biological regulatory systems, the probability that several variables change values simultaneously is negligible *in vivo*. So, we ask transitions to modify only one variable at a time.
- in asynchronous mode, only one variable is allowed to change values at a given time. Réné Thomas uses this transition type to model the dynamics of regulatory network.

Assuming for example that, when $(x,y) = (0,1)$, the variable x is attracted toward $K_{x,\omega_x}=1$ and y is attracted towards $K_{y,\omega_y}=2$. The Thomas approach *does not allow* the diagonal transition from (0,1) to (1,2) (left part of Figure 4.5). Moreover, because we do not know if x or y will reach the real threshold first, the Thomas approach allows both transitions in a non-deterministic manner (right part of Figure 4.5).

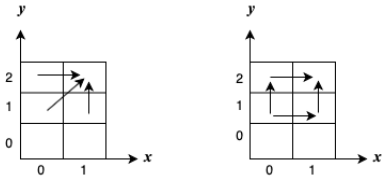


Figure 4.5: State transition type (i) Synchronous: x and y in state (1,0) change values at the same time (ii) Asynchronous: Desynchronisation of state (1,0) to allow either x or y to change values. The Thomas approach is asynchronous.

When we say a variable is attracted towards a certain value for a given set of resources, three obstacles are imposed on that "attraction" which means that it may or may not go in that direction. We explain this with Figure 4.6 as follows:

- we can modify only one value of a variable at a time in the current state; that is transitions are asynchronous. This reflects the non-deterministic behaviour of variables in biological systems as we cannot predict which variable is going to change first.

  The probability of crossing one of the three surfaces where only one variable changes is 1 which explains the change of only one value for a variable. A simultaneous change of two values means we are going to reach the intersection of at least 2 surfaces and the probability of attaining this set of points is 0. The probability is null because the surface of all the points is null. Thus, we ignore this possibility.

- since we perform a discrete simulation of continuous change in concentration values, we are not going to leap over several values at any moment. Therefore, the next state must belong to one of the neighbouring cubes; any change in one of the variables will proceed by only one unit at a time for modelling a continuous phenomenon.

- when we shift from one cube to another, we go above a certain threshold and consequently, the set of resources may change and thereby affect the set of corresponding K parameters. As a result, the value towards which we were initially attracted may change direction in due course.

Unfortunately, we can visualise the state transition for a maximum of 3 variables only. When a regulatory network contains more than 3 variables, it is more difficult to visualise the state transition graph. This is where we find it interesting in constructing a software platform for verification and validation purposes called DyMBioNet, as we will demonstrate in Chapter 6 (section 6.1).

4.3.1 Identifying the parameters eligible for resources

For a given state $\eta \in \zeta$, and a variable $v \in V$, v can change state under one of the following conditions:

1. First of all, η being given, we have the state of all the predecessors p_i of v. Thus, we can determine the set ω of resources of v.

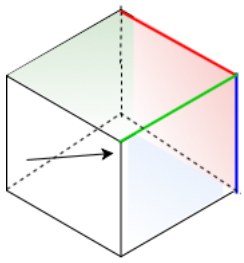


Figure 4.6: If the global K vector at the state represented here by a cube (3 variables) attracts all points in the direction given by the black arrow then all trajectories will cross one of the three grey surfaces of the cube. The three coloured edges of the cube show the places where continuous trajectories would modify several discrete variables at the same time. The union of the three edges is a set of points of surface 0. The probability of crossing them in the cube is 0.

- if p_i is an activator of v and $\eta_{p_i} \geq s_i$ then $p_i \in \omega$
- if p_i is an inhibitor of v and $\eta_{p_i} < s_i$ then $p_i \in \omega$
- else $p_i \notin \omega$

Consequently, we can look at the value of the parameter $K_{v,\omega}$.

2. if $K_{v,\omega} > \eta_v$, then there is a transition $\eta \to \eta^{'}$ where $\eta^{'}{}_x = \eta_x$ except in v where $\eta^{'} = \eta_v + 1$ (asynchrony).
3. if $K_{v,\omega} < \eta_v$, then there is a transition $\eta \to \eta^{'}$ where $\eta^{'}{}_x = \eta_x$ except in v where $\eta^{'} = \eta_v - 1$.

This calls for some comments:

- If several variables satisfy condition 2 or condition 3 for a given state η, then there are several transitions starting from η to $\eta^{'}$ (non-determinism).
- Transitions are limited to modify only *one* variable at a time ($\eta^{'}$ coincides with η except for one variable). This is the asynchronous approach of R.Thomas.
- $\eta^{'}$ is not directly equal to $K_{v,\omega}$. It is only equal to (η_v + 1) if $K_{v,\omega} > \eta_v$ and (η_v - 1) if $K_{v,\omega} < \eta_v$. This reflect the fact that the production of a gene v (or the degradation of its product) is a continuous phenomena, thus one cannot jump several values : $\eta^{'}_v$ goes toward $K_{v,\omega}$ step by step.

In this way, we can generate the total number of possible parameter values for the whole system by using the formula: $\prod_v (d_v^+ + 1)^{2^{d_v^-}}$ for $v \in V$. For the toy example, the total number of dynamics is (2^{2^1}) x (3^{2^2}) = 432.

4.3.2 The notion of multiplexes

Often, knowledge from the biologists allow us to simplify the biological network and consequently reducing the number of parameters, and the number of dynamics. The dynamic behaviour of a variable (vertex) depends on the number of predecessors (n) it has in the directed graph and it is controlled by a family of parameters that contains 2^n parameters, where n is the in-degree of the vertex. In order to reduce the complexity of a system, a very efficient approach is to minimise the number of predecessors for a variable. Consequently, this will reduce the number of resources and the number of kinetic parameters. For example, reducing the in-degree of a variable by 1, divides the number of its parameters by 2.

Let us illustrate this with an example with four variables as in Figure 4.7. In this system, the variable x has three possible influences on it: a and b as activators, and c as inhibitor. In the classical Thomas-Snoussi framework, this context will generate 2^3 parameters for x, one for each subset of predecessors, as follows: $\{\}, a, b, c, ab, ac, bc$, and abc.

We now assume that from some biological knowledge, objects a and b collectively act on x and that the absence of one paralyses the other object (e.g because their proteins must make a complex a-b before

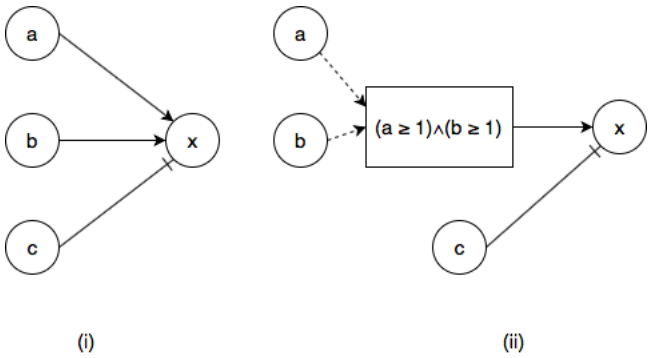


Figure 4.7: Three variables acting on x. If we know that the two variables a and b need the other one to act on x, we group them in a multiplex (right).

acting on x). There are no biological reasons to include any parameter where we have either a or b alone. Another possibility is when we know that a or b alone is sufficient to act on x. If a does not need b to act on x and the same is true for b, then, this can be represented by the logical formula $(a \geq 1) \vee (b \geq 1)$. Using these kind of reasonings, we can largely reduce the number of influences on a variable. An extension of the classical Thomas framework has been made in this direction to regroup collaborative variables ; a and b in this case.

Bernot et al [**?**] proposed the notion of *multiplex*, m, to regroup the coupling actions of a set of resources by a logical formula, ϕ. In this example, we will have the conjunction of a and b in the multiplex $(a \geq 1 \wedge b \geq 1)$ in Figure 4.7(ii)). This technique minimises the number of parameters as we are now limited to only two resources: c and the multiplex. This results in x having four parameters: $\{\}$, c, $multiplex$, $\{c, multiplex\}$. To have a clearer picture, multiplexes will be assigned a meaningful name as shown in Figure 4.8. Logical formulas are not only limited to conjunction of variables: a mixture of other connectives ($\neg$ for negation, $\vee$ for disjunction) are available to build complex formulas.

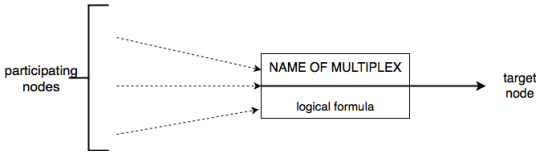


Figure 4.8: Multiplex notation: dotted arrows are used for variables involved in the logical formula with the name of the multiplex in block letters

Multiplexes can only be *satisfied* or *not satisfied* based on their logical formula. This means that the status of a multiplex is always evaluated to a truth value immediately. A multiplex *is not* a variable and will *not* be asynchronously updated based on its resources.

Remark: We no longer have *activation* or *inhibition* when we use multiplexes. These regulations are encoded in a logical formula. A formula prefixed with a negation means inhibition. Activation formula will normally follow a "$\geq$" sign and an inhibition will be assigned a "$\neg$" sign such as $\neg(c \geq 1)$ (we do not use $c < 1$ in order to make inhibitions explicit with a negation sign). Figure 4.9 shows how Figure 4.7(ii) is formally encoded with multiplexes.

Definition 4.3.2. (Languages describing formulas for multiplexes of $(V \cup M)$) - Given V as the set of variables v in discrete domains $[0, b_v]$ and M the set of multiplex name. The language describing the multiplex formulas of $(V \cup M)$ is inductively defined by:

- the atoms are the identifiers of M and the atomic formulas of the form $(v \geq s)$ with $v \in V$ and $s \in [1, b_v]$
- the formulas are of the form $\neg\phi$, $\phi \vee \psi$ or $\phi \vee \psi$ where ϕ and ψ are formulas.

Definition 4.3.3. Interaction graph with multiplexes − An interaction graph with multiplexes is defined by $\sum_m = (V \cup M, E)$, where $V \cup M$ represents the set of vertices of the graph and E is the set of the edges, with:

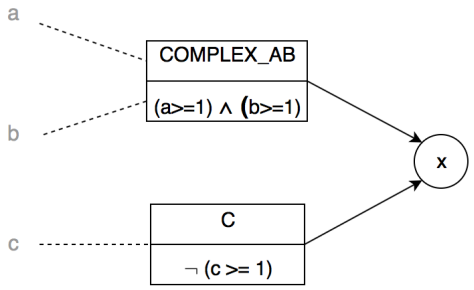


Figure 4.9: Multiplexes with logical formulas and names: multiplex C is prefixed with a negation meaning it is an inhibition. The multiplex $COMPLEX\_AB$ indicates that a and b are activators.

- V, the set of variables v in the discrete domain $[0,b_v]$,
- M, the set of multiplexes m with formulas ϕ_m, where ϕ_m are formulas on $(V \cup M)$
- $A \subset M$ x V
- There must be no cycle in the graph that contains only multiplexes (in particular, ϕ_m, the formula of the multiplex, cannot contain the name m itself).

Using Definitions 4.3.2 and 4.3.3, we transform the interaction graph of Figure 4.4 into the interaction graph with multiplexes in Figure 4.10. From the Definition 4.2.1, the edges link the variables and they are labelled with the atomic formulas. Now, the edges are from multiplexes towards the variables (solid lines) but the edges from variables to multiplexes are dotted lines as the variables are already included in the multiplex formulas (which means the variables form the predecessors of the multiplex). We add the dotted lines to see the predecessors and at the same time it is easier for the biologists. For example, if we consider the following formula $(v_1 \geq 1) \wedge (v_2 \geq 1)$ of the multiplex m, we can directly interpret that v_1 and v_2 are the predecessors of m.

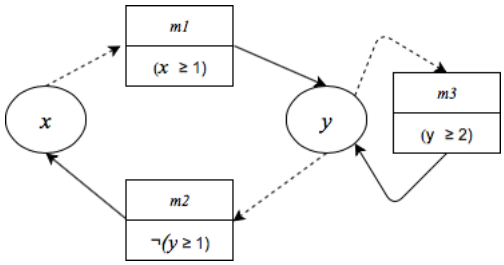


Figure 4.10: An interaction graph with multiplexes as resources. ¬ means inhibition. Doted lines are only drawn to facilitate the global view: They can be deduced from the multiplex formulas.

There are few design principles about the choice of multiplexes that must be considered when designing biological regulatory networks:

1. Consider Figure 4.11. We have two activators x and y acting on z, which are replaced by their corresponding multiplexes, for example, $m1(x \geq 1)$ for x on z and $m2(y \geq 1)$ for y on z. If, from some biological knowledge, we know that x and y are dependent on each other to act on z (for example they form a complex which act on z), then the multiplexes $m1$ and $m2$ must be regrouped into only one multiplex to get $(x \geq 1) \wedge (y \geq 1)$ to encode the cooperative reaction of x and y. This has two advantages: first, it reduces the number of resources on z and second, reduces the number of K parameters for z (see Figure 4.11).

2. It may happen that the multiplex can act both as an activator and an inhibitor at the same time but on different variables (see Figure 4.12). This is an exceptional case in which we must formally have have two versions of the multiplex with two different logical formulas (φ for activation and $\neg\varphi$ for inhibition). In the DyMBioNet software, the two multiplexes are implemented under different names. However, when drawing the interaction graph, we use only one name and the outgoing edges from the multiplex are labelled with a sign to indicate activation $(+)$ or inhibition $(-)$.

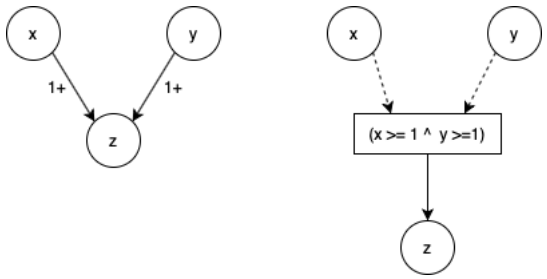


Figure 4.11: The individual reaction of x over z and y over z are merged into a multiplex with a logical formula.

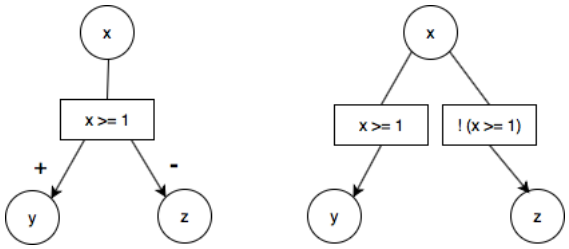


Figure 4.12: Separate multiplexes are incorporated in the regulatory graph: one for activation and one for inhibition (prefixed by a $\neg$ sign)

So, the overall objective is the repartitioning of $\neg, \wedge$ and $\vee$ in the logical formulas for each atom, to illustrate the type of cooperation.
However, there are certain rules that must be adopted when implementing multiplexes:

- a multiplex can have one atom ($x \geq 1$) or several (like $(x \geq 2) \wedge (y \geq 1)$) for implementing cooperation separated by $\wedge$ or $\vee$
- if ever there is a variable acting only as "relay" between two other variables, and if it does not have any other significance both in the design and action, then it can be converted simply as a multiplex in which we put the type of action in terms of a logical formula. This must be validated by the biologist. In Figure 4.13, z is replaced by a multiplex.

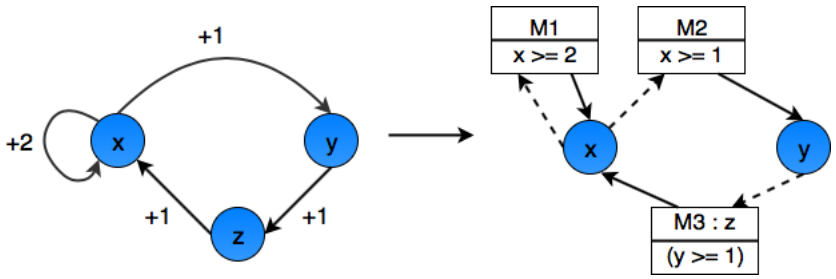


Figure 4.13: Interaction graph between x, y and z: y activates z which in turn activates x. This clearly means that y indirectly activates x. If z does not have any other biological relevance in the graph (and it does not influence other variables), it can be replaced simply by a multiplex ($M3$). For sure, y could have been chosen instead of z and the choice of multiplex vs variable is a matter of modelling choice.

4.3.3 Formal definition of a dynamical system

A regulatory network is formally defined by integrating the kinetic parameters with the interaction graph.

Definition 4.3.4. (Regulatory network). A regulatory network $(\sum_m, \mathcal{K})$ is given by the interaction graph with multiplexes in which we associate the family of kinetic parameters $\mathcal{K} = \{K_{v,\omega} \mid v \in V$ and $\omega \subseteq {\sum_m}^{-1}(v)\}$, where ${\sum_m}^{-1}$ are the predecessors representing the set of multiplexes influencing v.

Now, with the integration of multiplexes in the interaction graph, the resources for each variable are the

multiplexes, so that $\omega \subseteq M$ for all $K_{v,\omega}$. For our toy example, the parameters for x are $\{K_{x,\{\}}, K_{x,\{m2\}}\}$ and for y, the parameters are $\{K_{y,\{\}}, K_{y,\{m1\}}, K_{y,\{m3\}}, K_{y,\{m1,m3\}}\}$.

Definition 4.3.5. (State transition graph) − A state transition graph is based on a regulatory network $(\sum_m, \mathcal{K})$ and is defined by the following:

- the set of vertices is the set of possible states ζ
- the transitions between the current state $\eta = (\eta_{v_1}, \eta_{v_2}, .., \eta_{v_n})$ and the next state $\eta' = (\eta'_{v_1}, \eta'_{v_2}, .., \eta'_{v_n})$ are such that:
  1. there exists a unique i such that $\eta_{v_i} \neq \eta'_{v_i}$; this means that only one variable can change value for each state transition. This is a called asynchronous state transition. In case, there are many variables that may change values, then there are as many transitions starting from η..
  2. given v_i such that $\eta_{v_i} \neq \eta'_{v_i}$ and that $K_{v,\omega}$, the parameter acceptable for v. We have $K_{v,\omega} \neq n_{v_i}$ and if $K_{v,\omega} > \eta_{v_i}$ then $\eta'_{v_i} = \eta_{v_i} + 1$, and if $K_{v,\omega} < \eta_{v_i}$ then $\eta'_{v_i} = \eta_{v_i} - 1$. This means the change in the value of the kinetic parameter is by a step of 1 unit.

Since the variable modifications along transitions depend on the K values, then if some parameter values are unknown, there is not only one state transition graph but there exists as many as the possible correct values of K.

4.3.4 Identification of parameters

The study of the dynamics of a network means the study of the sets of state transitions graphs, which can be very large. So, in order to be coherent with biological properties under investigation, the major difficulty lies in the identification of a subset of these state transition graphs. A challenge is to obtain a subset that is sufficiently easy to be analysed by a human being to verify credible biological hypothesis. In other words, finding this subset means interpreting the values of the K parameters.

In some cases (as we will see in Chapter 7 and 8), the biological knowledge (with some hypothesis) can be helpful in finding these kinetic parameters with a possibility to reduce the number of parameters.

Generally, the identification of kinetic parameters is a major problem in biological networks as in other complex systems. For a biological network, this depends on the:

- expertise of the biologists to extract the maximum information on the system and consequently on its dynamics (for example properties observed in experiments like oscillations, steady states, etc can be helpful for the modeller)
- applications of formal methods to use these information for finding the parameters and also to cross-check biological properties

4.4 Kinetic parameters for network dynamics

In the modelling of network dynamics, finding the values of kinetic (K) parameters that best fit experimental data and biological knowledge, is the main challenge. This is because they are difficult to measure directly experimentally and as such, we fix these values by indirect deductions from the biological knowledge about the system.

Contrarily to differential equations, we have a limited number of K parameter values that are integers within the bound of the variables. Nevertheless, they may be unknown and one tries to identify them using formal methods. These formal methods are discussed in section 4.6.

Snoussi condition

According to Snoussi [110], the set of resources, ω, has an influence on the determination of the K parameters. When there are more resources, the value of the K parameters tend to be larger (or at least not lower). For our toy example in Figure 4.3, the list of Snoussi constraints on K parameters are as follows:

– $K_{x,\{\}} \leq K_{x,\{m2\}}$, for variable x

– $K_{y,\{\}} \leq K_{y,\{m1\}} \leq K_{y,\{m1,m3\}}$ and $K_{y,\{\}} \leq K_{y,\{m3\}} \leq K_{y,\{m1,m3\}}$, for variable y

However, there is one counter-example where the condition of Snoussi is not applicable. Consider Figure 4.14 in which we have variables x and y activating z separately. If, from a certain biological experiment, we found that x and y form a complex X-Y which does not influence z, then this contradicts the Snoussi's condition since condition in which x and y are present as resources will be neglected.

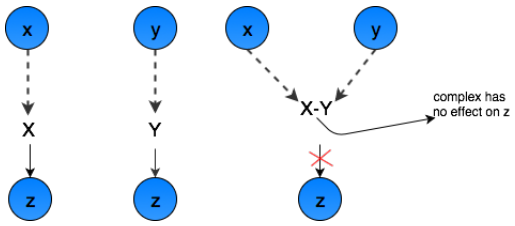


Figure 4.14: One counter-example of Snoussi's condition. x (respectively y) produces a protein X (respectively Y). Alone X or Y activates z but their products produces another complex X-Y that does not activate z. So, in presence of x, the "activator" y appears to be an inhibitor because it captures the activator X without activating z.

Remarks: There are some cases where we can have resources that are contradictory. We explain the term *"Contradictory"* with an example.

Consider Figure 4.15, where we have three multiplexes $M1$, $M2$ and $M3$ with the formulas $x \geq 1$, $\neg\ (x \geq 1)$ and $\neg\ (x \geq 1) \wedge (y \geq 2)$ respectively. This means that the variable z has 2^3 resources and a priori 2^3 parameters. The set of resources for z are as follows: $\{\}$, $M1$, $M2$, $M3$, $\{M1, M2\}$, $\{M1, M3\}$, $\{M2, M3\}$ and $\{M1, M2, M3\}$.

- if we take $M1$ and $M2$ as resources for example. $x \geq 1$ in $M2$ is the negation of the formula in $M1$. So, they both cannot occur at the same time (we cannot say the formula is true in one multiplex and false in another multiplex at the same time). The same reasoning applies for $\{M1, M3\}$ and as such resources $\{M1, M2\}$ and $\{M1, M3\}$ are eliminated from the list of resources.
- if we take the example of $M2$ and $M3$ as resources. The formula $\neg\ (x \geq 1)$ in $M2$ is present also in $M3$. This means that $M3$ cannot act as a resource without $M2$, eliminating $\{M2, M3\}$ as resources.

Overall, 3 resources out of 8 are eliminated and we are left with these valid resources for z: $\{\}$, $M1$, $M2$, $M3$, $\{M2, M3\}$, for which the K values must be identified.

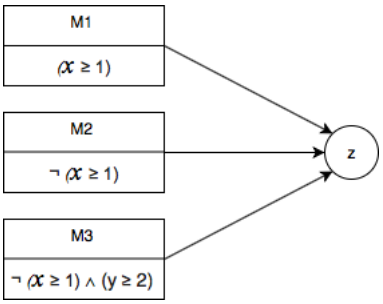


Figure 4.15: Unsatisfiability in the set of resources. $\{M1, M2\}$ and $\{M1, M3\}$ cannot be considered as resources.

4.5 Transition graph for modelling network dynamics

After the parameters have been identified, the next step is to define the dynamics of the network, using a state transition graph. To facilitate this process, we use a resource table in which we list all the resources for each variable at any state of the system.
The change in the values of a variable allows the system to transit from one state to another over

time. Using our toy example (Figure 4.10), we construct the following resource table for x and y using multiplexes.

x	y	Resources for x	Resources for y
0	0	$m2$	–
0	1	–	–
0	2	–	$m3$
1	0	$m2$	$m1$
1	1	–	$m1$
1	2	–	$m1, m3$

Table 4.1: Table of resources for x and y using multiplexes as resources from Figure 4.10.

x	y	$K_{x,\{\omega\}}$	$K_{y,\{\omega\}}$
0	0	$K_{x,\{m2\}}$=1	$K_{y,\{\}}$=0
0	1	$K_{x,\{\}}$=0	$K_{y,\{\}}$=0
0	2	$K_{x,\{\}}$=0	$K_{y,\{m3\}}$=1
1	0	$K_{x,\{m2\}}$=1	$K_{y,\{m1\}}$=1
1	1	$K_{x,\{\}}$=0	$K_{y,\{m1\}}$=1
1	2	$K_{x,\{\}}$=0	$K_{y,\{m1,m3\}}$=1 or 2

Table 4.2: Table of K parameters for x and y. x has a maximum of two K parameters and y has a maximum of 4 K parameters.

Using information from Table 4.2, we can build a state transition graph. Figure 4.16 shows the state transition graph for x and y. We follow exactly the desynchronisation principle already defined at the beginning of Section 4.3, as well as limiting transition length to 1.

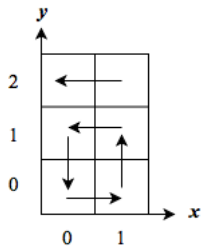


Figure 4.16: State transition graph showing the dynamics of x and y. Oscillation between x and y between their respective thresholds of 0 and 1.

4.6 Classical methods for the identification of parameters

4.6.1 The notion of cycles

We define a *cycle* as a succession of actions between variables in the interaction graph which starts from one variable and ends with the same variable without crossing twice any variable. This also includes a variable acting on itself. For example in Figure 4.3, $x \to y$ and $y \to x$ represent a cycle and the auto-activation of y is another one. In general, there are two types of cycles:

- Positive cycles
  A positive cycle is one in which there is an even number of negative actions (inhibitions). We give two examples in Figure 4.17: (i) there are two positive actions: x activates y and y activates x (ii) there are two negative actions: x inhibits y and y inhibits x. It has been proved that positive cycles are *necessary conditions* for the state transition diagram to lead to several "basins of attractions": i.e sets of states in which the system remains forever as shown in Figure 4.18.

  Notice that positive cycles are *not* sufficient to generate several basins of attractions. They are only *necessary* conditions. Parameter values introduced in Section 4.4 decide if the cycle is effective or not.

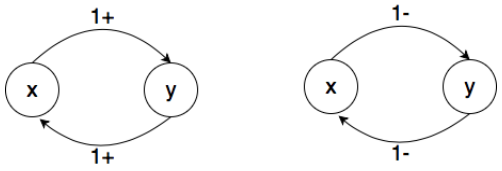


Figure 4.17: (i) Only activators (ii) Only inhibitors

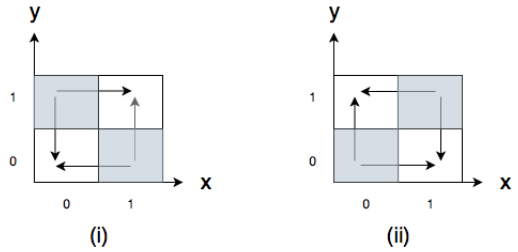


Figure 4.18: Basin of attractions (i) For (+,+) cycle (ii) For (−,−) cycle (white areas)

- Negative cycles
  A negative cycle is one in which there is an odd number of negative actions as shown in Figure 4.19. In this example , we have a negative loop where either x activates y and y inhibits x (in black) or x inhibits y and y activates x (in red). A negative circuit is a necessary condition to observe oscillations, as shown by the black arrows and red arrows.

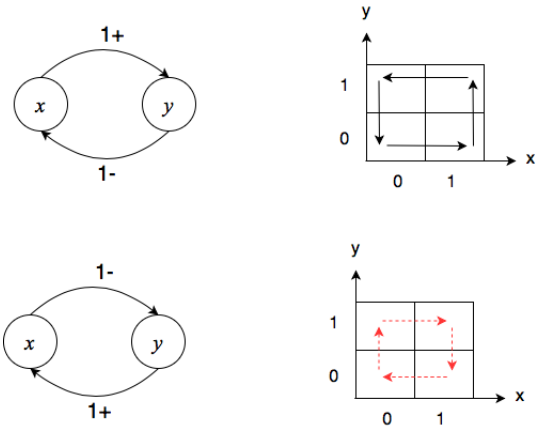


Figure 4.19: Negative circuit can generate oscillations. Black lines for x activates y and y inhibits x. Red dotted lines for x acting as inhibitor on y and y is an activator of x.

Similarly, notice that negative cycles are *not* sufficient to generate several oscillations. They are only *necessary* conditions.

In section 4.4, we gave arbitrary values to the K parameters. In fact, usually we do not know the actual values of K parameters. We should therefore start by using existing knowledge to infer a set of K parameters which is biologically relevant in the model. We can use initial notions like steady states, "bassin of attraction" to get part of "relevant" K values. These are "hand-made" techniques which are helpful at the start of the Thomas modelling framework.

More often, in biology and other similar fields, measuring the parameter values directly is impossible with wet experiments. For this reason, we revert to computer tools to find the parameter values via indirect reasoning. We check temporal properties using formal languages which perform exhaustive explorations of all possible parameter values. Several techniques are available and we briefly discuss here three of them which are popular in the study of regulatory networks: CTL, Hoare logic and constraint solving.

4.6.2 CTL

This classical logic is insufficient to model the non-deterministic properties of Thomas's regulatory networks. Starting from an initial state, t, the tree-like structure that is generated by the non-deterministic

possibilities that exists for transiting between states better captures this non-deterministic characteristic. Going from one state to another can have several possibilities based on the set of resources (and the values of the K parameters) at that particular moment. There are certain biological properties that are observable *in-vitro* or *in-vivo* over time for example, the oscillatory properties of certain biological entity, the occurence of one event following another one and so on, and these can be easily captured using temporal logic.

Many temporal logic variants are available (LTL, CTL, CTL* and so on) and we will use CTL because it is a suitably branching time logic with efficient model checking capabilities [114, 115, 116]. As shown in Chapter 3, in addition to using propositional logic for expressing biological properties, CTL always uses two additional operators: a quantifier operator followed by a temporal operator. By giving certain initial condition(s) and the property or list of properties we want to observe (written in a well-formed CTL formula), SMBioNet enumerates all the K parameter values which satisfy these temporal specifications. This is handled properly via model checkers like NuSMV.

The verification of these CTL properties is done using a software platform which helps the modeller to give as input a well-formed CTL formula (and the state transition graph) and returns whether the formula is satisfied or not. Using our parameter identification tool SMBIoNet (in which model checking is integrated), SMBioNet takes as input the CTL formula and traces systematically all the credible K parameters which satisfies the given CTL property. If some K parameters are known, we specify them in the software and we let the model checker enumerates the set of remaining missing K parameters.

For our toy example of Figure 4.3, let us suppose we do not know the K parameters for x and y, and there is some observation that x and y oscillate. This additional knowledge of oscillations allows us to translate it into the CTL formula: $(x = 0 \Rightarrow AF\ (x = 1)) \wedge ((x = 1 \Rightarrow AF\ (x = 0))$. SMBioNet uses this formula and the boundary of each variable ([0,1] for x and [0,1] for y) to enumerate all K parameters that will satisfy this CTL formula.

In Figure 4.3, if the biologists have observed that x and y oscillate when $y < 2$, then we can translate this knowledge in CTL :

$(y = 0 \Rightarrow \mathrm{AF}(y = 1)$

$(y = 1 \Rightarrow \mathrm{AF}(y = 0)$

If the conditions of Snoussi are verified, then SMBioNet determine the following K parameters:

For x :

$K_x = 0$

$K_{x,y} = 1$

For y :

$K_y = 0$

$K_{y,x} = 1, 2$

$K_{y,y} = 0, 1, 2$

$K_{y,xy} = 1, 2$

And this coherent with Table 4.2 for example.

We have use CTL extensively to enumerate all the 100 K parameters for our metabolic model enriched by a bibliographic knowledge from biological literature.

4.6.3 Hoare Logic

Here, we discuss a different approach from computer science based on Hoare logic and its associated weakest precondition calculus that generates constraints on these parameters. Hoare logic was initiated by Flyod and Hoare [117], to provide a proof system for imperative program correctness. A program is considered **correct** if it produces the expected output based on the inputs. In [112], Bernot et al showed that this proof technique, if "genetically modified", can find interesting applications in systems biology.

Most of the time, biological experiments (with certain initial conditions) offer the possibility for molecular biologists to observe some traces (transcriptomic or proteomic levels) of the system with a certain expected observable outputs. This scenario is almost identical to extracting programs from experiments. A computer program takes a certain input(s), processes the inputs to produce a given set of output(s). Using expected properties of the outputs, if we go backward by using the changes in the variables during the program, we can deduce the preconditions before the program executed to get these expected properties of the outputs. In the "genetically modified" Hoare logic, this process can be written as a triple:

$$\{\text{Initial conditions}\} \xrightarrow{Biological-traces} \{\text{Observed final properties}\},$$

whose equivalent is expressable mathematically as a Hoare triple [111]:

$$\{P\}\ \mathbf{S}\ \{Q\}$$

where P represents a set of preconditions, Q a set of postconditions and S the set of traces during the experiment.

Hoare logic avoids building the complete state graph by working only on the traces, the observable final state and putting constraints on K parameters. In doing so, it provides a fast computation time to find the set of consistent parameters. Next, we give some formal definitions helpful to write the triple properties of Hoare.

Considering any value of $v \in V$ in a regulatory network, v can evolve only in two conditions:

- v increases if $\eta_v < K_{v,\omega}$; that is $\eta_v^{'} = \eta_v + 1$ (where η_v represents the current state of v and $\eta_v^{'}$ represents the next state). In the trace, this is represented by $v+$, that is v is attracted towards a higher value.
- v decreases if $\eta_v > K_{v,\omega}$; that is $\eta_v^{'} = \eta_v$ - 1. In the trace, this is represented by $v-$, that is v is attracted towards a lower value.

Definition 4.6.1. (Trace specifications of discrete regulatory networks) − Let $N = (V, M, E, K)$ be a regulatory network. The set of trace specifications for N is inductively defined by:

- For each $v \in V$ and $n \in [0, b_v]$, the expressions $v+$, $v-$ and $v := n$ are atomic trace specifications (respectively increase, decrease or assignment to a specific value n during experiment).
- If e is an assertion for N, then the expression assert(e) is an atomic trace specification (see [112] for a complete syntax of the assertion).
- ϵ is called the empty trace.
- Several traces can be grouped sequentially together using quantifiers $\forall$ and $\exists$.

We reuse the toy example in Figure 4.4 to illustrate how Hoare logic facilitates the identification of parameters for the variables x and y. Assuming that following some experiences carried out by biologists, we found the following traces and output:

$$\left\{ \quad \right\} x+; y+; x-; y - \left\{ \begin{array}{l} x = 0 \\ y = 0 \end{array} \right\}$$

Starting from the output of $x = 0$ and $y = 0$, we proceed backward step by step using the traces to determine the initial values of x and y. Since there are 4 observations, we have four steps as follows:

- Before the last observation, y decreased ($y-$), therefore we must have: $y = 1$ and $x = 0$, and moreover (the genetically modified part) because y has decreased, $K_{y,\omega_1} < y$. At this point, $x = 0$ and $y = 1$ thus the set of resources of y is $\omega_1 = \{\}$ (because $x = 0$). So, we get $x = 0 \wedge y = 1 \wedge K_y < y$, which is equivalent to $x = 0 \wedge y = 1 \wedge K_y = 0$. We will similar explanations for the remaining three observations.
- Next, in the trace, we have $x-$ which means $y = 1$ and $x = 1$, and because x has decreased, this means $K_{x,\omega_2} < x$. At this point, $x = 1$ and $y = 1$, and the set of resources of x is $\omega = \{\}$, we get $x = 1 \wedge y = 1$ and $K_x < x$, which is equivalent to $x = 1 \wedge y = 1 \wedge K_x = 0 \wedge K_y = 0$.

- Next, in the trace, we have $y+$ which gives us $y = 0, x = 1$ and moreover since y has increased $K_{y,\omega_3} > y$. At this point, $x = 1$, $y = 0$, then the set of resources for y is {x}. So, we get $x = 1 \wedge y = 0 \wedge K_{y,\omega_3} > 0$ ($K_{y,x} > 0$).
- Last, we have $x+$ observed in the trace from which we must have $x = 0$ and $y = 0$, and moreover since x has increased $K_{x,\omega_4} > x$. At this point, with $x = 0$ and $y = 0$, the set of resources for x is ω_4={y}. So, we get $x = 0 \wedge y = 0 \wedge K_{x,y} > x$, which is equivalent to $x = 0, y = 0$ and $K_x = 1$.

Remark : We have the same results as in CTL and this is normal since we wanted to verify the oscillation of x and y.

In this thesis, we finally have been fortunate enough to identify all the 100 K parameters of our model directly from the basis of biological literature and CTL formulas. It was not necessary to use Hoare logic. This technique is suitable in the long run if there are many unknown parameters and we tag it so that we can consider it in any future endeavours. In the general case, the constraints can be more complicated and this is where the use of *Constraint solving* is important. The next section gives a small rapid flavour of what constraint solving does.

4.6.4 Constraint Solving

Constraint solving is a computer science approach to find values of variables that satisfy some relationships between variables in the form of constraints [108]. It consists of the following:

- A finite set of variables which stores the solution. For our example, this set includes $\{x, y, K_x, K_{x,y}, K_y, K_{y,x}, K_{y,y}, K_{y,xy}\}$.
- A set of discrete values known as domain for each variable. In our example, we have the following domain for the variables: $x \in \{0, 1\}$, as well as all the kinetic parameters of x, and $y \in \{0, 1, 2\}$ as well as all its kinetic parameters.
- A finite set of constraints which will help in finding the solution (in our case finding the K parameters).

Two problem solving domains where constraint solving have proved to be successful are: boolean and linear domain. In the boolean domain, a variable can be true or false. In the linear form, the relationships can be expressed as systems of equations and inequalities (for example $K_{x,y} > 0$).

4.7 Conclusion of the chapter

In this chapter, we have covered the framework of Réné Thomas well adapted for the *regulations* of biological networks with a *qualitative* vision. Our study is on the regulation of the energy and biomass metabolism for which formal qualitative frameworks are the best suited formalisms.

We have presented two versions of the Thomas's framework: the classical one (without multiplexes) and one with multiplexes (less K parameters). The name of multiplexes in the second improved version allowed an easy interpretation of the type of interactions between variables. We will see in Chapter 5 how and when we choose between a variable and a multiplex. In the discrete formalism of R.Thomas, the values of the kinetic parameters are normally small integer values for which the identification is easier than the classical differential equations (where the values are real). Nevertheless, the identification of these kinetic parameters, even if integers, represent the main difficulties in the modelling of biological networks.

With R. Thomas approach, biological networks are represented using state transition graphs and therefore benefit from formal methods. We have three formal methods: CTL, Hoare logic (modified version) and constraint programming. In all three cases, the biological knowledge from experiences are fundamental for either writing CTL formulas, translating into Hoare Triples or expressing in terms of constraints.

In the particular case of our model and the high abstraction used, all the K parameters have been identified using information from biological literature. But, we have used CTL to validate the set of values of the K parameters.

CHAPTER 5

A METHODOLOGY FOR THOMAS MODEL DESIGN

Introduction

The activities for the modelling of dynamic networks have some similarities with the activities for the conception of a software in software engineering. In software engineering, a panoply of useful methodologies (software life cycle models, rapid prototyping, Agile methodologies [119]) are used. Traditionally, to achieve good software quality, methodologies have been instrumental in the software development life cycle and for good project management for decades. For example, the V-model in software development (which is inspired from the general V-model in technology) has a set of procedures starting from requirements analysis of a software to its validation by end users. In requirements analysis, the needs of the user are collected (what is the problem that is being addressed). This gives an exhaustive description of the software expected set of functionalities. After this crucial step that defines *what* the software is supposed to do, the software design process starts in order to choose *how* the software will do. Successive steps are often followed, depending on the size of the software : from 0 to several intermediate specifications progressively introducing "how solutions", and a last detailed specification containing all the main choices. The detailed specification contains modules that facilitate a multi-team development of the software. This division of tasks allows multiple developers to work on the problem. As each module is developed, their unit testing can be done. Once this verification process is completed, modules are progressively integrated to make the whole software and integration testing is performed. At this stage, system testing is carried out to assess the global functioning and the product is delivered to the client. A final step validates all the functionalities with the initial set of objectives proposed by the user often called "functional testing". These rigorous steps make sure all the stakeholders sub products in the system life cycle are checked.

In general, a methodology can be described as a blueprint containing well-defined procedures to achieve a particular task. Many advantages exist when using a methodological approach: smooth teamwork on a collaborative project, a faster development lifecycle of a given product, reverting to any particular step in case something goes wrong, and the possibility to apply the methodology to a range of types and sizes of problems. A well chosen methodology strongly helps to build a product of good quality with respect to the user specifications, which are initially developed before designing the model. We are going to adopt a similar strategy, which starts with user requirements followed by development and testing.

We propose a full methodology in this chapter which has been successful in the design of our coarse-grained model of the regulation of the energy and biomass metabolism network. All its steps are applicable to any other network using the Thomas framework. In this methodology, we have exploited to the maximum the particularities of the Thomas' framework namely the notion of thresholds, the precise meaning of parameters, the ability to perform intensive model checking and simulations. In this chapter, we present a global view of the main steps of the methodology:

1. The opening step aims at better defining the biological problem into consideration, leading to an inventory of variables we need to make explicit. We also provide "types" to variables, depending on the biological context.

2. In step 2, we extract the minimum number of threshold values for each variable by asking the biologist the exhaustive set of targets of each variable. The variables may be boolean or multivalued, and the order between thresholds is validated with biological justifications from literature or from experimental data. This is a delicate step and sometimes requires revising the variables, and the context, to get sensible information at the proper abstract level.

3. In biology, there exist many combined regulations which can co-exist and this is the objective of step 3 where we discuss how we deal with these regulations using multiplexes. Again here, the biologist is central to be able to tell the biological importance of each multiplex and the formulas involved in it. At this stage, it stands to reason on the choice of the multiplex. Also, we decide if a variable is really needed or if it can simply be represented as a multiplex (in which case, the model spares the state of a variable, which will facilitate further studies).

4. The importance of a validation matrix, inspired from the functional testing of the final validation step in software engineering, is explained in step 4. The validation matrix is used to give the main behaviours that the model must exhibit according to biological knowledge. This matrix is designed before the identification of K parameters and as such it is constructed independently. The properties used in step 5 to identify the missing K parameters should be independent of this matrix.

5. The thorough identification of a maximum possible number of K parameters using biological knowledge for a given model is elaborated in step 5. For the remaining unknown K parameters, formal techniques, like model checking and Hoare logic, are available to complete the set of parameter values.

6. In step 6, we see the usefulness of simulations and how we get initial hints on the mathematical model and whether those observations are possible with respect to the biological model. At the same time, we can extrapolate those observations and transform them into appropriate CTL formulas which can be validated using model checking tools (further explained in Chapter 6), participating to the predictive capabilities of the model.

7. The last step of this methodology validates the model using the validation matrix. We use fair path CTL to make sure all trajectories are treated equally and are reachable. This step is independent of the feasibility study in the previous step and as such is a way of validating known properties in a time-independent manner. If validation fails, one must backtrack and a careful analysis of the possible reasons of invalidity helps to choose at which step one needs to backtrack.

The remainder of this chapter exactly follows these steps of the methodology.

5.1 Inventory of main variables

Generally, in the design of any network, we must first construct a list of basic components making the nodes of the network. This is to make sure every sensible concept is incorporated in the design process. In a typical biological network, these components can take the form of proteins, genes or biological processes, and this is highly dependent on the problem under consideration, the biological hypotheses to check and more generally the discussions we have with the biologist. One must be careful to make a choice of variables as abstract as possible, in order to better capture the main causalities in the model.

At a particular point of time, in order to gain some new insights about the dynamics of the network, we must introduce the external environment to which the network will be confronted. The integration of the environmental factors is helpful to simulate different scenarios and thus provides a more detailed view about the compatibility of the futur model with the biological system it represents. This gives a great deal of flexibility to the modeller, when validating the network in terms of which control variables can be turned ON or OFF (signalling their presence/absence in the environment; for example, the presence of oxygen to mimic aerobic respiration). These variables are called **environmental** or **input** variables. They can represent drugs, nutrients, growth factors and many alike. Input variables can also be multi-valued, for example, glucose intake can be low(0), normal(1) or in excess (2).

In this variable identification process, which is the starting point of the methodology, it may happen that if the network is large, we are obliged to categorise the variables, more precisely than as "input variables" vs "internal variables". The advantages are many-folds: each category can represent a topological interest; for example cell compartments (cytoplasm or mitochondria), biological processes or a group of proteins having similar cellular functions (for example enzymes). When tracing the interactions between these variables in a graph, we can use different colours to represent these variable types.

At the end of this step, we have an overview of all the variables and how they abstract a given biological function or molecule. The interdependency between all the variables results in an intermediate and informal graph. Upon consultation with the biologist, this graph helps us to make sure that we have

not missed variables of interest and also if there is any possibility to regroup those variables whose functionalities resemble. For example, variables having the same influence and exhibiting similar behaviours can be grouped together.
Also, let us consider we have an intermediate variable (say z) between two variables (like x and y in Figure 5.1 a). If z has no outgoing edge other than y, then z can probably be ignored, as it is considered as a relay, so that one can consider that x is influencing y "directly" (Figure 5.1 b). This simplifies the interaction graph, reduces the final number of variables, and offer a better abstract view of the system.

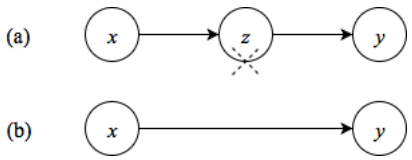


Figure 5.1: (a) z is acting as a "relay" between x and y (b)z is removed as it has no outgoing edge to other variables in the system.

5.1.1 Input variables

To address the issue of external environment, input variables (sometimes referred to as environment variables) are incorporated in the network. They have no predecessors. This is particularly useful when we need to simulate the real network within its environment and as such they are needed in the validation matrix (see section 5.4). Depending on the network, these input variables can be binary (a "0" means the input variable is absent in the environment) or they can be multivalued to express multiple concentration levels; for example a low, medium or high level of presence.

Using input variables, we can control under which condition(s) a variable switches from one threshold to another. Let us assume that the presence of c triggers the expression of x (Figure 5.2), then this will modify the number of K parameters of x as discussed in the next section.

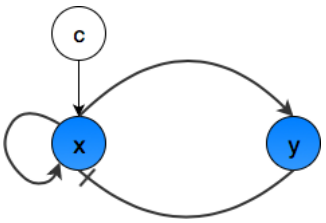
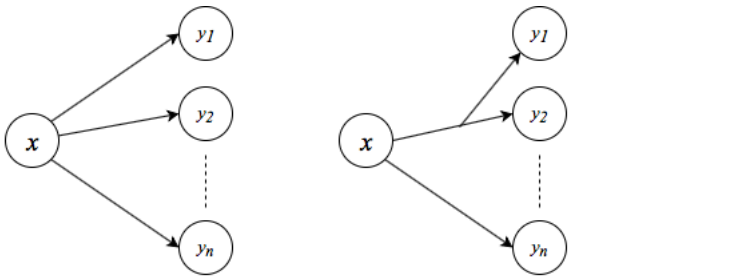


Figure 5.2: Introducing the input variable c

5.2 Finding the abstract thresholds

Once we have gained empirical knowledge of the system and its variables, the next step is to see how each variable acts on its successors under different conditions. In the Thomas design methodology, this is referred to as the interpretation of *threshold*.
As mentioned in section 4.1.2, Thomas framework was originally limited to the ON and OFF of genes, that is, a purely boolean approach. There is an extension which allows for more than a boolean value for each variable; thus giving this flexibility to have a multivalued variable. From the preceding informal graph, we have a clue on the links between the variables. We use it to identify all the targets for each variable and these targets represent the outgoing arcs for the variable (see Figure 5.3 a).

Once the targets are exhaustively identified, one needs to know at which threshold the variable has an impact on each target. To achieve this, we follow a simple thought experiment : we assume that the product of the variable is entirely absent. Then, the technique is to assume that this product increases slowly and to ask the biologist to list in order of occurrence the targets that are activated or inhibited. It may happen that the same threshold applies for different targets, which also forms part of discussions to have with the biologist. In this case, the outgoing edges having the same thresholds are grouped together (see Figure 5.3 b). Therefore, the number of thresholds for a variable is always lower or equal to the number of outgoing arcs of that variable after regrouping. Whether we have the same or different



(a) Outgoing edges to identify thresholds for x

(b) Similar thresholds are grouped so that only one outgoing edge represents that threshold

Figure 5.3: (a) Using outgoing edges to identify thresholds for each variable. (b) $x \to y_1$ and $x \to y_2$ have the same thresholds and therefore are grouped together.

thresholds, we must provide rigid biological justifications.

The difficulty but also crucial element all along this thought experiment is that, during this process, the biologist must avoid taking into account transitive interactions of the targets among themselves as shown in Figure 5.4 (avoid the interaction of y_i over y_j irrespective of the length of the paths - dotted edges). This can affect the determination of the threshold order of x over y_i. So, we can say it is a strict one-to-one relationship that must be considered at a given time and for each target, we assume the other targets stay constant (they are "freezed"). This thought experiment [121] we carry out with the biologist offers a solid way to reason locally for each variable (5.4) and gives a kind of guarantee that we are not mistaken in this identification task.

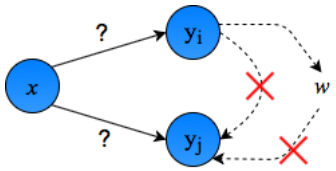


Figure 5.4: Finding the threshold for the variable x. Assuming the action of x over y_i arrives before that of y_j. We must avoid seeing the interaction of the variable y_i on y_j, when determining the threshold of x over y_j.

In practice, we do this thought experiment twice; in the second one, we assume that the product of x is saturated and that it decreases slowly. Of course, we must get the reverse threshold order else it would be a clue that the biologist has probably taken into account a non local system behaviour in his/her thought experiment.
An important point to note here is that this assignment of threshold on the targets is not quantitative and therefore it does not make any sense to add intermediate values. For example $x \geq 1$ and $x \geq 2$ does not mean the quantity of x but a threshold above which x acts (in Figure 5.5 x acts at different thresholds on y and on itself). From a formal point of view, the thresholds will be helpful to identify which atom to put in the multiplex (see Chapter 4). Later, we reuse this notion in the identification of K parameters for the dynamics of the network towards which value a particular variable will have the tendency to go. The central point in this section is the *local thought experiment* which must be set as a reminder each time questioning the biologist.

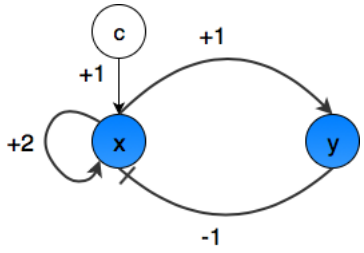


Figure 5.5: After identifying the thresholds, we obtain an interaction graph showing the threshold value on all edges between variables.

5.3 Inventory of multiplexes

The informal graph gives us a clue on the interactions between all variables in the system. The inventory of multiplexes depends on the predecessors of variables (see Figure 5.6) and possible combinations of predecessors are possible with respect to biological questions. We give three examples to illustrate this as follows :

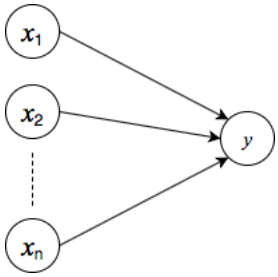


Figure 5.6: Predecessors of y. If there are no cooperation between the predecessors, then y will have 2^n possible set of resources.

1. Let us say that the two predecessors x_i and x_j (with thresholds 1 and 2 respectively) need to form a complex which will activate y, then this complex is encoded in a multiplex with the formula $(x_i \geq 1) \wedge (x_j \geq 2)$ as shown in Figure 5.7. This means that both variables x_i and x_j are needed; the absence of either one will not activate y, so it is encoded by a conjunction.

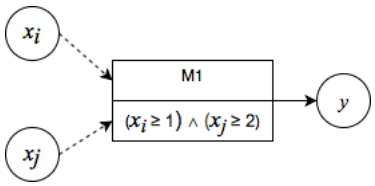


Figure 5.7: The presence of both x_i and x_j are needed to activate y.

2. Let us say we have three predecessors acting on y as follows : 2 activators, x_i and x_j , which form a complex and 1 inhibitor x_k, and that x_k has a stronger influence on y. This more elaborated multiplex can be formulated as shown in Figure 5.8.

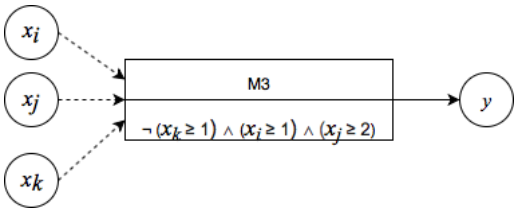


Figure 5.8: The inhibitor x_k has a greater influence than the two activators x_i and x_j.

5.4 Validation matrix

In software engineering, validation via testing is crucial to improve software quality and is an important ingredient in the software life cycle. It can take the form of either functional test or structural test, both having a specific role. Classically, in software engineering, functional test cases are proposed after writing the software specifications. These functional tests are obtained by inventorying, on the one hand, the test of functionalities that the software is supposed to perform according to the specification, and on the other hand, the list of different contexts and particular cases that are mentioned in the specifications. As such, we make use of a sort of requirements traceability matrix, where each cell contains (if applicable) one or several test cases. In our methodology, we will generalise the concept and use temporal properties

instead of simple test cases. We use the term *validation matrix* as it will be used as a backbone for validating the model.

When validating a biological model, we adopt the same strategy except that a cell for us will contain a formula that encodes a desired behaviour. The equivalent of the software specifications are the biological knowledge about the global behaviour of the real biological model (chapter 2) in which the knowledge is expressed in terms of the variables of the system using temporal logic. At a later stage, we can first use simulations to test these observations and secondly use proof techniques like standard model checking. As we will see later, we will use fair path CTL.
The validation matrix is written before the identification of the kinetic parameters, just like functional test cases are written before the realisation of the software in software engineering.

For a biological model, therefore, the validation matrix is built as follows:

- the rows represent the context in terms of the external environment which is based on the combination of the status (absence / presence or level of expression) of the input variables and the initial conditions of certain main variables. For example, from Figure 5.9, we see line 1 having a context where the input variable is absent (c=0) and the initial condition is $x < 2$.
- the biologically observable variables represent the columns but there are some exceptions. It may happen that the cooperative action of certain variables are known in which case we represent this collective behaviour in one of the column. For example, if we have three variables a, b and c for which we are certain that under some context at least one of these is expressed but we do not know which one precisely at any time; in this case we can name the column as *abc* and the corresponding cells can host the formula $AF(AG(a \geq 1 \vee b \geq 1 \vee c \geq 1)$.
- each cell in the matrix represents the behaviour of the corresponding variable (or cooperation of variables) in terms of trajectory properties with respect to the corresponding given context. These trajectories can for example take the form of oscillations or tending towards a particular value. We formalise these biological observations in their CTL equivalent which can be validated using SMBioNet. In Figure 5.9, from $c = 0$ and $x < 2$, we require for x and y to oscillate.

For some contexts, we may have no knowledge about the properties of some variables. If so, we ignore the cell. In Figure 5.9, if ever there are ambiguities or we are unsure about the behaviour of a given variable, we leave the corresponding cells empty. In such cases, we must discuss with the biologist to have a plausible justification on this exception.

At a later stage, after the K parameters are identified, we use these parametric information to cross check each cell of the validation matrix; for example using model checking or any other formal methods.

We illustrate a sample validation matrix (Figure 5.9) of the running example in Figure 5.5.

CONTEXT		X	Y
C=0	X < 2	*OSC (0,1)*	*OSC (0,1)*
C=0	X = 2	*–> 2*	*–> 1*
C=1	X < 2	*OSC (0,1)*	*OSC (0,1)*
C=1	X = 2		*–> 1*

Figure 5.9: Validation matrix with x and y as main variables, c as boolean input variable; x<2 and x=2 representing the initial states. '→' means tends towards a particular value; OSC(0,1) means oscillation between 0 and 1.

5.5 Identification of K parameters

The identification of K parameters is the most important and challenging step in the analysis of the dynamics of any biological network. More often, even if the network is a well-studied one, the list of K parameters are still difficult to derive. This process of identification becomes worse with large number of

variables. Let us remind that the number of possible parametrisation is a double exponential expressed as : $\prod_{v \in V}(b_v + 1)^{(2^{in\_deg_v})}$, where b_v is the boundary and in_deg_v is the in-degree of each variable v.
When the biologist is unable to establish certain parameter values due to insufficient knowledge, then these remaining parameters could be identified by model checking solvers, provided that the number of unknown parameters remain reasonable. So, even if we use the full strength of formal methods, the identification process remains complicated.
For these reasons, before undertaking any formal verification techniques, we must exploit to the maximum the knowledge of the biologist for possibly known K values. Undeniably, the main challenge in this identification process remains on how to extract these parameters from the biologist knowledge.
From the beginning of this thesis, we were aware of the large dimension of the regulatory network of energy and biomass metabolism, with a significant number of variables. Prior to discussion with the biologist, we decided to put in place a strict methodology which we describe in this section. We will use Figure 5.10 as an example to identify the K parameters for u. Likewise the identification of thresholds, we will also apply thought experiments to ease the identification of K parameters with the biologist. Swiftly following on from this observation, it is important to note that:

- for each variable of the model (here u), we successively study all the $2^{in\_deg_v}$ possible combinations of available resources ($\{\}$, $\{r_1\}$, $\{r_2\}$, $\{r_1, r_2\}$ for u). Each of these $2^{in\_deg_v}$ cases gives rise to a new thought experiment.
- for each of these thought experiment, we stay in the *local* interactions of the variable under consideration (here u) and we make sure the biologists do not take into account the system globally. This literally means that we assume that all variables that can participate to the set of resources of u remain at their current values for an infinite time. From Figure 5.10, we assume that the truth values of Φ_1 and Φ_2 remain constant forever.
- once we are in the local context, that is, once the truth values of the possible resources of u have been fixed, we ask questions towards which value (the possible threshold values) the given variable (u) will tend to evolve with respect to the availability of these resources; that is which of its targets (t1 to t5 in Figure 5.12) will be reached. Remember that the different threshold values for the variable have been well-identified (section 5.2). We show the targets for u and their corresponding thresholds in Figure 5.11. The targets of u having similar thresholds are grouped as shown in Figure 5.12.
- avoiding to take into account the system globally implies that feedback loops must not be taken into account in this thought experiment, as shown in Figure 5.13.
- moreover, intermediate variables acting on the same targets must be ignored as well, as shown in Figure 5.14 (bottom red arrow)

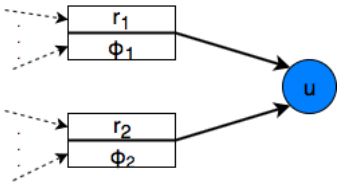


Figure 5.10: Considering the variable u and all the resources(r_1 and r_2) acting on it. Each resource r has a logical formula which is satisfied or not.

Once we have the list of resources, the next step is to reuse the outgoing arcs (targets and their associated thresholds). As already mentioned, these values on the arcs are not quantitative and as such this only means the order in which the multiplex(es) are activated.

Having these pairs of incoming and outgoing arcs allow us to query the tendency of the variable to evolve asymptotically according to the availability of a given set of resources. As a reminder, the n incoming arcs which are multiplexes can be satisfied or not and, as such, there are 2^n possibilities. We omit the formula in the multiplexes and consider only their names to allow proper reasoning for the biologist. For each proposed K parameter value, we must provide appropriate biological justifications with corresponding bibliographic references.

Now, let us see how to tackle the identification of all K parameters for the variable u. Since u has two resources r_1 and r_2, this gives us four possible K parameters as follows :

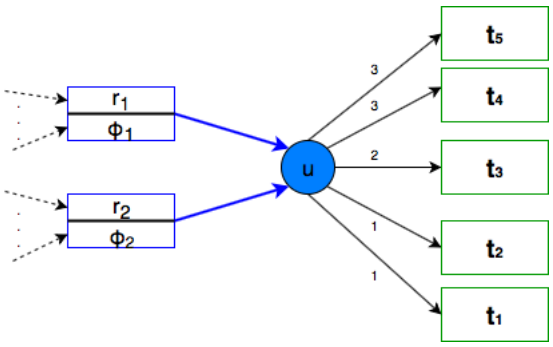


Figure 5.11: Representing all the targets of u and their corresponding thresholds according to the knowledge of the network.

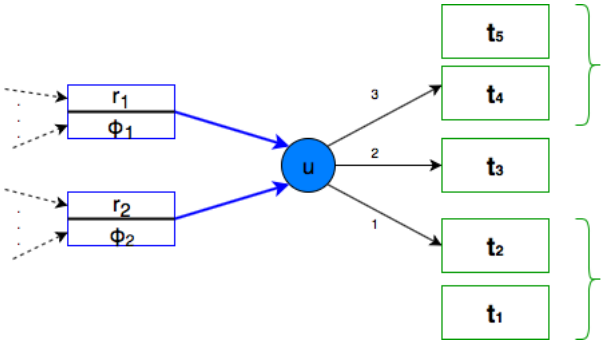


Figure 5.12: Regrouping all outgoing edges having same threshold.

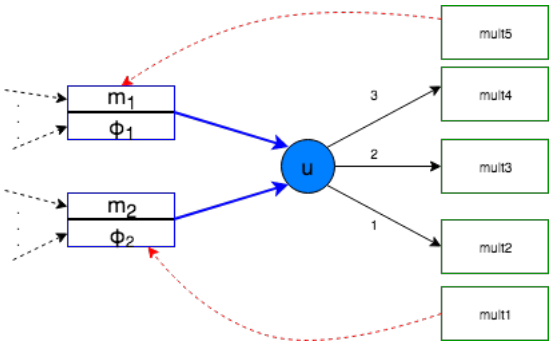


Figure 5.13: For identifying the K parameters for u; feedback loops (top and bottom red arrows) must not be considered during the thought experiment.

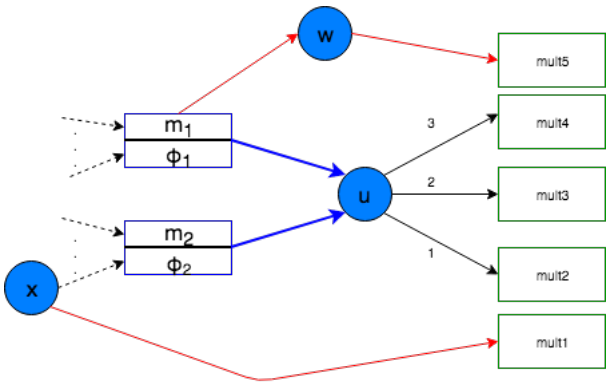


Figure 5.14: Identifying the K parameters for u; intermediate variable w must not be considered. Indirect variables are also ignored (x acting on $mult1$) during the thought experiment.

- No resources are present : $K_{u,\{\}}$
  We ask the biologist, assuming that the products of u are at a level where there are no resources

acting on u : are there any targets that will be influenced ?. If the biologist says no, this means that u is insufficient to act on its targets and we set the value of the parameter, $K_{u,\{\}} = 0$ (see Figure 5.15).

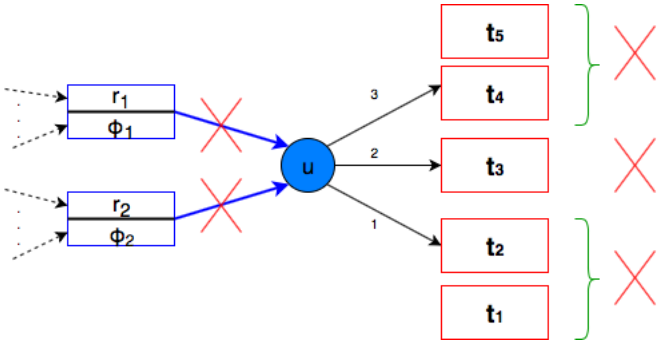


Figure 5.15: No resources acting on u.

- Only r_1 is present : $K_{u,\{r_1\}}$
  If only r_1 is present as a resource to u, then we ask the biologist what are the targets that are influenced. If the biologist response is only t_1 and t_2, for example, then this means that the value of u will move towards the threshold 1 and consequently the parameter $K_{u,\{r_1\}}$ will be equal to 1 (see Figure 5.16).

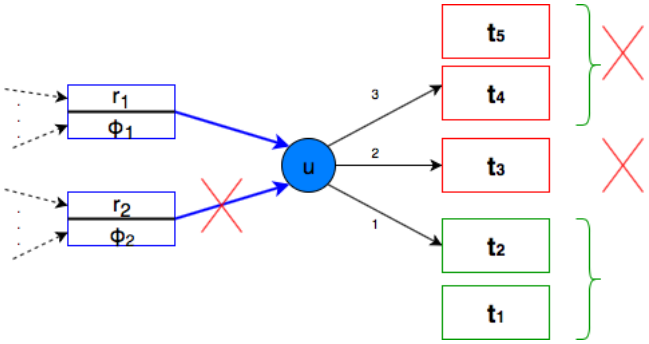


Figure 5.16: Observing the effect of r_1 only on u. We must not consider r_2 as a resource of u.

- Only r_2 is present : $K_{u,\{r_2\}}$
  If only r_2 is present as a resource to u then if the biologist response is that only targets $t1$,$t2$ and $t3$ is affected, then the corresponding parameter $K_{u,\{r_2\}}$ will take the value 2 (see Figure 5.17).

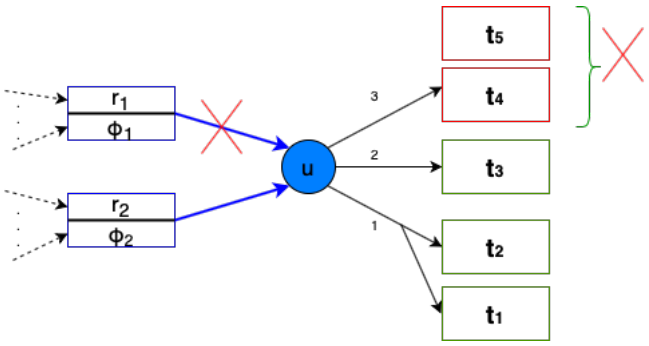


Figure 5.17: Observing the effect of r_2 on u. We must not consider r_1 as a resource of u.

- Both resources r_1 and r_2 are present : $K_{u,\{r_1,r_2\}}$
  Assuming the presence of both r_1 and r_2 will cause the products of u to influence all its targets, then the parameter $K_{u,\{r_1,r_2\}}$ will reach the maximum value of 3 (see Figure 5.18). If not, it means that the influences that u to t_4 and t_5 are not functional.

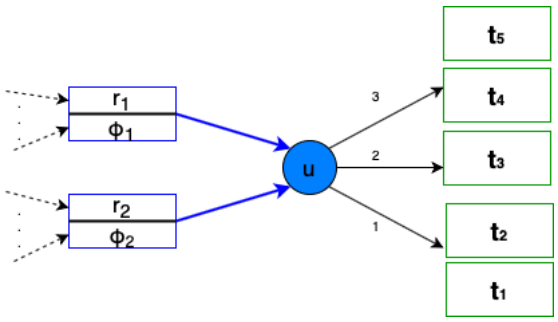


Figure 5.18: Considering both r_1 and r_2 as resources.

It may happen that the biologist response is "u acts on t3 but not on t1..." In this case, it means that the threshold caracterization of step 2 has to be reconsidered: the order of thresholds may be wrong, a new variable may be missing, or possibly different resources may be abusively abstracted into a single multiplex".

At this stage, if some responses are missing due to insufficient biological knowledge, then we left the identification of this parameter to model checking (see chapter 6, section **??**).

At the end of this exercise, it is still possible that we are left with few unknown parameters for which we can assign arbitrary values, simulate and discuss the results with the biologist. If the results are relevant to the question asked, then we can use these values. If we are still uncertain on some values, we then write the biological observable properties of certain variables in the form of CTL. We already have a software called SMBioNet (6.2.3), which uses NuSMV models checking to find these possible values. To do this, we make an inventory of certain known observable properties according to the network, we write them in the form of CTL and feed them to SMBioNet.

5.6 Preliminary validation with simulations

At this stage, we have a good understanding of the static representation of our model together with almost all parameter values, resulting in a set of fully functional regulation graphs. In order to understand the dynamics of the model under different environmental pressures, we have two solutions:

- either by simulating in order to obtain representative traces of the behaviour of the model, these traces can be a good basis to discuss further with the biologists
- using model checking techniques in order to establish behavioural properties expressed in CTL.

In this section, we will see the importance of simulation, which rapidly gives an indication of the behaviour of variables (x and y in Figure 5.19), based on the combination of truth values of the input variable c (here c is boolean). The advantage of simulation is its graphic representation of all the variables and how they evolve over time which resemble usual curves obtained from experiments. The simulation step gives results which can be rapidly cross-checked with known biological observations (up to a rescaling of time, as Thomas framework does not model time delays), and assess whether they are possible in the given biological context. Therefore, we get a first feeling of the mathematical model, allowing to rapidly exhibit the main misbehaviours in the considered model, and correct them. Also, these observations can provide us with new intuitive global view of the model behaviour and help us construct appropriate temporal logical formulas expressed in CTL that can be checked later on, with model checking tools.

However, Thomas models possess non-deterministic behaviour and, as such, simulations only show partial views of the dynamics. Consequently, we can have to wait for long until simulations exhibit some biologically relevant result. This can be explained by two reasons:

- Firstly, if there are circular loops or oscillations, it may happen that we enter these loops, and take a long time until we get out and follow any other trajectories. At first, this can give a perception that there is only oscillation and can misguide the modeller or biologist. We illustrate this by two diagrams in Figure 5.20 and 5.21.

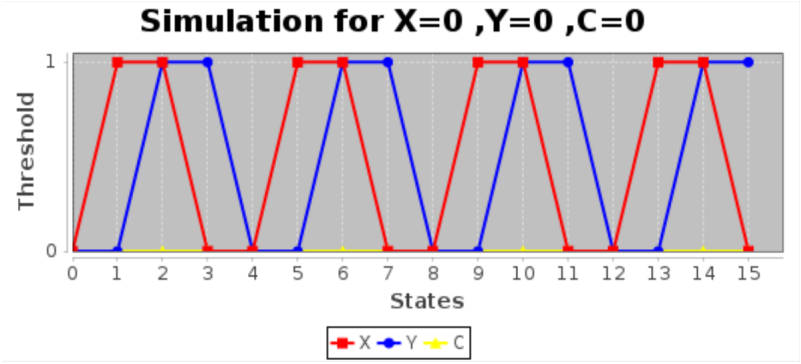


Figure 5.19: Oscillation of x and y in the absence of c (c=0).

We can see that in 5.20, a shift from state x=1 to x=2 reaches stable state instantly. In 5.21, we have to wait for a longer time to see that shift, as we enter the loop for both $x(0,1)$ and $y(0,1)$.

- We also take the opportunity to illustrate this non-determinism within a regulatory graph in Figure 5.22. Assuming we reach a certain state η, from there we have 10 possible transitions towards η_1 to η_{10}. We assume there is a loop from η_i to η for i=1 to 9; then many simulations will make us believe that the system always oscillates.

  Moreover, apart from the oscillations, we also have a large number of variables which can enforce this non-deterministic simulation. First, the probability that a given transition is chosen is random and may not be equitable. Even if we introduce a kind of uniform distribution between the transitions, it will take time to go through all the variables updates. Another scenario can happen in which we transition to η_{10} in the first instance, and we reach the stable state without observing any oscillation.

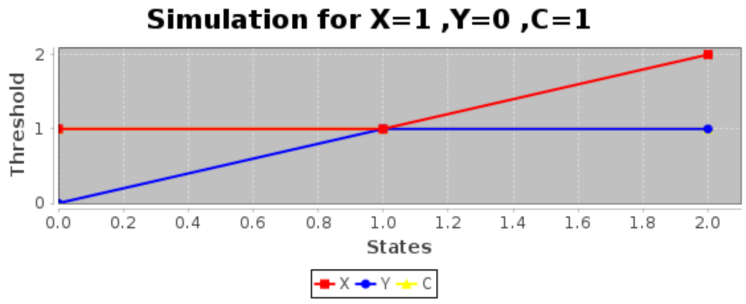


Figure 5.20: Oscillation of x and y in the presence of c (c=1) but stable state is reached immediately.

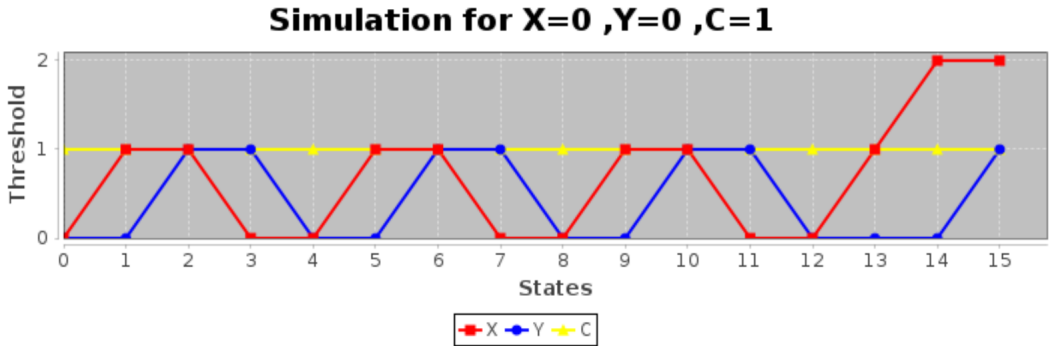


Figure 5.21: Oscillation of x and y in the presence of c (c=1). Due to non-determinism, we can wait a long time to see the transition from x=1 to x=2

For this reason of non-determinism, apart from simulation, we have a complementary validation method by using model checking with CTL. This allows us to verify temporal properties of variables. In

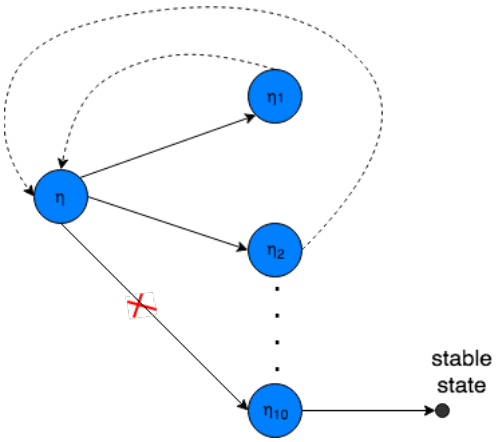


Figure 5.22: Non-determinism: one or more trajectories may never be reached due to many feedback loops from η to η_1 to η_9 and back, and only one trajectory from η to η_{10} leading to a stable state that several simulations can miss.

the next section, we oversee these two advantages of CTL and its variant called **fair path CTL** which introduces some sort of equity in all trajectories.

5.7 Validations using fair path CTL

CTL allows us to study the dynamics of a model in terms of global properties of the tree trajectories in an infinite time interval. It is a way of reasoning about non-deterministic behaviours. Given a certain property expressed in a well-formed CTL formula, the model checker (integrated in SMBioNet) tells us if it is satisfied or not. Unfortunately, there are cases where standard CTL is misleading. We give two examples in Figure 5.23 and Figure 5.24.

In Figure 5.23, standard CTL takes into account the paths that stay in the four states (0,0), (0,1), (1,0) and (1,1), x and y oscillating between the values 0 and 1. So, CTL considers the case where the path cannot escape from this oscillation. As such, the trajectory will never see x change value from 1 to 2. In fact, such a path in the trajectory graph is *unfair* because it crosses the state (x=1, y=0) an infinite number of times without choosing the transition towards x=2. So, standard CTL will consider that the state (x=2,y=1) is not always reachable.

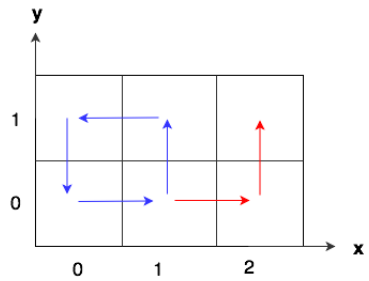


Figure 5.23: According to standard CTL, two possible qualitative dynamics are observed with a bifurcation occurring at $x = 1$, $y = 0$. First, the blue oscillation means we can stay infinitely in the loop and a second possibility of attaining the stable state (via the red arrows). The oscillatory behaviour is unfair, as it neglects a possible transition an infinite number of times.

Consider the state transition graph in Figure 5.24 in which we have three variables x, y and z in a three dimension state graph. Notice the two loops (oscillations of x and y) in both plan $z = 0$ and $z = 1$. Suppose, we start from $z = 0$. If the transitions run for an infinite time, it is reasonable to ask that, at some point, z will switch from 0 to 1 because it is always attracted towards 1. Nevertheless, if we write the CTL formula AF (AG($z = 1$)), the model checker returns false. This is because it may happen that we stay in the plan $z = 0$ infinitely and this is not 'fair'.

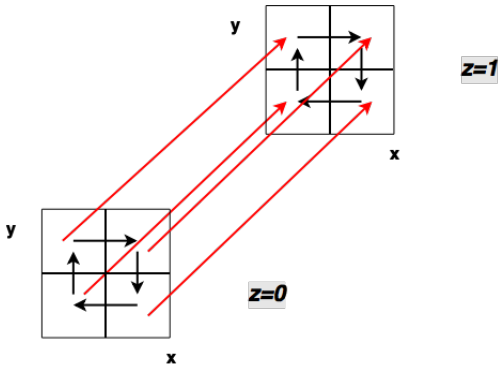


Figure 5.24: CTL fairness : In this example, if we leave the system dynamics for a long time, we biologically consider that Z will eventually reach its value 1. But, the CTL formula AF (AG ($z = 1$)) returns false since there is possibility to stay in the plan Z=0 for an infinite number of transitions.

For the same reasons, many CTL formulas of the form AF(..) or A [..U..] become false, due to these spurious infinite cycles where the choice of transitions is unfair. This is an unwanted artefact with respect to real biological behaviours. An effective solution is to change the semantics of CTL by ignoring unfair paths and this allows us to restrict the search to fair paths only. To achieve this, we will use a fair CTL described in this section. On top of this, it works irrespective of the number of variables and trajectories.

In 2008, Adrien Richard wrote a note which has not been published, defining the mathematical semantics of fair path CTL and the conversion of any fair path temporal formula into standard CTL. We attach the paper in Annex 11.6 but nevertheless, we give some hints about the fairness of trajectories and the possible conversions of three types of trajectory properties that are often observed in biological experiments: the usual boolean oscillation, the possible oscillations of a multivalued variable and finally the tendency of a variable to converge towards a fixed value.

A primer on CTL has already been covered in Chapter 3 (section 3.5). As a reminder, we recall that a CTL modality is a couple of a quantifier symbol (**E**(xist), **A**(ll)) followed by a temporal symbol (ne**X**t, **F**uture, **U**ntil, **G**lobal).

In fair path CTL, A and E are converted to A' and E' respectively that quantify only on fair paths. Given a fair path CTL formula Φ', its standard CTL equivalent formula Φ is defined inductively as follows :

1. if Φ' is of the form $A'F(\Psi')$ then Φ is the standard CTL equivalent of $\neg E'G(\neg\Psi')$
2. if Φ' is of the form $A'G(\Psi')$ then Φ is the standard CTL equivalent of $\neg E'F(\neg\Psi')$
3. if Φ' is of the form $A'(\Psi'_1 U \Psi'_2)$ then Φ is the standard CTL equivalent of $A'F(\Psi_2) \wedge \neg E'(\neg\Psi_2$ U $\neg\Psi_1 \wedge \neg\Psi_2)$
4. if Φ' is of the form $E'F(\Psi')$ then $\Phi \equiv EF(\Psi')$
5. if Φ' is of the form $E'G(\Psi')$ then $\Phi \equiv E(\Psi'$ U $AG(\Psi'))$
6. if Φ' is of the form $E'(\Psi'_1 U \Psi'_2)$ then $\Phi \equiv E(\Psi'_1 U \Psi'_2)$

A path is considered *fair* in a system if it never enters a state infinitely often and always ignores one of the transitions from that state. With this transformation of CTL formulas (Figures 5.25 to 5.27), it is not necessary to invent another CTL model checking algorithm to treat fairness of trajectories. We will be using these fair path CTL extensively for validations of biological properties in Chapter 8.

Each cell in the validation matrix is verified by using these CTL translations.

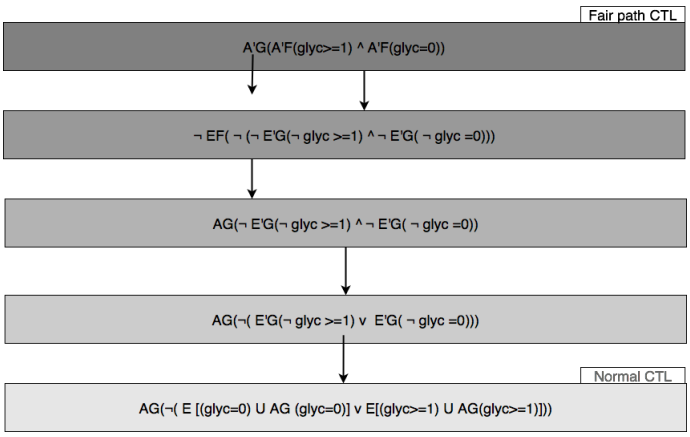


Figure 5.25: Oscillation of variable *glyc* between 0 and 1 : Fair path CTL conversion into normal CTL equivalent

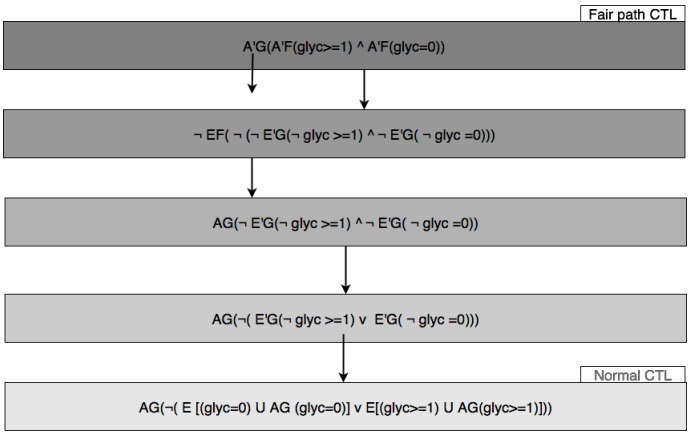


Figure 5.26: Standard CTL equivalent of fair path CTL for oscillation between 0 and n (n > 1).

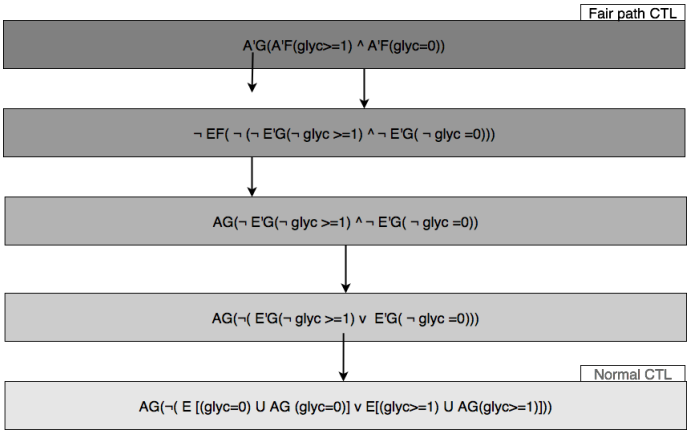


Figure 5.27: Standard CTL equivalent of fair path CTL of the variable x tending towards a specific threshold (here 2).

5.8 Conclusion of the chapter

In this chapter, we design a methodology dedicated to the design of Thomas framework models. To our knowledge, it is the first fully defined methodology dedicated to this formal framework. All the important steps of the methodology have been documented and we gave sample illustrations. These steps are composed of the static (sections 5.2 to 5.4) and dynamic (section 5.7) implementations of the biological regulatory model as well as how we can verify the model prior to suggest biological experiments. In between, we proposed a *validation matrix*, inspired from software engineering, which provides an efficient tool to cross-check properties of each variable under each context.

We have shown how simulations can be useful to verify some initial well-known properties of a given system. The in-built non-deterministic aspect of the simulations motivates to validating certain properties using CTL and the variant fair path CTL (detail is available in the Annex). This validation technique has been instrumental in the elaboration of all biologically sensible trajectories (irrespective of spurious oscillations). In the next chapter, we will describe how we implemented all these functionalities in our tool called DyMBioNet which allows to perform simulations as well as fair path CTL verifications.

CHAPTER 6

DYMBIONET

6.1 Introduction

Small biological networks can be designed and verified on paper and blackboard but this method is not feasible for large networks. When the number of variables is significant, we are confronted with a huge number of possible state transition graphs and checking certain network properties can be complicated and time consuming. More often, the extra but crucial step in modelling approach is casting the model into a formal, computable form that can be analysed rigorously using simulation and other mathematical methods 3.2. Furthermore, multiple simulations are needed to cater for main possible observable properties or to refute a model, and this is where a software platform proves to be valuable.

On top of this, visually representing the network and being able to observe the dynamic evolutions of the network is an important step to initiate discussions with the biologists. The advantages of simulations are therefore many folds: the network can be graphically represented, simulation of any size can be done repeatedly, we can observe known properties as well as emerging properties, we can get used to the network and attain faster diagnostics. We can also be exposed to counterexamples obtained by simulation or model checking verification results. With the help of a simulator, the user can replay the violating scenario to obtain useful debugging information. The model (or the dynamic property) can then be adapted accordingly.

In the first section of this chapter, we will have a brief overview of four software that have been developed for modelling complex networks using formal methods. We then give reasons why we have not use them in our context. In later sections of this chapter, we will dive into the core functionalities of DyMBioNet and show how it can be used to construct, analyse and simulate a biological network. We continue to elaborate on how CTL can be helpful to establish important general biological properties inside DyMBioNet.

6.2 Existing software tools for the Thomas framework

Some qualitative modelling tools already exist for the modelling of complex biological networks [122]. We mention in this section, the advantages and drawbacks of four main tools namely: GINSim, GNA and SMBioNet, which are based on discrete formalisms.

6.2.1 GINsim

GINsim (Gene Interaction Network simulation) is a computer tool for the modelling and simulation of genetic regulatory networks. GINsim allows the user to specify a model of a genetic regulatory network in term of asynchronous, multivalued logical functions, and to simulate and/or analyse its qualitative dynamical behavior [131] as shown in Figure 6.1. This tool is platform–independent (Linux, Mac OS 10.3+, Windows).
GINsim is based on a compilation of Thomas networks with known parameter values into standard Petri nets. Consequently, it takes benefit of the whole corpus of analysis tools for Petri nets. The remarkable result, proved by Chaouiya in [157], is that no inhibitory arcs are needed to perform the translation, so that all decidability results are preserved. This provides a good theoretical ground and at the same time accounts for the popularity of GINsim. While this method is powerful, the complexity incurred by this transformation into Petri nets is a major drawback. Moreover, it is somewhat paradoxical to translate Thomas networks, where there is no concurrency on resources, into Petri nets. The complexity of the

translation is precisely to remove the concurrency.
Last but not least, GINsim performs verifications and simulations only when all the parameter values are fixed (as the translation into Petri nets needs the parameter values). So, within our setting, where we need to discover the parameter values, GINsim would be of low help.

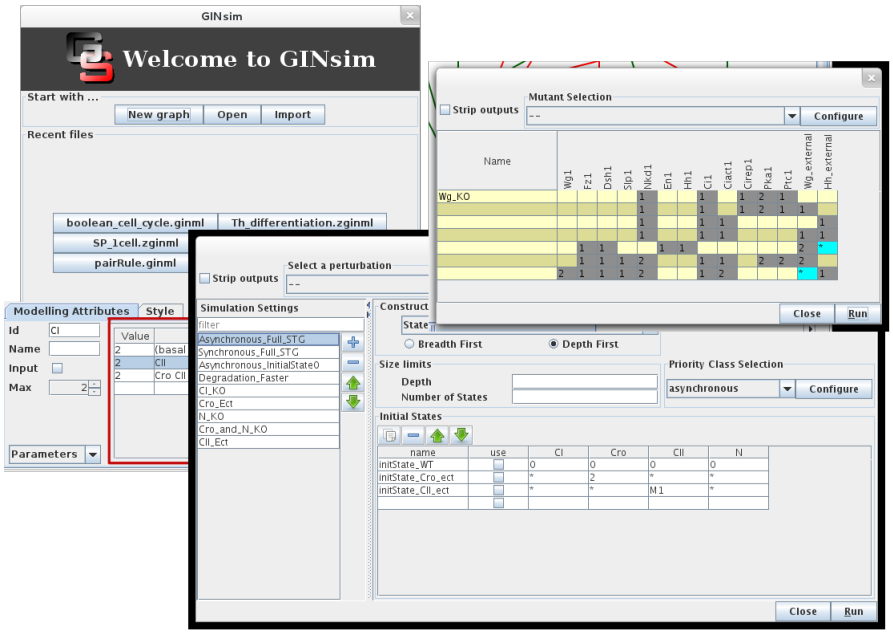


Figure 6.1: User interfaces of GINSim. The tool allows to create a new network, import as well as save recently created networks (top left). Each node in the network can be configured with an initial number of tokens (bottom left). Various options (synchronous, asynchronous, etc) are available to simulate the network (right).

6.2.2 GNA

The aim of Genetic Network Analyzer(GNA) is to assist biologists and bioinformaticians in constructing a model of a gene regulatory network using knowledge about regulatory interactions in combination with gene expression data. GNA consists of a simulator for the qualitative modelling of genetic regulatory networks based on piecewise-linear differential equations and uses the approximation of Filippov [123] to obtain a discrete modelling equivalent.

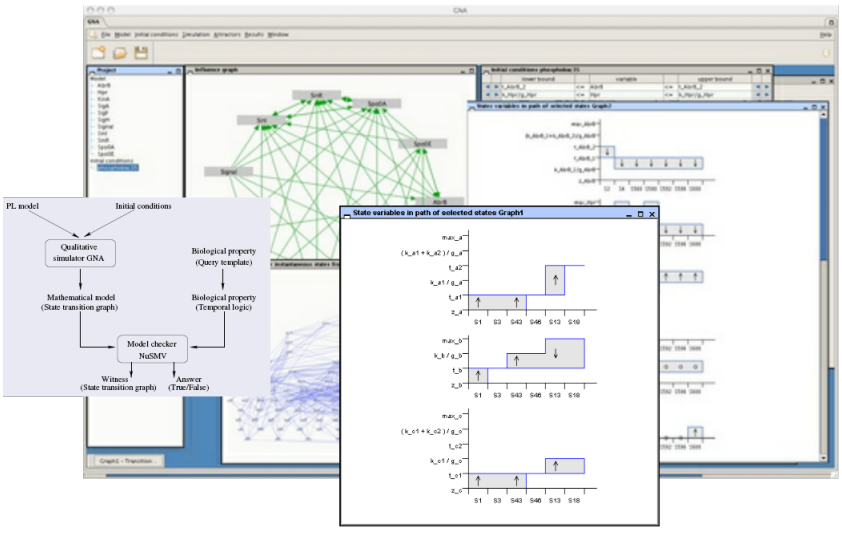


Figure 6.2: User interfaces of GNA

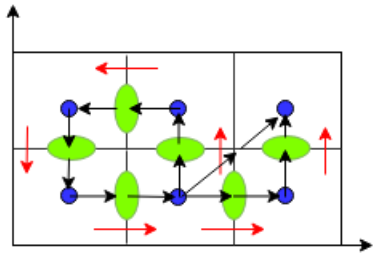


Figure 6.3: Singular states in GNA

Despite using a qualitative modelling approach, the methodology used by GNA is not the same as we adopted in this thesis. Our discrete formalism is based more on the logical aspects of nodes in the graph. We rely heavily on the discovery of values for kinetic parameters for system dynamics which we cannot see in GNA. The implementation of CTL is somewhat comparable to our proposed software, where both use symbolic checker NuSMV, for validating CTL formulas. Figure 6.2 shows sample user interfaces of the GNA tool with several useful features, to list some: the model can be constructed from scratch and the tool provides the possibility to import / export existing models; the ability to visualise the regulatory networks with an indication of all steady states; and finally, the specification of the dynamics in temporal logic.

Instead of using discrete values for its kinetic parameters, the user of GNA specifies inequality constraints [128]. The use of inequalities for thresholds and the application of differential equation for dynamic modelling of the network make GNA different from our approach which uses a logical implementation with discrete variables. For this reason, we prefer to extend our own platform to cater for these differences.

6.2.3 SMBioNet and TotemBioNet

SMBioNet which stands for *Symbolic Model checking of Biological Network* is a command line tool created by Adrien Richard in 2007 [129]. It is mainly used for the modelling of gene regulatory networks based on an extension of R.Thomas' approach that integrate multiplexes. It acts as an interface between the model and the associated model checking tool, NuSMV. Recently, several extensions of SMBioNet have been developed namely: HHLforBioNet and HyMBioNet [127]. In terms of functionality, it ressembles GNA with two differences: temporal model checking in the form of CTL is implemented in SMBioNet and we do not have singular states in SMBioNet. Nevertheless, the disadvantages of SMBioNet is the lack of visual simulation capability and it does not take into consideration fair CTL paths, which in return could give rise to erroneous interpretation of the evaluation results. Since SMBioNet is an integral part of DyMBioNet, it is further discussed in section 6.5.2.

TotemBioNet is the successor of SMBioNet. Its aim is to combine Hoare Logic and CTL in order to improve the efficacy of the enumeration phase that permit to automatically output all the correct parameter valuations. As we will not use Hoare logic for our model of metabolism regulation, we have only integrated SMBioNet into our DymBioNet software.

6.3 Description of the sample model

In this section, we describe the sample network we will be using throughout this chapter as reference to show some of the functionalities of DyMBioNet and at the same time demonstrate our methodological approach (we called it *model x-y* throughout this text).

6.3.1 Main variables

We use a simple network with 2 main variables: x and y. We assume they represent two biological entities whereby x is an activator of y and at the same time it is self-activator, and y is an inhibitor of x. We also put a control variable, c, which is an activator of x and is boolean.

6.3.2 Thresholds

x activates y at a threshold of 1, there is an auto-activation of x at threshold of 2 whereas y inhibits x at a threshold of 1. As a reminder, whenever there is a unit threshold, it may be represented with

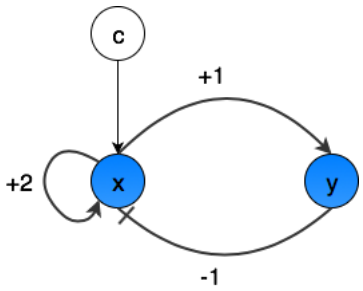


Figure 6.4: Interaction graph between x and y

the activation or inhibition sign only. Overall, x has a boundary of 2 and y a boundary of 1. All these information are summarised in Table 6.1

Variables	Threshold Levels	Boundary
x	0,1,2	2
y	0,1	1
c	0,1	1

Table 6.1: Information deduced from Figure 6.4.

6.3.3 Multiplexes and the regulation graph

The three actions will be modelled with the following three multiplexes for which we give arbitrary names: ACT1 (for $x \to y$), ACT2 (for $x \to x$) and INH (for $y \to x$) and ACTC (for c $\to$ x). In reality, multiplexes represent biological events.

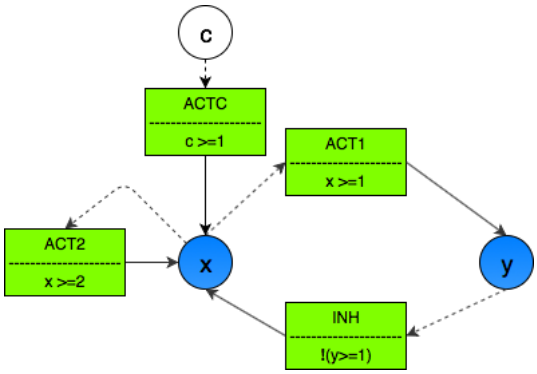


Figure 6.5: Regulation graph with multiplexes. Note that the negation sign "!" to encode sign to denote inhibition of y over x.

6.3.4 Kinetic parameters

The next step in our methodology is to build the table of resources as well as finding the right kinetic parameters for both x and y. For x, we have 8 $K_{x,...}$ parameters and kindly note that c is an activator of x only above a threshold of 1 as shown in Figure 6.2. We have only two K parameters for y. When there is no resource for y, its K parameter $K_{y,\{\}}$=0 and when there is x as resource, it will tend towards 1; that is $K_{y,\{X\}}$=1. Usually kinetic parameters are extracted either from direct biological knowledge, or from indirect deductions issued from known behaviours of the global system, mostly using temporal logic. For this simple example, let us assume that all kinetic parameters can be directly deduced from biological knowledge, as summarized in Table 6.2.

6.3.5 Validation matrix

Based on the information we have, there are two contexts describing the absence and presence of c. Moreover, we assume here that biologists consider that, for each of these two contexts, two sets of initial

c	x	y	$Resources for X$	$K_{X,\{\}}$
0	0	0	–	1
0	0	1	y	1
0	1	0	x	2
0	1	1	x,y	2
1	0	0	c	0
1	0	1	c,y	2
1	1	0	c,x	2
1	1	1	c,x,y	2

Table 6.2: Table of resources from Figure 6.4.Let us remind that : (i) y is a resource of x if y is absent (the absence of an inhibitor is a resource equivalent to the presence of an activator). This explains the appearance of y in the column $K_{X,}$ when y=0. (ii) x is a resource of y when it is above 1.

states result in two different behaviours. Consequently, in Figure 6.6, we split each context according to these two sets (x<2 and x=2)."

CONTEXT		X	Y
C=0	X < 2	*X OSC (0,1)*	*Y OSC (0,1)*
C=0	X = 2	*X –> 2*	*Y –> 1*
C=1	X < 2	*X OSC (0,1) (Normal CTL)*	*Y OSC (0,1), using Normal CTL*
C=1	X = 2	*X –> 2 (Fair CTL)*	*Y –> 1 (Fair CTL)*

Figure 6.6: Validation matrix for x and y with some fictitious observations. OSC means a variable can oscillates between two given values and "$\rightarrow$" means a variable tends towards a value. These two notions can also be encoded in CTL as we will see in Chapter 9.

Alternatively (and more formally), instead of splitting each context into two lines, one can group them together using preconditions. For example the two first behaviours described in the x column can be grouped together as "$(x < 2 \Rightarrow x$ OSC(0,1))" and "$(x$=2 $\Rightarrow (x \rightarrow 2)$".

Lastly, the choice of expressing the properties using CTL or Fair Path CTL depends if one wants to express a strongly general property, or a property that is true "to the limit" when time goes for a sufficiently long time. This is a technical choice, and by default we almost always use Fair Path CTL because biological observations are almost always done after the system stabilised its behaviour.

6.3.6 Simulation

The next step in our methodology is to use this set of kinetic parameters to construct the state transition diagram from which we can trace the dynamic of the network. It is important to point out that (i) an oscillation indicates the presence of a negative cycle, and (ii) a basin of attraction indicates a positive cycle as shown in 6.7.

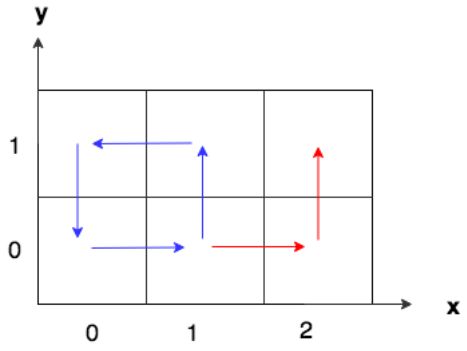


Figure 6.7: State transition graph showing the dynamics between x and y, when c=1.

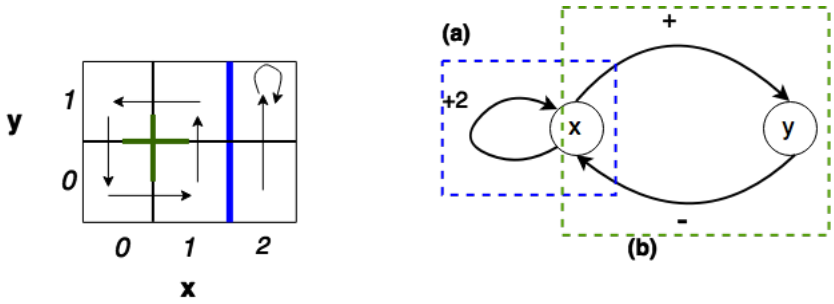


Figure 6.8: State transition graph showing the dynamics between x and y, when c=0.

6.4 Conception

The latest extension added to SMBioNet for the needs of our research is the development of a user-friendly environment to accompany researchers in the modelling of biological regulatory networks, called DyMBioNet, short for *Dynamic Modelling of Biological Network*. It comes with a unified graphical interface that allows the modelling of networks of any size. It is bundled with a couple of functionalities accompanying the users from modelling to visualising the evolution of the network by tuning parameters of the variables. DyMBioNet offers also a user-friendly interface for simulations of Thomas networks, which was surprisingly missing in SMBioNet, moreover it takes advantage of the model checking capabilities of NuSMV through SMBioNet but provides an additional feature to deal with CTL fairness. This feature is detailed in section **??**.

DyMBioNet has been conceived following the classical software design principles: modularity, flexibility and reusability, by applying object-oriented guidelines. It has two parts: information about the model is stored in an XML file, and secondly all the modelling and GUI interfaces are done using Java. The basic DyMBioNet engine is showcased in Figure 6.9. On the left hand side, we have the two file formats that are used by DyMBioNet for storing and processing biological networks data. Simulations, visualisations and reporting functionalities are showed on the right hand side. This conception section first explains the core engine in terms of Java classes followed by a brief explanation of the XML format.

DyMBioNet has been created using the Java language which is among the most popular and preferable language in bioinformatics for creating GUI interfaces for modelling and visualisation purposes. It is a full fledged object-oriented programming language with the following advantages:

1. Platform-independent
   Java's slogan WORA (Write one, run anywhere) makes it a particularly good choice for the development of bioinformatics tools which can run on any platform. This is possible due to its JVM (Java Virtual Machine JVM) which is shipped in almost every OS. Compiled Java classes (called bytecodes) are interpreted by the JVM removing the need to compile it for a specific OS and therefore renders Java as cross-platform.

2. Open-source
   Java is an open-source programming language under the GNU / GPL.

3. Huge library API support
   File manipulation, graphs, GUI are among the basic API bundled in Java specifications making it a complete language for simulation software.

6.4.1 Core classes

A class is a blueprint or prototype that defines the attributes and methods common to all objects of a certain kind. In the biological context, objects can represent a biological entity (eg. a gene), variables represent properties of entities (eg. thresholds of genes) and methods represent their behaviour (eg. activation or inhibition). In graph terminology, nodes and edges are the two important aspects that make the graph. In our formalism, we added a third aspect, multiplex, to make the metabolic graph. These three classes and their signatures are detailed below.

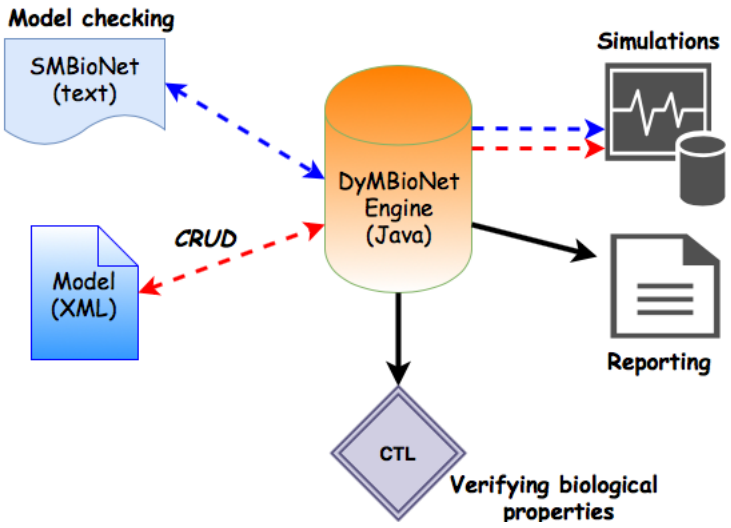


Figure 6.9: DyMBioNet engine

(i) Node : a node can represent a biological entity, a gene or a species in ecology. The Node class contains useful regulatory information expressible using the following attributes:

- a name written using letters or a mix of alphanumerical letters
- a boundary which is an integer data type strictly positive
- a couple (x,y) representing x and y coordinates for positioning the node in the graph; this is to avoid overlapping nodes in the display. Initially, a node is assigned random coordinate
- a color to identify the current threshold level of a node
  Noteworthily, this initial value (and color) is likely to change demonstrating how each node evolves in the network over time.

(ii) Edge : An edge represents any link between a given node and its counterparts (dotted line from node to multiplex and solid line from multiplexes to nodes). The Edge class has the following signature:

- a name : written using letters or a mix of alphanumerical letters (as before); this name is useful when simulating to know incoming and outgoing arcs
- fromNode : a string representing the source node
- toNode : a string representing the destination node

(iii) Multiplex : The attribute that is unique to multiplexes is their logical formula. Otherwise, they are fixed with a couple of (x,y) coordinates accompanied with a name which can represents important biological information like LIPIDS or CITRATE, in the case of metabolism.

- a name written using letters or a mix of alphanumerical letters
- a propositional logical formula which can contain a single atom or several atoms
- a couple (x,y) representing x and y coordinates for positioning the multiplex between the nodes in the graph

6.4.2 Interface classes

These are set of classes used to describe all the interfaces that allow the user to interact with the model. Three most important classes falling in this category are detailed in this section.

(i) Network class
This class is central to create, modify and delete biological properties of nodes, edges and multiplexes. Following are some examples of methods:

- Network(fileName, fileDesc)
  the basic constructor allowing to create an empty model given its name and description for future references

- addX(attributes)
  where X represents a Node, an Edge or a Multiplex; the same naming is used for delete and update
- addParameter(..) and updateKParameter(..) are the set of methods for creating and deleting K parameters for each variable

(ii) DyMBioNet class
This is the main entry point to the software when the application is launched. It represents the dashboard of the software.

- createGraph(..)
  This method will create the graph with the set of nodes, edges and multiplexes by retrieving data stored in the XML file.
- showGraph(...)
  This method will display the graph on the interface.
- explore(...)
  This method is used to simulate the network with initial parameters provided by the user.

(iii) KineticScreen class
Kinetic parameterisation is done in this class and is divided in two parts. One with an outline of the set of resources for the variable under investigation in a tabular form. The second shows the variable graphically which allows the user to reason locally with all its incoming and outgoing arcs.

- KineticScreen(..)
  Default constructor to show the interface for displaying the list of K parameters which can be updated directly.
- generateKineticGraph(..)
  Display the set of resources of a particular node to facilitate the user with the task of identifying the K parameters.
- isActivator(..)
  This boolean method is to verify the formula of a given multiplex to check if it an activator or inhibitor.

The complete code for the above-mentioned classes are found in Annex11.8 together with other java classes in the package fr.unice.

6.4.3 Model format

Many possibilities exist to store biological models and their related information of interest; for example using text files, a local database, or the widely used XML format. Text files contain delimiters and information stored are unstructured making it difficult to be interpreted and managed. The use of a local database is highly dependent on a web server for interfacing with the software. Updating network information is cumbersome and the migration of the networks would require backing database tables which is not too practical. Notably, XML becomes a good choice as a modelling format and its practicality is outlined hereafter. This short section is by no means a complete guide to XML or any if its validity format, but instead is intended as a broad overview of how it is used to represent the information of variables and their interactions in the biological network.

XML, which stands for e**X**tensible **M**arkup **L**anguage, has been here since more than a decade from now. In our context, its usefulness is justified as follows:

- Simplicity - Information coded in XML is easy to read and understand, plus it is understood by almost all programming languages (through API)
- Openness - XML is a W3C standard, endorsed by software industry market leaders.
- Extensibility - There is no fixed set of tags. New tags can be created as and when needed. This is particular interesting in our case: for example a <node> element for representing a variable, a <mult> tag linking it to a multiplex, etc.
- Self-description - Each tag or attribute speaks clearly about its meaning in the file. For example a <nodes> tag refers to the list of nodes in the graph whereas a <node> tag refers to a particular node with some fixed attributes.

Figure 6.10 shows the generated source code for the toy example of x-y-c. We provide some important points of the source code as follows:

```
<?xml version="1.0" encoding="UTF-8" standalone="no"?>
<network>
<description>This model is used to represent the interaction between two variables
X and Y.
Three interactions are available:
1. an auto-activation of X at threshold of 2
2. an activation of X over Y at threshold of 1
3. an inhibition of Y over X at threshold of 1
We name the multiplexes as ACT2,ACT1 and INH respectively</description>
<nodes>
<node default="0" id="X" kparam="1" thres="2" weight="1" xcoor="100"
ycoor="200">X<K id="KX00" value="0"/>
<K id="KX01" value="1"/>
<K id="KX10" value="0"/>
<K id="KX11" value="2"/>
</node>
<node default="0" id="Y" kparam="1" thres="1" weight="1" xcoor="100"
ycoor="150">Y<K id="KY0" value="0"/>
<K id="KY1" value="1"/>
</node>
</nodes>
<edges>
<edge fromNode="X" id="XACT2" toNode="ACT2">XACT2</edge>
<edge fromNode="ACT2" id="ACT2X" toNode="X">ACT2X</edge>
<edge fromNode="Y" id="YINH" toNode="INH">YINH</edge>
<edge fromNode="INH" id="INHX" toNode="X">INHX</edge>
<edge fromNode="X" id="XACT1" toNode="ACT1">XACT1</edge>
<edge fromNode="ACT1" id="ACT1Y" toNode="Y">ACT1Y</edge>
</edges>
<mults>

<mult formula="!(Y&gt;=1)" id="INH" xcoor="200" ycoor="50">INH</mult>
<mult formula="(X&gt;=2)" id="ACT2" xcoor="152" ycoor="142">ACT2</mult>
<mult formula="(x&gt;=1)" id="ACT1" xcoor="165" ycoor="138">ACT1</mult>
</mults>
<trans/>
<simulation/>
<ctl formula=""/>
<normalNode size="4"/>
<multNode formula="1" size="100"/>
<defaultNode>0</defaultNode>
</network>
```

Figure 6.10: Sample XML file for building the model X-Y.

- The first line of the network file has always the following line : `<?xml version="1.0" encoding="UTF-8" standalone="no"?>` ; meaning an XML file
- All XML files have a root element which signals the start of the document and always end with that element : `<network>...</network>`
- The list of nodes are enclosed inside the `<nodes> </nodes>` element
- A node (or variable) obeys the following structure: `<node default="0" id="X" kparam="1" thres="2" weight="1" xcoor="198" ycoor="211">x</node>`; default="0"; meaning its default threshold level; a node has an id which represents its name; if its K list of parameters has been set, then kParam = 1 otherwise it is 0; *thres* means its threshold value followed by its x and y coordinates.
- The set of K parameters for a variable is also enclosed inside the <node> element; `<K id="KX000" value="0"/>` for a given K parameter, the id is written as K followed by the name of the variable and ends with the binary value of the truth table (X has three values which will generate eight binary combinations from 000 (means 0) to 111 (means 7); the value is stored in the value attribute).

- Like nodes, edges also are enclosed inside the opening and closing <edges> tags. An edge is therefore an element with the following attributes: its name is specified in the id attribute; the two endpoints of the edge are specified in the fromNode and endNode attributes. An edge is represented with the following signature: *<edge fromNode="Y" id="YMULTYX" toNode="MULTYX">YMULTYX</edge>*.
- Multiplexes also are enclosed inside their respective tags as follows : `<mults><mult formula="(y>=1)" id="MULTYX" xcoor="257" ycoor="245">MULTYX</ mult>`;
  a multiplex has a name (id), it can be specified on the graph using x and y coordinates plus a logical formula enclosed in the formula attribute.

Two important requirements for XML documents are : they must be valid (respect certain grammar rules) and second they must be well-formed (build on pre-defined XML syntax). There are two ways of checking the validity of the XML file : using a DTD or an XML Schema.

This is important to highlight:

- to avoid serious compiling issues (eg. other developers integrating in the software life cycle) in the future development of DyMBioNet;
- for clarity to inform developers that there are certain data structure to follow when using or modifying the core XML file;

These two data structures are briefly explained in the subsections that follow.

6.4.3.1 DTD

A Document Type Definition (DTD) is a specific document defining and constraining definition or set of statements. DTD usage has the advantage of simplicity but lacks data typing. A sample DTD document equivalent to the source code in Figure 6.10 is shown in Figure 6.11, which are self-explanatory.

6.4.3.2 XML Schema

XML Schema offers a more powerful way of content definition with flexible data types than DTD. Sample of the XML Schema is shown in Figure 6.12 and 6.13. An element is *complex* when it either contains other elements or many attributes. All elements are complex in our model.

6.5 Functionalities

The objectives of DymBioNet is to offer important tools for simulation, model checking through proofs(using CTL) and visualisation of biological networks. These tools allow the modeller to have a fluid dialogue with the biologists when discussing both the statical and dynamical part of these networks. In this section, we will show with examples how these tools are integrated in DyMBioNet.

6.5.1 A visual-interface for building the network

The software interface provides a visual support to initiate discussions with the biologists. As such, the interaction graph can be done incrementally and nodes as well as multiplexes can be added as and when required. This is crucial step as more often we can be mistaken about particular information or merely miss fine-grained details, and these can later be added without disrupting the whole graph. In other words, the graph can be incomplete at the beginning, leaving space for scalability and this is never a modelling problem. Additionally, we can name multiplex without any fixed formula and without justifying its theoretical importance. This can provide understanding of the network in the meantime and as soon as information is available, these details can be integrated easily.
DyMBioNet offers users the facility to create a network by providing information on nodes, edges and multiplexes as shown in Figure 6.14, 6.15 and 6.16 respectively. When creating the model from this interface, the corresponding attributes for the nodes, edges and multiplexes are input by the user. This will generate the corresponding .xml file on the fly and any change in the nodes, edges and multiplexes information, will be updated automatically in the .xml file. Arbitrary coordinates are specified for all these three graph entities which can later re-position by the user to make the graph more visually appealing.

```
<?xml version="1.0" encoding="UTF-8"?>
<!ELEMENT Network (Description, nodes, edges, mults, trans, simulation, ctl,
normalNode, multNode, defaultNode)>
<!ELEMENT Description (#PCDATA)>
<!ELEMENT nodes (node+)>
<!ELEMENT edges (edge+)>
<!ELEMENT mults (mult+)>
<!ELEMENT node (#PCDATA|K)*>
<!ELEMENT edge (#PCDATA)>
<!ELEMENT mult (#PCDATA)>
<!ELEMENT trans EMPTY>
<!ELEMENT simulation EMPTY>
<!ELEMENT ctl EMPTY>
<!ELEMENT normalNode EMPTY>
<!ELEMENT defaultNode (#PDATA)>
<!ELEMENT multNode EMPTY>
<!ELEMENT K EMPTY>

<!ATTLIST node default  CDATA "0"
                    id              CDATA "0"
                    kparam   CDATA "0"
                    weight   CDATA "0"
                    thres    CDATA "0"
                    xcoor    CDATA "0"
                    ycoor    CDATA "0">

<!ATTLIST K    id              CDATA "0"
                    value    CDATA "0">

<!ATTLIST edge id              CDATA "0"
                   fromNode  CDATA "0"
                   toNode    CDATA "0">

<!ATTLIST mult id              CDATA "0"
                    formula  CDATA "0"
                    xcoor    CDATA "0"
                    ycoor    CDATA "0">

<!ATTLIST normalNode size     CDATA "0">

<!ATTLIST multNode  formula CDATA "0"
                            size  CDATA "0">

<!ATTLIST ctl formula CDATA "0">

<!ATTLIST K id          CDATA "0"
                value       CDATA "0">
```

Figure 6.11: DTD sample of our model XML file.

6.5.2 Importing SMBioNet files in DyMBioNet

The model format used by SMBioNet is a plain text file where details containing the gene regulatory network (with all the genes represented by variables (VAR) and their corresponding threshold accompanied by kinetic parameters (REG)]) are used as input parameters. A CTL section allows the user to enter a given set of CTL formulas to calculate all possible paths in the regulatory graph which valid those CTL formulas [129, 130]. The CTL can also be helpful to generate all K parameters which satisfy this formula. Figure 6.17 shows the equivalent SMBioNet file representation of the network of Figure 6.4. The text file contains four sections as follows:

- VAR
  This block represents the list of variables (nodes) in the graph with their respective thresholds. This information gives us clues on the number of states in the network. Here variable X contains 3 possible values from 0 to 2 and Y contains 2 possible values from 0 to 1. C does not appear in the list as it does not has any incoming arrows (called resources).

```
<?xml version="1.0" encoding="UTF-8"?>
<xs:schema xmlns:xs="http://www.w3.org/2001/XMLSchema"
elementFormDefault="qualified">
  <xs:element name="network">
    <xs:complexType>
      <xs:sequence>
        <xs:element ref="description"/>
        <xs:element ref="nodes"/>
        <xs:element ref="edges"/>
        <xs:element ref="mults"/>
        <xs:element ref="trans"/>
        <xs:element ref="simulation"/>
        <xs:element ref="ctl"/>
        <xs:element ref="normalNode"/>
        <xs:element ref="multNode"/>
        <xs:element ref="defaultNode"/>
      </xs:sequence>
    </xs:complexType>
  </xs:element>
  <xs:element name="description" type="xs:string"/>
  <xs:element name="nodes">
    <xs:complexType>
      <xs:sequence>
        <xs:element maxOccurs="unbounded" ref="node"/>
      </xs:sequence>
    </xs:complexType>
  </xs:element>
  <xs:element name="node">
    <xs:complexType mixed="true">
      <xs:sequence>
        <xs:element minOccurs="0" maxOccurs="unbounded" ref="K"/>
      </xs:sequence>
      <xs:attribute name="default" use="required" type="xs:integer"/>
      <xs:attribute name="id" use="required" type="xs:NCName"/>
      <xs:attribute name="kparam" use="required" type="xs:integer"/>
      <xs:attribute name="thres" use="required" type="xs:integer"/>
      <xs:attribute name="weight" use="required" type="xs:integer"/>
      <xs:attribute name="xcoor" use="required" type="xs:integer"/>
      <xs:attribute name="ycoor" use="required" type="xs:integer"/>
    </xs:complexType>
  </xs:element>
  <xs:element name="K">
    <xs:complexType>
      <xs:attribute name="id" use="required" type="xs:NCName"/>
      <xs:attribute name="value" use="required" type="xs:integer"/>
    </xs:complexType>
  </xs:element>
  <xs:element name="edges">
    <xs:complexType>
      <xs:sequence>
        <xs:element maxOccurs="unbounded" ref="edge"/>
      </xs:sequence>
    </xs:complexType>
  </xs:element>
  <xs:element name="edge">
    <xs:complexType>
      <xs:simpleContent>
        <xs:extension base="xs:NCName">
```

Figure 6.12: XML schema sample of our model XML file

- REG
  In this section, we described the list of regulations (activations and inhibitions) of the underlying network. Indirectly, this represents the list of multiplexes in the network. Each line contains the name of the multiplex followed by its formula and the target(s) on which this multiplex is acting. If the multiplex is prefixed by a ! sign, then this indicates an inhibition.

```
            <xs:attribute name="fromNode" use="required" type="xs:NCName"/>
            <xs:attribute name="id" use="required" type="xs:NCName"/>
            <xs:attribute name="toNode" use="required" type="xs:NCName"/>
          </xs:extension>
        </xs:simpleContent>
      </xs:complexType>
    </xs:element>
    <xs:element name="mults">
      <xs:complexType>
        <xs:sequence>
          <xs:element maxOccurs="unbounded" ref="mult"/>
        </xs:sequence>
      </xs:complexType>
    </xs:element>
    <xs:element name="mult">
      <xs:complexType>
        <xs:simpleContent>
          <xs:extension base="xs:NCName">
            <xs:attribute name="formula" use="required"/>
            <xs:attribute name="id" use="required" type="xs:NCName"/>
            <xs:attribute name="xcoor" use="required" type="xs:integer"/>
            <xs:attribute name="ycoor" use="required" type="xs:integer"/>
          </xs:extension>
        </xs:simpleContent>
      </xs:complexType>
    </xs:element>
    <xs:element name="trans">
      <xs:complexType/>
    </xs:element>
    <xs:element name="simulation">
      <xs:complexType/>
    </xs:element>
    <xs:element name="ctl">
      <xs:complexType>
        <xs:attribute name="formula" use="required"/>
      </xs:complexType>
    </xs:element>
    <xs:element name="normalNode">
      <xs:complexType>
        <xs:attribute name="size" use="required" type="xs:integer"/>
      </xs:complexType>
    </xs:element>
    <xs:element name="multNode">
      <xs:complexType>
        <xs:attribute name="formula" use="required" type="xs:integer"/>
        <xs:attribute name="size" use="required" type="xs:integer"/>
      </xs:complexType>
    </xs:element>
    <xs:element name="defaultNode" type="xs:integer"/>
  </xs:schema>
```

Figure 6.13: XML schema sample of our model XML file (continued).

- PARA
  The kinetic parameters and their corresponding values for each variable are specified in this section. Each kinetic parameter takes the form $K_{set\_of\_resources} = value$

- CTL
  Here, the set of properties that will verified in the whole graph are listed using CTL.

Figure 6.17 shows the equivalent SMBioNet file of the running example X-Y.

To benefit from the complete set of functionalities of DyMBioNet, existing networks from SMBioNet can be imported and integrated in DyMBioNet, which additionally gives the user some freedom to specify

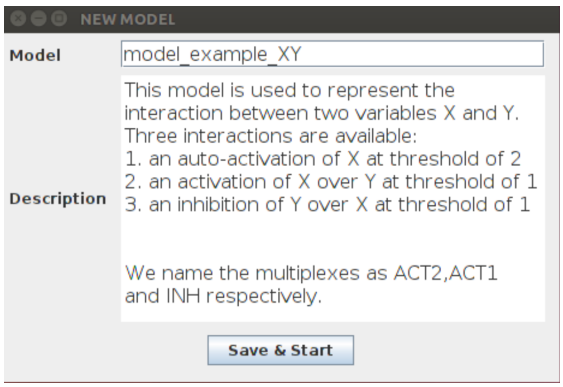


Figure 6.14: Creating a model from scratch with the name and description of the model.

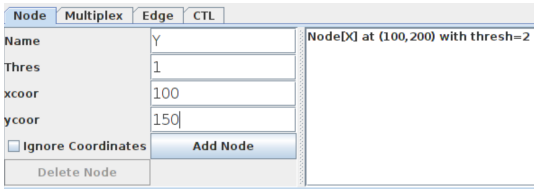


Figure 6.15: Creating a node by specifying its attributes.

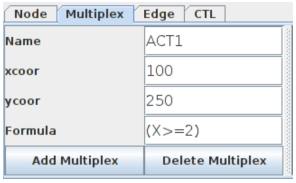


Figure 6.16: Creating a multiplex with its logical formula.

```
VAR
x = 0 2;
y = 0 1;
c = 0 1;

REG
inh [!(y>=1)] => x;
act2 [(x>=2)] => x;
act1 [(x>=1)] => y;
multcx [(c>=1)] => x;

PARA

# Parameters for X
K_x=0 ;
K_x+inh=2 ;
K_x+act2=2 ;
K_x+act2+inh=2 ;


# Parameters for Y
K_y=0 ;
K_y+act1=1 ;


CTL
(x=0 & y=0) -> !(E[EF(!(y=1)) U (AG(EF(!(y=1))))])
```

Figure 6.17: SMBioNet file of the running example.

coordinates of the nodes and multiplexes. This action will generate a new file (with a different extension from *.txt to *.xml). It is important to note that the minimum basic requirements of the SMBioNet file are the variables and regulation(activations and inhibitions). Missing K parameters as well as CTL can be added afterwards. DyMBioNet then exploits this XML file to perform simulations which SMBioNet

cannot handle. These simulations facilitate the observation of well-know properties as well as emerging ones. This transformation from SMBioNet to DyMBioNet format is explained in Figure 6.18 and detailed as follows:

- Line 11 and 16 contains the transformation of variables (VAR) into <node> elements
- Lines 29-31 represents the regulations (REG identifier) which is mapped into multiplexes in the XML file
- Line 11-14 encode the set of parameters for X (PARA) into a set of <K> elements (the number of K elements depend on the number of incoming degree of the variable)
- Line 16-17 encode the K parameters of Y

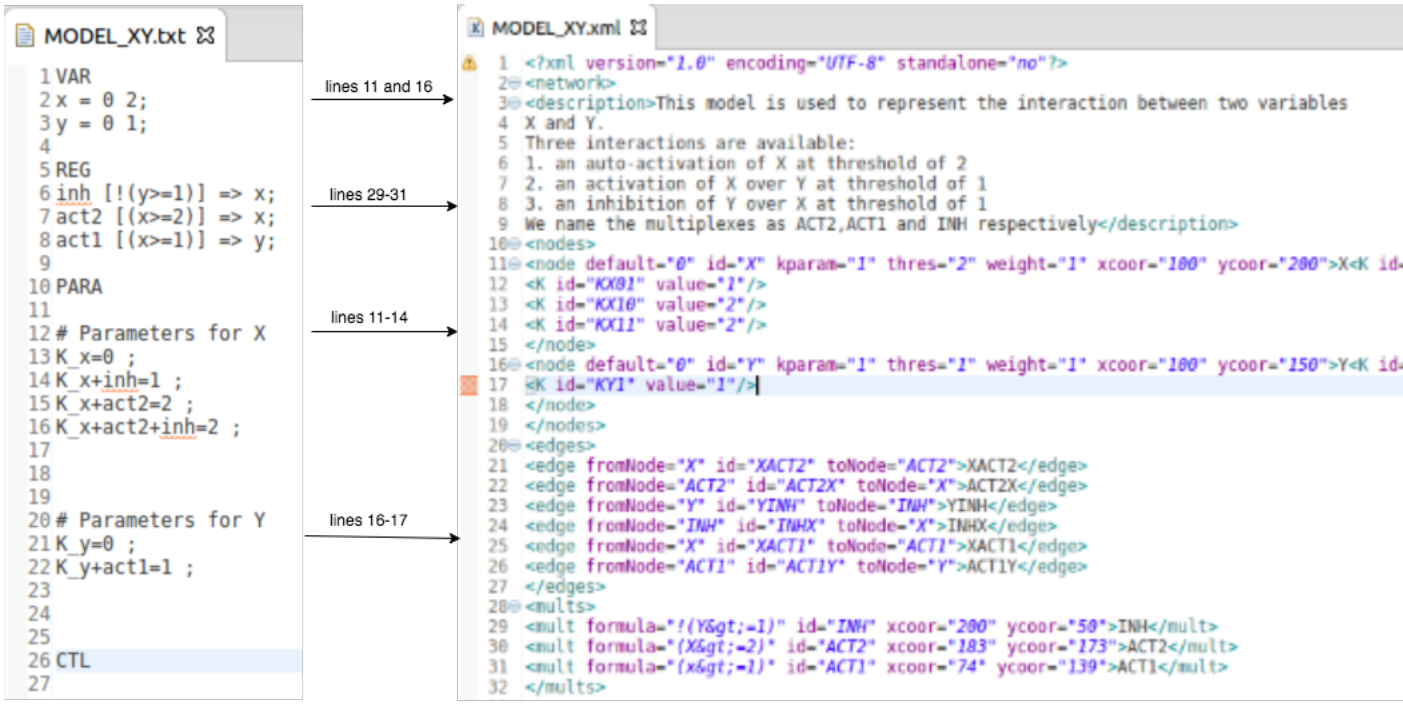


Figure 6.18: Conversion of SMBioNet text file to its equivalent XML file in DyMBioNet using "Open->SMBioNet" menu.

However, in SMBioNet, if the kinetic parameters are unknown, it is automatically been proposed by a set of all possible values according to the questions asked [which results in many models] or the number of model is one if all parameters are known [in our metabolic network].
The complete SMBioNet file for the energy metabolism network can be found in the Annex section 11.7.1.

6.5.3 Converting DyMBioNet files to SMBioNet format

SMBioNet is configured to run a text file with some pre-built specifications. A reverse conversion of DyMBioNet files (*.xml) to SMBioNet text files is possible (see menu in Figure 6.19). On top of that, we can run SMBioNet within DyMBioNet and visualise the output based on certain CTL formula. Sample valid models from SMBioNet are then listed in DyMBioNet by accessing the *Launch SMBioNet* option from the main menu. This is shown in Figure 6.20, listing the number of valid models out of the total number of models available.

6.5.4 Viewing network with thresholds and regulations

By default, DyMBioNet will display the network with its multiplexes, which already incorporates the sign for activation and inhibition. If the user wants to visualise the network with the sign, thresholds as well as regulations, the option *See w/o multiplexes* can be accessed as shown in Figure 6.21 and its results can be seen on the left hand side of Figure 6.23.

In case, there is more than one threshold for a given variable on a possible target, then we draw as many edges as there are different thresholds. For example consider Figure 6.22 where x can act on Z either as an activator above a threshold of 2 or as an inhibitor above a threshold of 1.

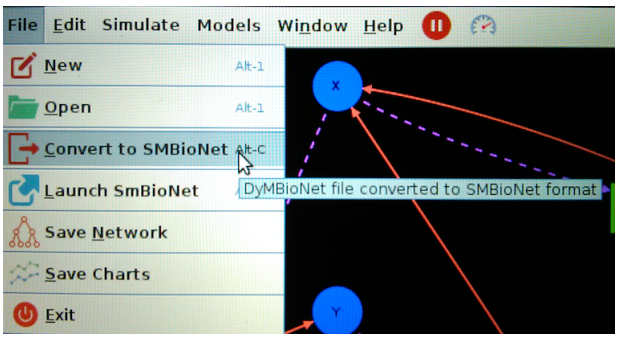


Figure 6.19: DyMBioNet to SMBioNet menu option.

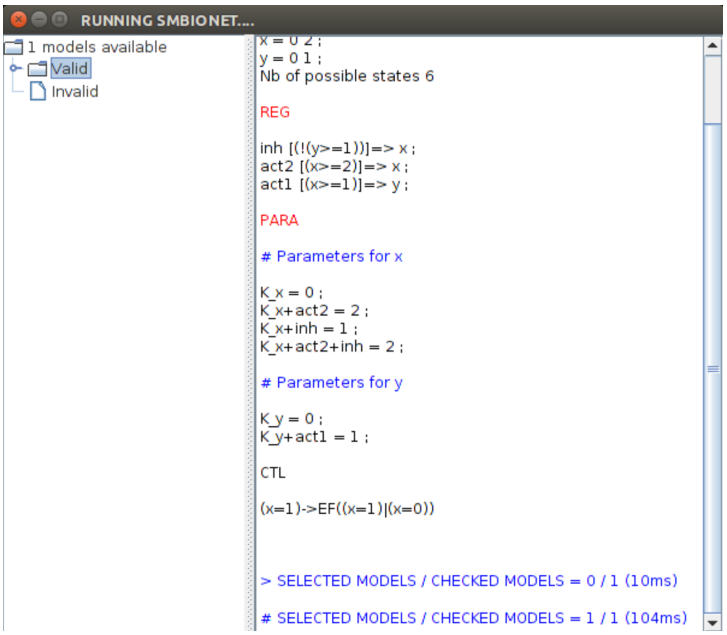


Figure 6.20: Launching SMBioNet within DyMBioNet.

Figure 6.21: Option to view thresholds and regulations of variables.

6.5.5 Viewing state transition diagrams

State transition diagrams can provide quick and concise information rapidly about the behaviour of the network and towards which state the network has the tendency to go. This is applicable when we have at most 3 variables. In such a case, the interface can allow the user to interpret stationary states as well as oscillations (see Figure 6.23) directly from the state transition diagrams from which important conclusions can be deduced rapidly. Nevertheless, when the number of variables exceeds 3, there is the state explosion problem as the number of states becomes exponential to the number of variables. This is not too practical to represent using the state transition diagram as it becomes unreadable. This is another reason to explain the importance of using CTL in such circumstances to study the dynamical properties of the system. This is why our software is limited to 3 variables as well and an example for a 3-variables network is available in Annex for illustrative purposes.

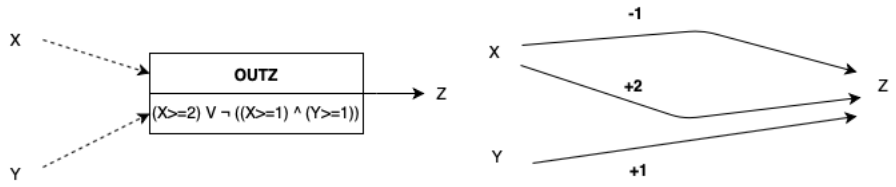


Figure 6.22: (Left) A multiplex where the variable X appears twice in the formula with different thresholds. In this example Z has only one possible ressource (OUTZ), thus 2 parameters K_Z.... (Right) Its translation into a Thomas network without multiplexes. Z has then 3 possible ressources, thus 8 parameters, and the respective values of the parameters have to reflect the truth table of the formula of the original multiplex.

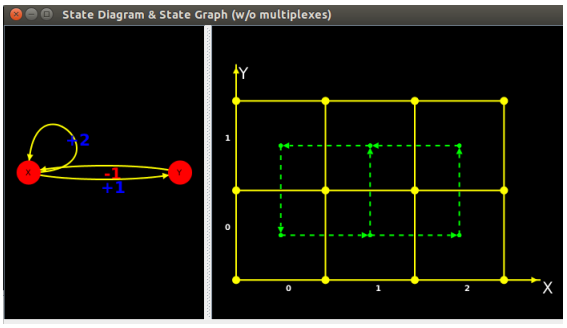


Figure 6.23: Regulations and state transitions

To cater for a large number of variables, GINsim, on the other hand adopts a different strategy. It takes a random order of all variables and repartition them arbitrarily on subgroups based on certain biological reasoning. This process can be repeated in a cascade manner until it is limited to a small groups and wherever it becomes difficult, certain intermediate transitions are skipped or represented by dotted arrows. But, it may be subject to errors in case there are missing regulations that have been ignored. At this stage, our model is too complex to have a visualisation of all state transitions and we prefer to use CTL to observe properties of the dynamical system which is further discussed in section **??**.

6.5.6 Network information

More information on the network can be viewed in this panel including the list of nodes and their respective thresholds and kinetic parameters(Figure 6.24 and 6.25). The threshold option enables the user to view all the outgoing arcs with a pair of threshold and sign. Similarly, using the K parameters interface, the user can view the set of K parameters for each variable, and selecting a particular row will display the necessary information in terms of resources. Modifying any K parameter, will automatically update the corresponding xml file.

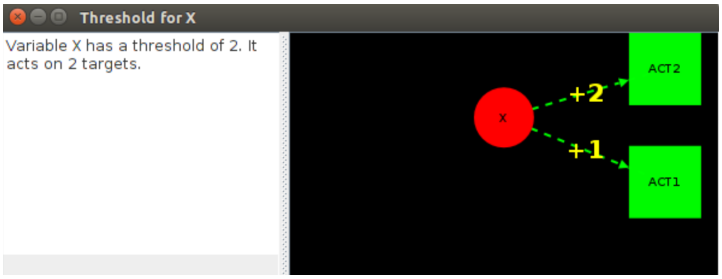


Figure 6.24: Thresholds

6.5.7 Analysing evolution of the network

We recall that a simulation is not limited by the number of variables compared to the state transition graph. In a simulation, we are able to capture the evolution of each variable/node and also main emerging properties of the network. Multiple combination of initial values for threshold are easily configured in

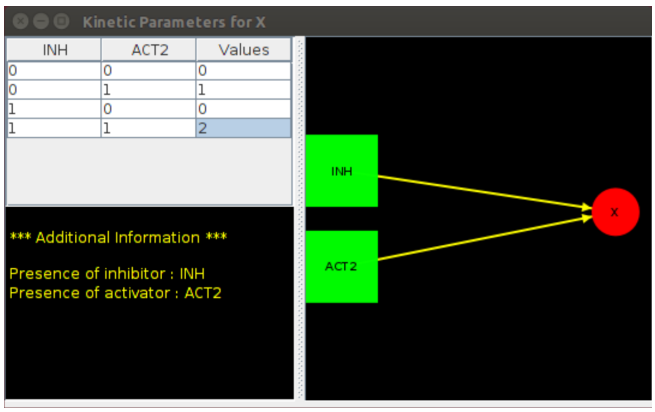


Figure 6.25: K parameters for X and Y

DyMBioNet to help carry out different simulations. The charts can be saved in *.png format. An option to choose variables of interest (checkboxes in the Graphs windows) is also proposed to the user. In the example given in Figure 6.26, we choose X=0 and Y=0 as initial conditions ans see how the network evolves over time. We can see that both X and Y oscillate alternately (they are in opposite phase). We can still put a meaningful title for the chart as it can be useful when presenting the chart in a report.

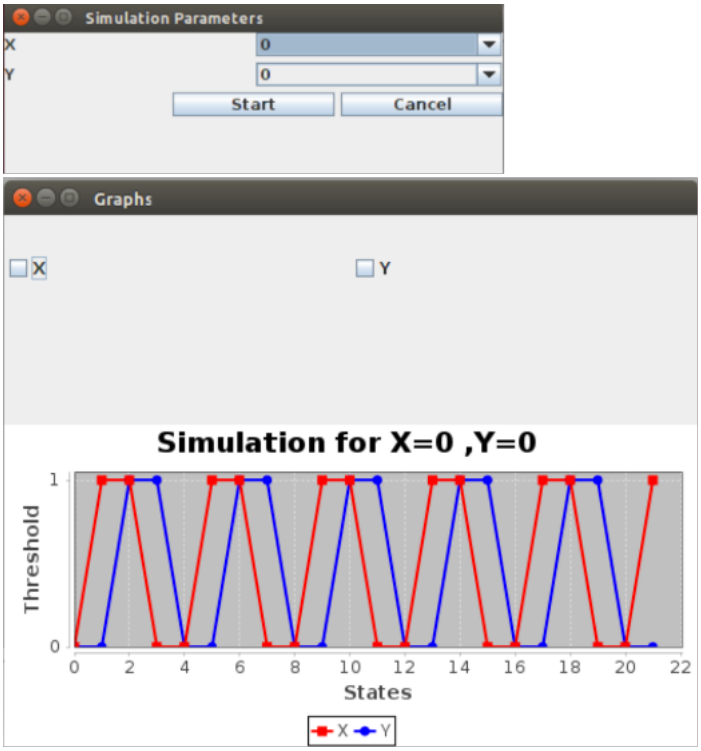


Figure 6.26: Dynamics of the model XY showing evolution of X and Y based on initial conditions X=0 and Y=0.

Since in biological network modelling, we are constraint to several simulations or several modelling scenarios, it is decisive that users are allowed to modify some network/chart parameters. The following modifications are available in DyMBioNet:

- Title/Axis of charts
- Colors of nodes based on threshold level
- Speed of simulation, in seconds
- Viewing specific charts data
- Position of nodes in the graph

- Initial states of variables

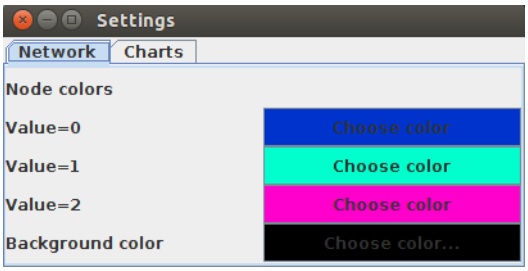


Figure 6.27: Network settings

6.5.8 Automating simulations

As soon as the model becomes gradually larger and complex, it is evident that simulations will be longer and can be frustrating for the modeller, especially when we have different input conditions. This hassle is eliminated in DyMBioNet to ease the work of the modeller. Instead, the user can specify all the required parameters for the given set of simulations and all the rest is taken care by DyMBioNet. In this case, the following input is required from the user (as shown in Figure 6.28): Number of simulations (this will generate tabs for each simulation), Number of states and Number of trials (number of times to run for each simulation). Once started, all observations are systematically recorded in *.png format for each simulation in the respective folder, which can be viewed and interpreted at a later time, as well as easily included in a scientific publication. We also have the possibility to save charts (observations) and network as images in *.png format (Figure 6.29). The advantages are two-fold :

- Firstly, these images can be included in any report/article to be published, avoiding the need to do screenshots each time the user wants to capture any important event in the network.
- Secondly, this functionality can be automated without user intervention. This means one can specify specific events (for example specific threshold of nodes) after which the network evolution/result can be saved. This prevents the user from waiting for a specific event to occur and pause the simulation to capture a screenshot of the graph and chart.

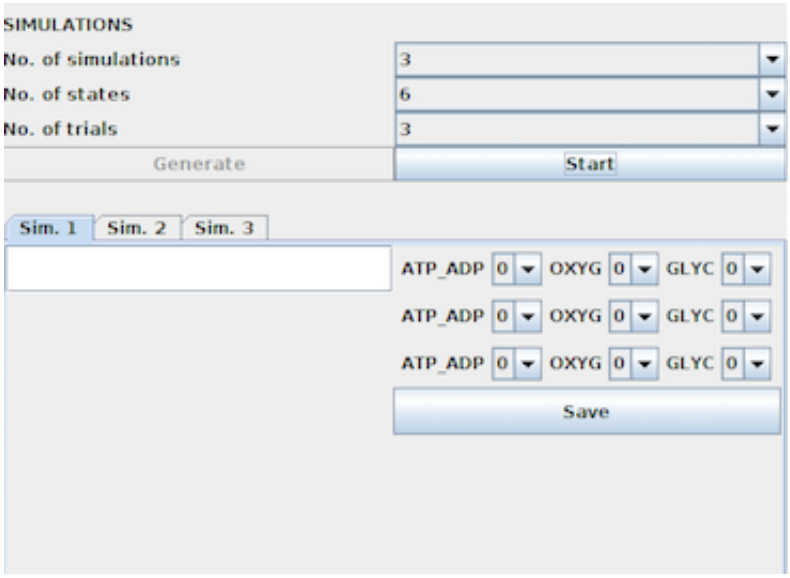


Figure 6.28: Automating simulations

For clarity, we take the running example where we can have several experiments based on the input conditions of C, X and Y (first column of validation matrix in section 6.6). Here, we have two sets of simulations to be carried out: C=0 and C=1 as detailed below. This automatic simulation becomes increasingly important when the number of variables is significant.

- Simulation 1 (C=0) and Number of trials = 2 (X<2 and X=2 which, for example, can be saved as c0_lowX and c0_highX respectively). A separate folder is created for each simulation.
- Simulation 2 (C=1) and Number of trials = 2 (X<2 and X=2)

While testing our model for different simulations (we perform more than 100 simulations manually), we were often confronted to spend a lot of time doing modifications on the input variables as well as which type of observations we expected. At some point of time, we also change one or two K parameters, which means we had to restart the 100 simulations. This motivated us to perform automatic simulations which was the last feature added to DyMBioNet.

We recall that SMBioNet already does the CTL verification task (see section 4.6.2 and 5.7) but does not integrate the fair CTL translation. We implement this functionality in DyMBioNet which does this translation of fair path CTL to its CTL equivalent before submitting as input to SMBioNet.

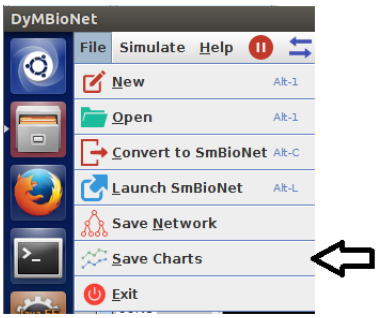


Figure 6.29: Saving charts / networks functionality

6.5.9 Documentation

Software documentation is an integral and decisive part of software engineering. It ensures good product quality, and maintenance in future versions. In DyMBioNet, Javadoc tool uses Java code to generate the API specifications, the software at least has a comprehensive API specification to bank on [**?**]. A comprehensive API specification is available in the Help section which is of paramount importance for assisting in designing the model as listed in Figure 6.30.

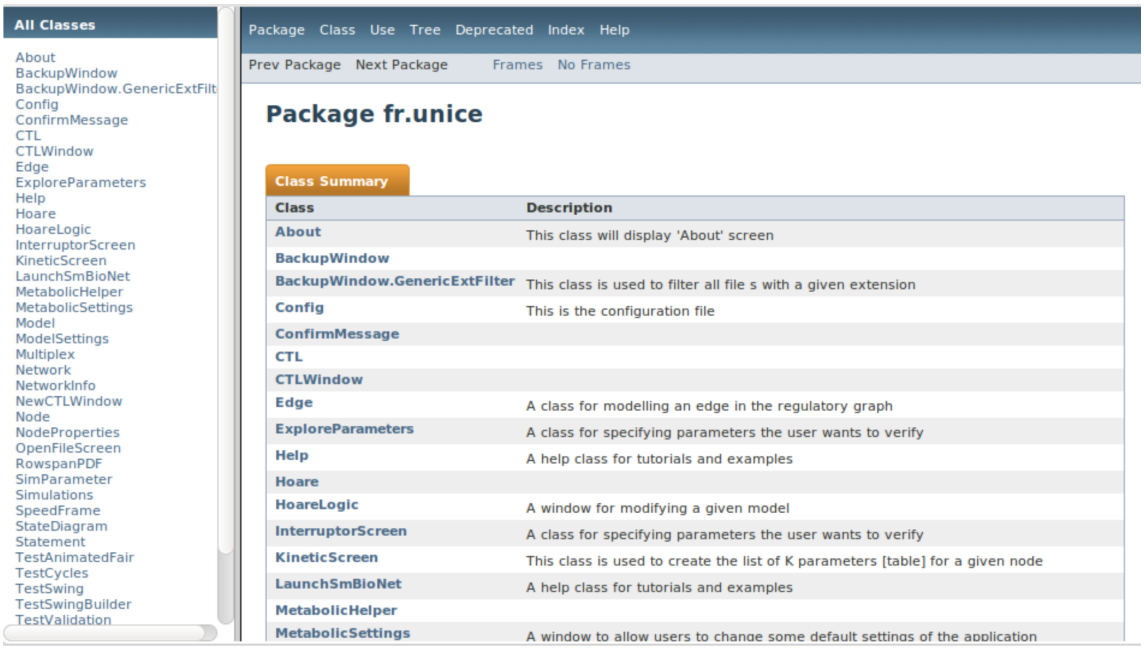


Figure 6.30: List of useful classes/methods

6.5.10 User documentation

As in all software platforms, a User documentation is available to accompany new users, with the different functionalities and it also include some demonstrated examples. The Help window shows five options as follows:

- Getting started
  In this option, users are presented with the different menus the software has to offer and how a network is constructed from start to finish.

- Basics
  The basics of the modelling approach are elaborated in this help menu with emphasis on : formal logics and Thomas modelling framework.

- Examples
  We explain a series of examples of 2-nodes network and 3-nodes network, how they can be constructed from XML files, their modifications and how we check CTL formulas to validate biological properties.

- Tutorials
  Sample tutorials are given in this menu with different scenarios allowing the user to get used to the modelling framework and how to tackle them.

- Answers
  We finally give answers to exercises we have in the tutorial section so that user can cross-check their answers.

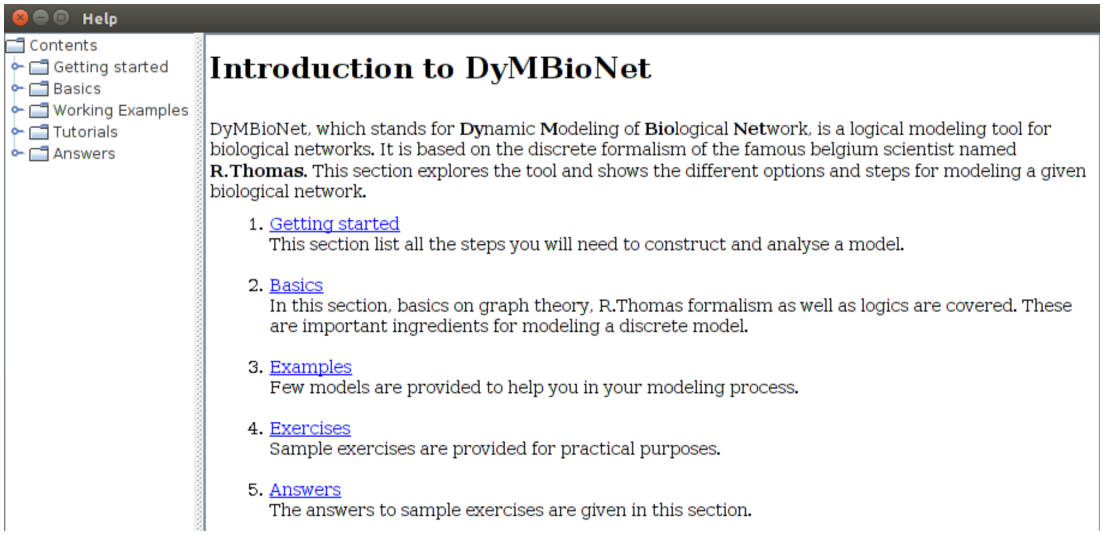


Figure 6.31: Help menu options

6.6 A scenario : a simple network with three variables

In this section, the sample network 6.4 is built by assembling the different nodes x, y and c with their respective biological information (thresholds, their typical actions and their K parameters). We follow part of our methodological approach and illustrate some sample steps. Figure 6.32 shows the appearance of the sample network in DyMBioNet: each variable(node) is represented by a circle and their interactions are pictured as multiplexes in rectangles containing the name of the multiplex and its formula, that formalizes the known cooperations or concurrencies with the appropriate formulas to indicate activations and inhibitions. On the right, each variable is configured using the threshold and kinetic options.

We also present two sample screens which allow us to (i) modify the threshold of a given variable (Figure 6.33) and (ii) visualise the network graphically (Figure 6.34).

For each node, we need to specify the coordinates (*which are optional*) in case of a small network and the boundary (maximum threshold).

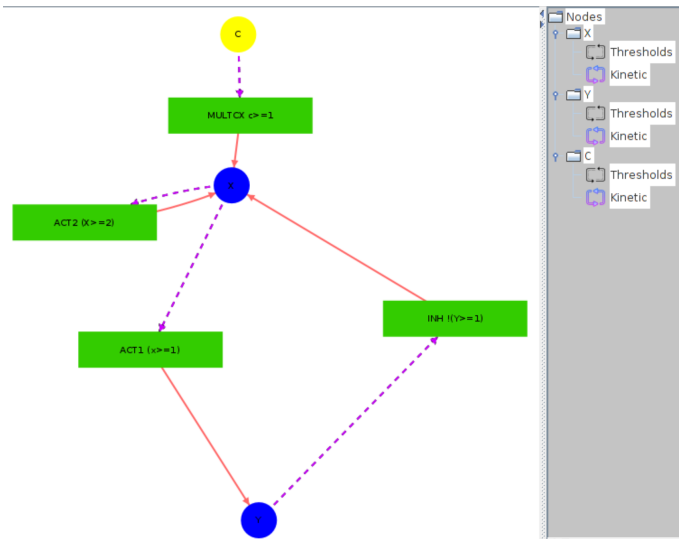


Figure 6.32: Regulation graph of X , Y and C

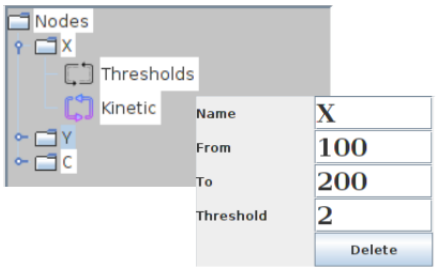


Figure 6.33: Threshold information of X

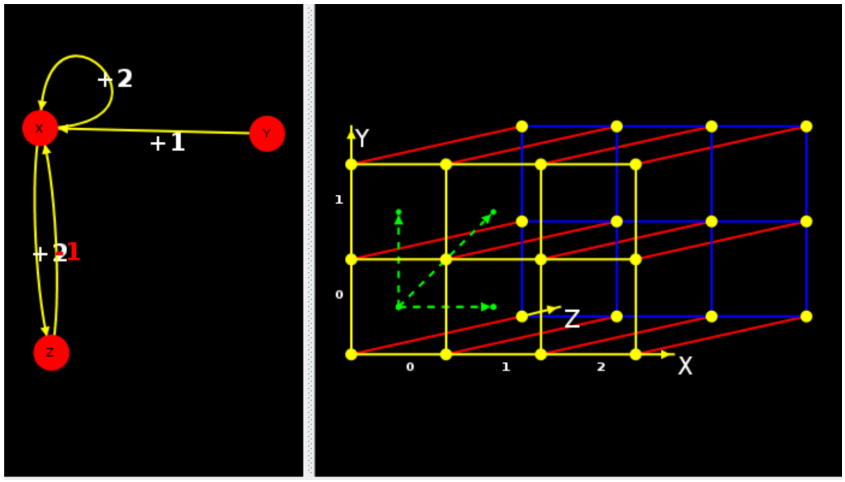


Figure 6.34: Regulatory network XYZ without multiplexes.

6.6.1 Adding known kinetic parameters

By default, each K parameter is assigned a value of 0 (column values in Figure 6.35). Users are free to modify the K values which are then automatically updated in the XML file. Selecting a row (a given K parameter) in the table of parameters will display additional information on the action of each source node and at the same time display on the graph on the right (see Figure 6.36). Variables not acting as resources are depicted with a cross on the arrow pointing to the target node.

6.6.2 Simulating the dynamics of the network and printing results

We treat two scenarios in this section: one where we have oscillations between x and y in Figure 6.37, and second where we have a basin of attraction in Figure 6.38 and 6.39.

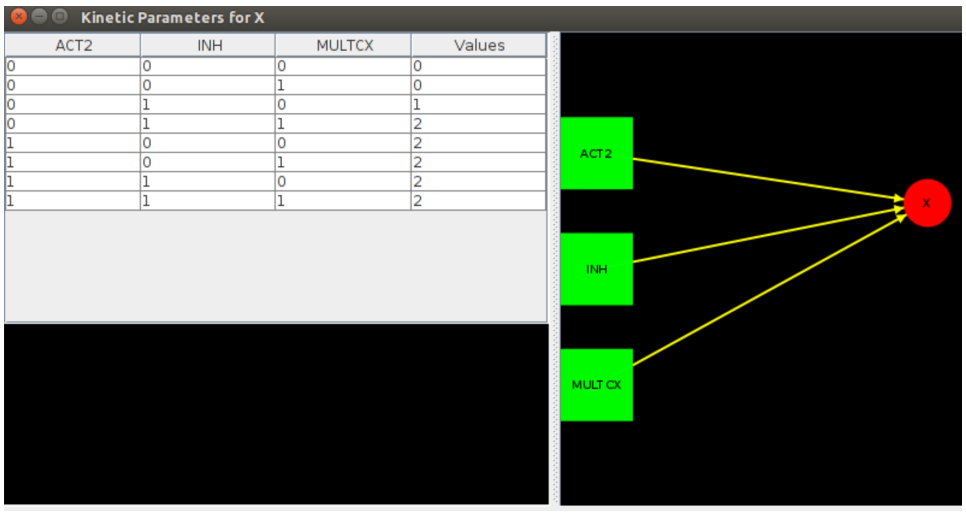


Figure 6.35: Table of all kinetic parameters for x

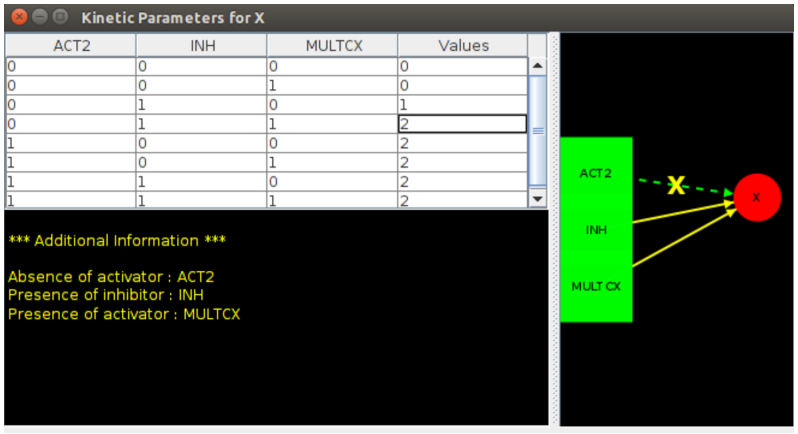


Figure 6.36: Kinetic Parameters for x with additional information on a given K param.

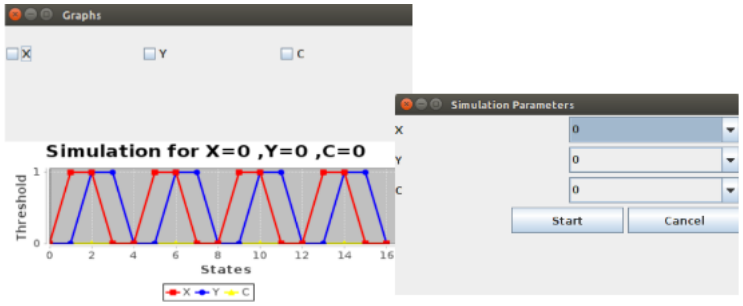


Figure 6.37: Simulation starting at x=0,y=0,c=0 and showing how oscillations prevent the variables from achieving stability.

Remarks:

1. we can do a simulation only if all the parameters are initialised
2. if some parameters remains unidentified, we can still test successively all the remaining parameters.
3. in this example, we have manually fixed all the possible values for the parameters.
4. by setting the values for x as 0 , y as 0 and c as 0, we observe long-term oscillation as shown in Figure 6.37.
5. by setting the values for x as 1 , y as 1 and c as 1, we either observe a short-term oscillation (Figure 6.38) or long-term oscillation until stable state is reached as shown in Figure 6.39.

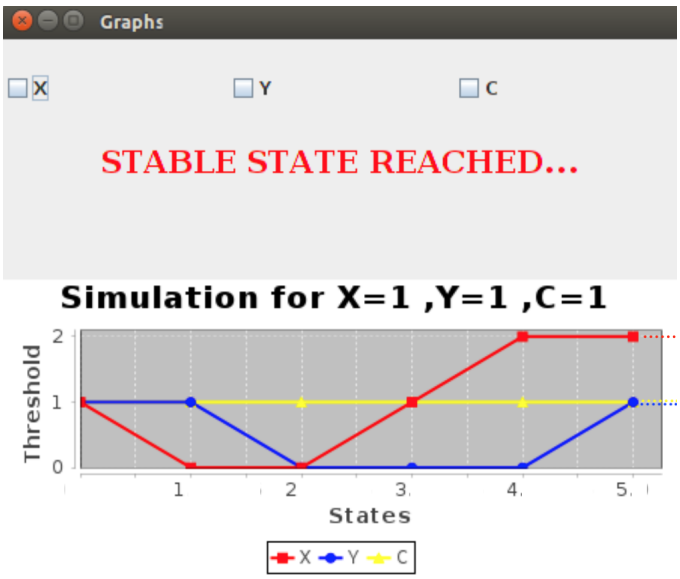


Figure 6.38: Simulation starting with x=1,y=1,c=1 and showing how stable states are achieved immediately (indicating bassin of attractions). This also means that all variables will thereafter maintain the same values; that is x will remain at 2 while y and c at 1 (see dotted lines).

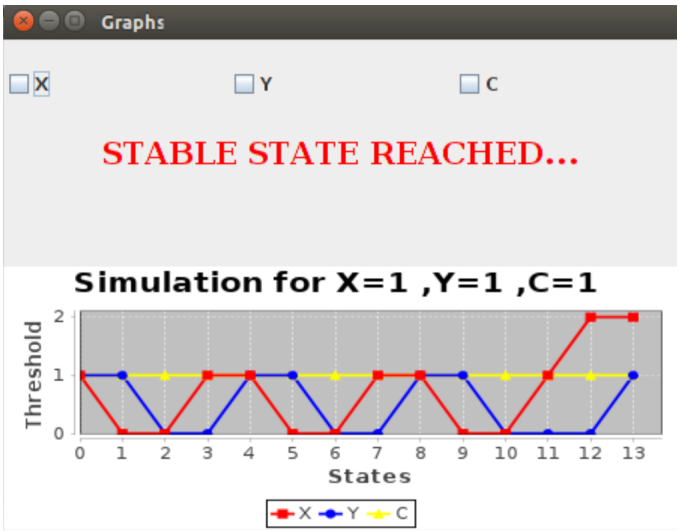


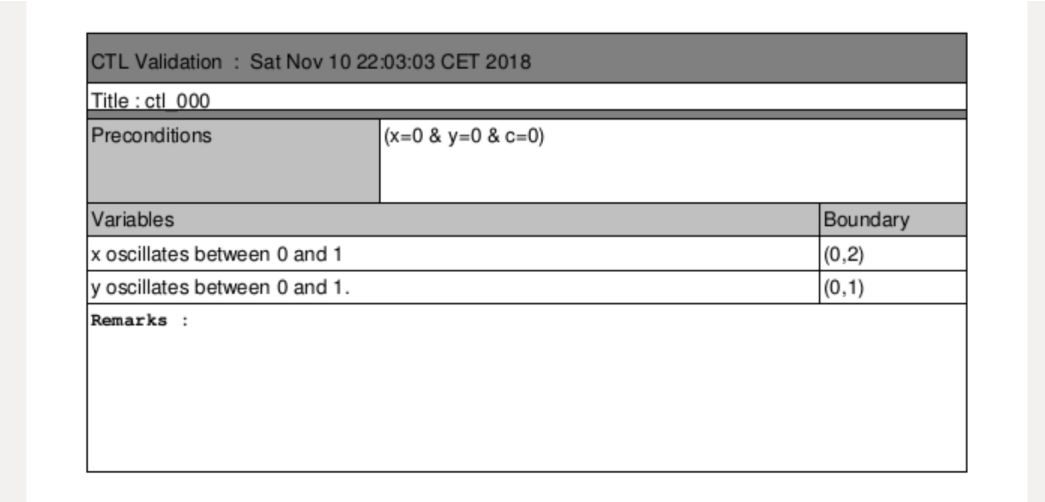
Figure 6.39: Simulation starting at x=1,y=1,c=1 and showing how stable states are achieved after a long time.

6. in fact the oscillation is not stable as it can be reached either in a short term or a long term depending on the arbitrary selection of values for the variables (for all possible transitions), and this represents a weakness for the simulation. We can wait for longer time before we attain the transition which allows the shift from this oscillatory behaviour to a stable state. This can leads us to two affirmations : we can believe that the system always oscillate when $x < 1$ at initial state, and also believe that there are two bassin of attractions (either with x=0 or or 1 on one side or with x=2 on the other side) when in fact we have only 1.

6.6.3 Adding CTL to verify biological properties

As we have observed from Figure 6.38 and 6.39, we can be exposed to cases where we have to wait longer to observe certain biological events. This is where we use model checking with CTL to write interesting CTL formulas to prove whether a behaviour (with certain preconditions) will occur. In Figure 6.41, we show how DyMBioNet facilitates writing CTL formulas. The encoded with our CTL formula is the following: if we start with initial conditions (x=0, y=0 and c=0), can we reach a state where x=1 ?. Since, these input conditions will generate oscillations of x and y (from Figure 6.37), DyMBioNet returns a 'YES' response. Otherwise, the other alternative is to specify only the input conditions, then DyMBioNet

calculates all the possibilities and generates the respective PDF file with all biological observations as shown in Figure 6.40, 6.42 and 6.43.



CTL Validation : Sat Nov 10 22:03:03 CET 2018	
Title : ctl_000	
Preconditions	(x=0 & y=0 & c=0)
Variables	Boundary
x oscillates between 0 and 1	(0,2)
y oscillates between 0 and 1.	(0,1)
Remarks :	

Figure 6.40: Simulation starting at x=0, y=0, c=0

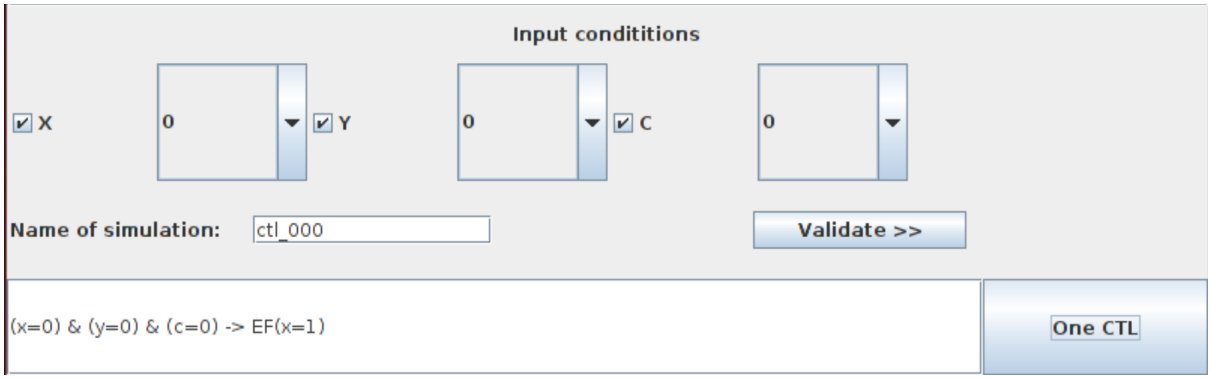


Figure 6.41: Running the CTL to check if oscillations of x and y are observed in the future with preconditions as x=0, y=0, c=0. This also proves that the absence of c prevents x to reach the value 2.

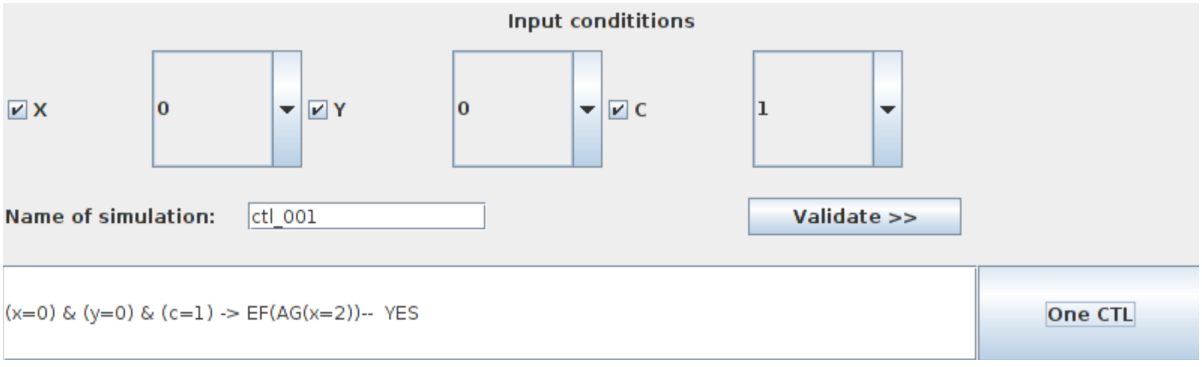
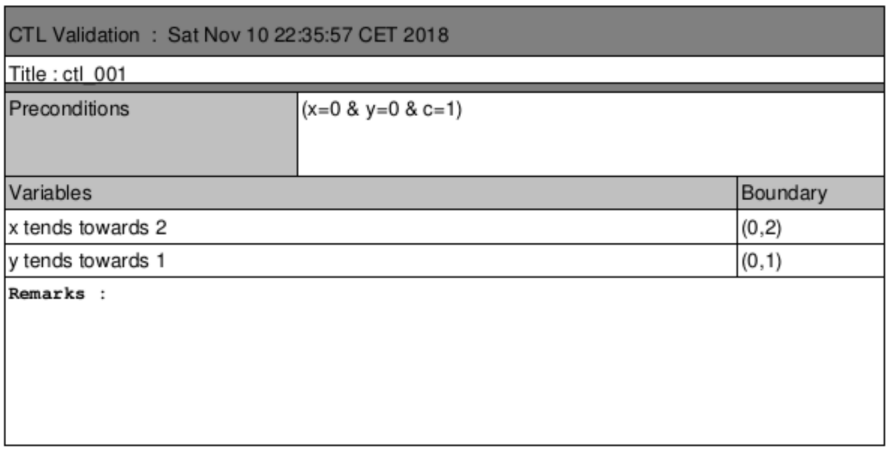


Figure 6.42: DyMBioNet interface to elaborate and check a CTL formula. Here we check if in the future x reaches the value 1 with preconditions as x=0, y=0, c=1

6.7 Conclusion of the chapter

This chapter portrayed the architecture and the usefulness of the software platform DyMBioNet as a simulation and visualisation tool for biological networks using the R.Thomas's approach. DyMBioNet



CTL Validation : Sat Nov 10 22:35:57 CET 2018	
Title : ctl_001	
Preconditions	(x=0 & y=0 & c=1)
Variables	Boundary
x tends towards 2	(0,2)
y tends towards 1	(0,1)
Remarks :	

Figure 6.43: Predictions starting at x=0, y=0, c=1

incorporates many functionalities to facilitate the task of the modeller in terms of constructing and simulating the dynamics of a given network through a user-friendly interface. The integration of SMBioNet to perform model checking of a biological network helps the modeller to refute or validate certain hypotheses.
DyMBioNet also accompanies the user according to the methodological approach we used from constructing to validating, and simulating the model. The software gives the user the interfaces to create the model from scratch and the user is allowed to modify the design at any moment. DyMBioNet offers:

1. a user-friendly interface to draw the interaction graph of the Thomas network with multiplexes
2. it generates proper XML files to memorize the networks under study and offers an easy way to classify them
3. it helps interactions with biologists in order to find the parameter values, offering different focused views
4. it performs simulations with easy to interpret visualisations of the results, including color codes to symbolize the expression levels of the variables w.r.t. their thresholds, as well as multiplex colors according to their truth values
5. it includes, and encapsulates within user-friendly interfaces, the main functionalities of SMBioNet, so that checking CTL formulas becomes easier
6. and it also incorporates a variant of CTL, which we call "fair path CTL" which only consider paths that do not infinitely ignore a possible transition in the state-transition graph of the models

In this chapter, we had the opportunity to demonstrate all these capabilities with a running example of three variables. We will equally show that the software can adapt well for any number of variables in Chapter 9.

CHAPTER 7

ABSTRACT GRAPH FOR THE REGULATION OF ENERGY METABOLISM

7.1 Introduction

In this chapter, we will show the methodology for constructing the abstract model of biological regulations which applies not only for gene networks but for metabolic networks as well. We reiterate our question of interest which is the regulation of the energy and biomass metabolism. As such, the notion of activator and inhibitor is adapted by using respectively the notion of "resource consumer" and "resource provider" in order to abstract the underlying mass action rules that governs metabolic fluxes in terms of regulations. We describe here five steps of our methodology (5) which we use to construct our metabolic graph (which can be extended to other regulatory graphs) :

1. Inventory of variables with respect to the scientific question and their classification in categories
2. Identification of the biological signals that may influence each target variables mentioned in step 1. (Definition of the multiplex, that is a meaningful name for each given biological signal and the group of variables that are part of this multiplex).
3. Defining the number of activation states for each variable mentioned in step 1: this is the number of outgoing arrows emerging from a given variable plus 1 (if counting background state 0).
4. Identifying activating threshold for each interaction between a variable and another variable or a multiplex.
5. Construction of the final graph.

7.2 Inventory of the pertinent variables

To address the energy and biomass regulations in our study, we categorise four types of variables for central carbon metabolism that enter into the description of the respiration-fermentative shift :

1. Metabolic pathways which are subdivided into catabolic and anabolic pathways
2. Molecular cofactors (ATP/ADP, NADH/NAD+)
3. Nutrients (glucose, O_2, amino acids and fatty acids) which are subdivided into internal metabolites and control nutrients

Next, we give the abstract and molecular definition of the variables associated to the four different classes.

7.2.1 Metabolic functions and pathways

From our coarse-grained and abstracted view of metabolism, we differentiate two types of metabolic pathways for representing our regulatory graph: catabolism and anabolism. The abstract definition below corresponds to the definition taken by the model. This abstract definition differs from the biological definition of the variable as at molecular level : a pathway may have many bifurcation points and might

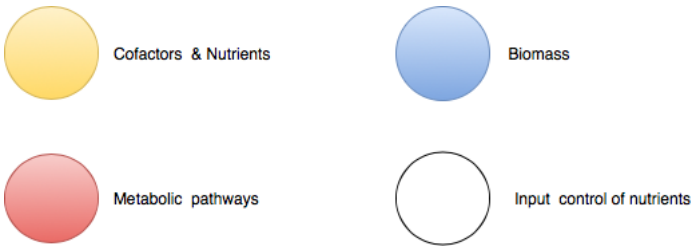


Figure 7.1: The color nodes for each class of variables used throughout the whole text.

promotes many other secondary pathways, and these are not considered in the model for the chosen granularity. The abstract definition of the pathway is just the primary role given by the model for each variable.

7.2.1.1 Catabolic pathways

We identified four catabolic pathway variables : Glycolysis, Krebs, Oxidative Phosphorylation and Fermentation.

- Abstract definition of Glycolysis (GLYC)
  Glycolysis is understood here as the degradation of glucose into pyruvate. During this process, it reduces NAD+ into NADH and produces two ATP from two ADP molecules. Note that glycolysis is not in this definition a provider of building blocks for amino acids and nucleic acids: the pentose phosphate pathway is instead taking resources from glycolysis. In our study, we assume there is a constant distribution between these two pathways, whatever the flow of glucose intake.

- Abstract definition of Krebs (KREBS)
  In the most abstract level, Krebs can be seen as a producer of mitochondrial NADH (from pyruvate fuelled by glucolysis) which acts as a resource for oxidative phosphorylation (PHOX). Krebs is therefore understood in its oxidative mode and not as its reductive branch.

- Abstract definition of Oxidative Phosphorylation (PHOX)
  PHOX is abstracted as a consumer of mitochondrial NADH to produce ATP.

- Abstract definition of Fermentation (FERM)
  The abstract definition of fermentation is very close to the biological definition : refuelling glycolysis cofactor NAD+ from NADH.

7.2.1.2 Anabolic pathways

To summarise, anabolism is synonymous to biomass, which we categorise in two forms : lipidic and non-lipidic. We have two anabolic functions that resume to biomass in the cells :

- Abstract definition of production and storage of lipidic biomass(LBP)
  This represents all complex lipids (ex all families of fatty acids) forming a certain percentage of biomass in cells.

- Abstract definition of production and storage of non-lipidic biomass(nLBP)
  This represents DNA/RNA which are precursors for protein synthesis contributing to another form of biomass.

7.2.2 Cofactors

Two cofactor pairs play central role in metabolism: ATP/ADP and NADH/NAD+. The first is the energetic money of the cell and the second can be seen as a reservoir of electrons and protons. NADH/NAD+ provides electrical energy in the respiratory chain (PHOX). In glycolysis and fermentation, it plays a dual role for oxidative and reductive reactions.

- Abstract definition of ATP/ADP (simplified as ATP in our model)
  In this model, the ratio ATP/ADP is supposed to be constant: if ATP is maximum, ADP is minimum. A “zero” level of ATP means that the cell cannot synthesize biomass.

- Abstract definition of NADH/NAD+ (simplified as NADH in our model)
  The NADH/NAD+ corresponds to both cytoplasmic and mitochondrial reduced and oxidized nicotinamide dinucleotide species. As for ATP/ADP, the ratio NADH/NAD+ is supposed to be constant: if NADH is maximum, NAD+ is minimum. A “zero” level of NADH means that fermentation or oxidative phosphorylation cannot work. A zero level of NAD+ means that glycolysis cannot work.

7.2.3 Nutrients

This section is divided into two parts : nutrients which are internal metabolites and nutrients which act as control variables for the energy metabolic network.

7.2.3.1 Internal metabolites

The cellular regimes for the regulation of the energy metabolism inside cells, called internal metabolites, are Glucose, O2 and Glutamine. They are abstracted as follows:

- Abstract definition of Glucose (GLC)
  Glucose symbolize the organic matter, i.e. the carbon source, the reservoir of protons and electrons needed for electric energy production.
- Abstract definition of O_2
  Oxygen is the acceptor of electrons used by the respiratory chain. Absence of oxygen is similar to hypoxic condition and prevents oxidative phosphorylation, and promotes fermentation as an ATP production pathway. The shift from respiration to aerobic glycolysis occurs even in presence of oxygen.
- Abstract definition of nitrogen and carbon donors (NCD)
  Nitrogen and carbon are essential precursors for the synthesis of amino acids and lipids. A majority of these nitrogen and carbon skeletons are derived from metabolic processes like glycolysis and Krebs, which we abstract here as NCD.

7.2.3.2 Input variables

In order to control the level of nutrients which are important to mimic cellular environments (like hypoxia, cancer microenvironment , etc), we use three input variables namely FA to mean Fatty Acids, AA to mean Amino Acids and $InO2$. We do not have an input for glucose since there are no actions (regulations) on glucose in our model. Therefore, glucose (GLC) is both a nutrient as well as an input variable.

Now, that we have the full list of variables, we need to see all the possible interactions between these variables to construct the metabolic network, and this is exactly what we will do in the next section.

7.3 Identification of regulation signals (metabolism)

The goal of this section is to identify regulation signals or more specifically to distinguish all biological regulation signals that influence a given variable of the system. This has to be done for all variables enumerated in the previous section. This is a preliminary identification as we are only here creating a group of variables for each regulation signal or regulation mechanism without mentioning at this stage the state condition of each variable for this regulation signal to occur. It is therefore an inventory of the phenomena that act on that given target variable.

This regulation signal can be triggered by a single variable or by several variables. We suppose this regulation to act as an on/off signal, for which the target variable switches to another state if in the group of variables that form the input signal (the multiplex), each variable is at the required activated state. If the notion of a "state" of a variable can yet be defined as its capacity or not to act on another variable, it is not possible at the moment to tell how many states a variable may have as it depends on the number of outgoing arrows in the network, that is on the number of distinct action this variable can do on another (single) variable to change its state.

For illustration, suppose a gene transcript with arbitrary 11 transcript levels numbered 0 to 10. Suppose that this gene transcript induces a single biological regulation signal if it reaches level 5. From levels 5 to 10, this gene transcript induces the same effect. This gene transcript has one threshold (corresponding to internal transcript level 5) which induces two actions: i) doing nothing (inactive state corresponding to

transcript level 0 to 4) ii) or doing something (active state corresponding to transcript level 5 to 10). In this example, the gene has two states (active/inactive). The number of states of a variable corresponds therefore to the number of different outgoing signals (outgoing arrows in the network) that this variable exhibits. We will first determine the number of states for each variable, that is, studying the outgoing signals for each variable.

The answer is subtle: a given variable may be involved in multiple regulation signals (often complex, i.e. involving many input variables, hence the name "multiplex" for expressing in a formal way these regulation signals). Worse, the threshold of this given variable might be the same for several multiplexes, i.e. regulation signals. Yet, these different regulation signals (i.e. different multiplexes) should be identified first before being expressed as "target variables" of this given "input variable". It is exactly what we are doing in this section 7.3: identifying the multiplexes (i.e. the biological phenomena of this regulation signal and variable involves) such that they could appear in the next section amongst the target variables (of a given input variable) for states identification of this input variable. At the moment, it is just enough to give a name to each regulation signal that a given input variable can induce and to collect all the variables involved in this signal.

Once the threshold will be known from the next section 7.4, the exact running logical condition of these multiplexes will be established (section 7.5). Note that a multiplex may contain a single variable with a precise condition for that variable (ex. Glucose >0 induces Glycolysis). So, nearly all signals can be expressed as a multiplex. Exception may concern linear signals in which a product linearly depends on the input concentration of substrate. Such steady state signals are not considered in our regulation network except to model the medium and high level of glycolysis which linearly depends on glucose input.

Throughout the whole text, we use the same schematic template to represent the influencers for other variables with three parts : the variable under investigation on the right, the direct influencers in the middle and finally on the left we have those variables which have cooperative actions on the variable through multiplexes (green rectangles). For readability, we list all the multiplexes in Table 7.1.

Table 7.1: List of multiplexes

Multiplex	Biological name	Description
COF	Cofactors	Cofactors (ATP and NADH) necessary for glycolysis
GR	Glycolysis regulation	Citrate, the first product of the citric acid cycle, can also inhibit PFK and as a result inhibit glycolysis (Glycolysis Regulation).
AnO	Acetyl-CoA and Oxygen	The transition from glycolysis to Krebs cycle is under the presence of Acetyl-CoA (Pyruvate) and Oxygen
BOX	$\beta - Oxidation$	Krebs can still function in the absence of glycolytic activities by the degradation ($\beta - oxidation$) of lipid biomass.
SAT	Saturation	The reverse of the Krebs cycle is possible by the degradation of lipid biomass (LBP).
PC	Phox-Control	The transition from Krebs to Oxidative Phosphorylation when oxygen is present.
EP	Excess Pyruvate	Shifting glycolysis to fermentation using pyruvate as an intermediate
LS	Lipids synthesis	Krebs at high level generate citrate which can be converted to acetyl-CoA for synthesising fatty acids.
PPP	Pentose Phosphate Pathway	The normal pathway for producing intermediates for nucleotides synthesis.
AAS	Amino Acids synthesis	High availability of Nitrogen and Carbon donors (ex glutamine) contributes to the synthesis of amino acids important for driving non-lipidic biomass production.

7.3.1 GLYC

Glycolysis is directly influenced by the input of glucose, the presence of cofactors (ATP and NADH) as well as the absence of the PFK1 : glucose drives glycolysis, ATP (respectively ADP) and NADH (respectively NAD+) are intermediate regulators for the chain of reactions occurring during glycolysis and finally PFK1 has a major role in the regulation of glycolysis (ex in the phosphorylation of glucose).

We display these interactions in Figure 7.2.

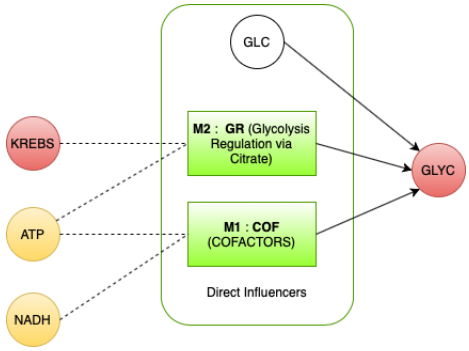


Figure 7.2: Influencers of glycolysis (GLYC)

7.3.2 KREBS

Here, we note two versions of Krebs : an oxidative and normal mode controlled by AnO (Acetyl CoA and oxygen ; that is normal glycolysis) or a reductive mode that can be controlled either by SAT (through NCD via &-KG) or by the degradation of biomass through BOX (beta-oxidation).

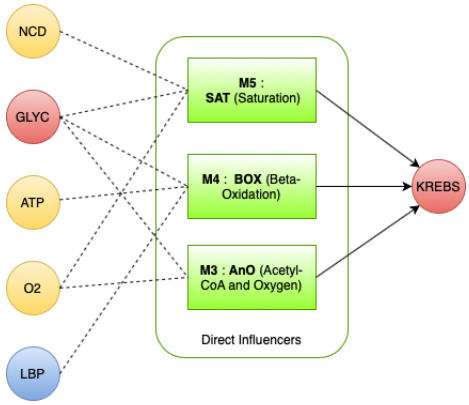


Figure 7.3: Influencers of Krebs (KREBS)

7.3.3 PHOX

Oxidation phosphorylation occurs only in the presence of oxygen. The sole and direct influencers of Oxidative phosphorylation are oxygen, ATP and NADH whose combined actions are merged in the multiplex PC (Phox-Control in Figure 7.6) as they are all pre-requisites for PHOX to happen.

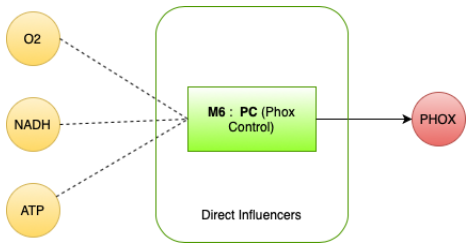


Figure 7.4: Influencers of Oxidative Phosphorylation (PHOX)

7.3.4 FERM

In our model, fermentation occurs only in the presence of excess pyruvate which results from the combined action of three variables : oxygen (its absence triggers fermentation but in the presence of high glucose milieu, fermentation process can be triggered even in the presence of oxygen), glycolysis (acts as donor of pyruvate) and NADH (see Figure 7.5).

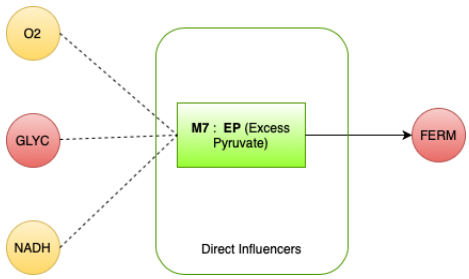


Figure 7.5: Influencers of fermentation (FERM)

7.3.5 nLBP

The building blocks for proteins are either through amino acids synthesis or from DNA and RNA through PPP (itself being a subway from glycolysis).

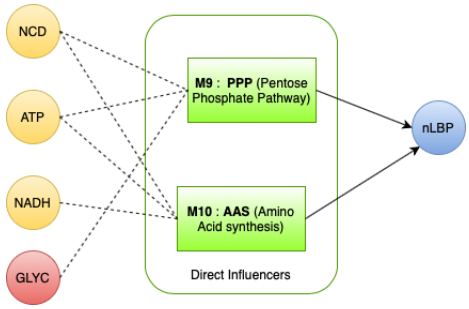


Figure 7.6: Influencers of Oxidative Phosphorylation (PHOX)

7.3.6 LBP

We abstract the two possibilities for lipidic biomass as either the degradation of storage form of lipids through the multiplex BOX or the production through the multiplex LS from NCD and fatty acids.

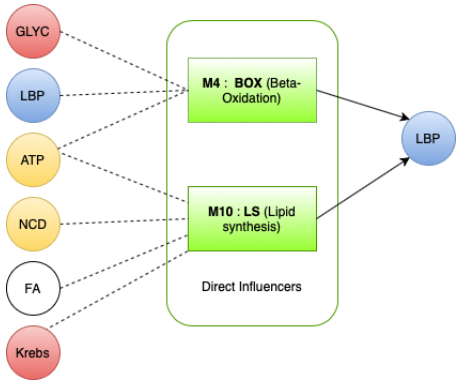


Figure 7.7: Influencers of lipidic biomass (LBP)

7.3.7 ATP

Energy in the forms of ATP can either be produced (through GLYC or PHOX) or consumed when the biomass machinery (both LBP and nLBP) is "ON".

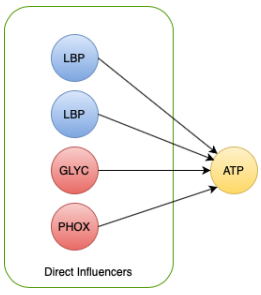


Figure 7.8: Influencers of ATP

7.3.8 NADH

In Figure 7.9, the direct influencers of NADH are the multiplex AAS (amino acids synthesis). Simultaneously, Ferm consumes NADH and Krebs is a feeder of NADH to PHOX.

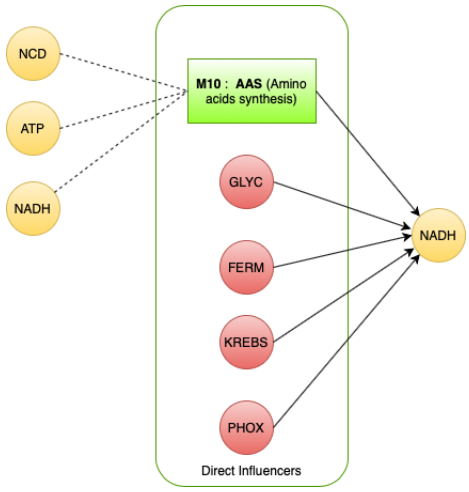


Figure 7.9: Influencers of NADH (NADH)

7.3.9 O2

Oxygen is under the control of either a unique provider : *Input of oxygen* or a unique consumer : *Phox*, as shown in Figure 7.10.

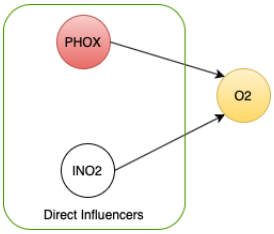


Figure 7.10: Influencers of Oxygen (O2)

7.3.10 NCD

The contributions of both nitrogen and carbon donors in the cells directly influence its storage form, and there are two cellular activities which can degrade it : either through reductive Krebs or it is used as a nutrient in the production of biomass. We display this in Figure 7.11.

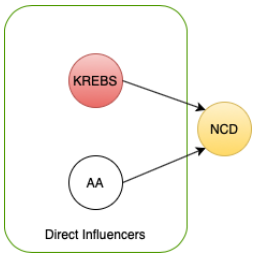


Figure 7.11: Influencers of NCD : The reservoir of carbon and nitrogen elements are filled from Krebs or Amino acids

Table 7.2 lists, for each given variable, the influencers of this variable, i.e. the individual variable or set of variables that may induce a switch on this given variable. Note that there are no influencers for *input* variables.

Table 7.2: List of influencers

Variables	Influencers	Multiplex	Comments
GLYC	GLC KREBS ATP NADH	M1 : [COF] M2 : [GR]	Cofactors necessary for Glycolysis Glycolysis regulation via citrate
KREBS	ATP GLYC O2 LBP NCD	M3 : [AnO] M4 : [BOX] M5 : [SAT]	Pyruvate and not HIF1 with oxygen Beta-Oxidation Saturation
PHOX	NADH/NAD+ O2 ATP	M6 : [PC]	Phox-Control
FERM	NADH/NAD+ GLYC O2	M7 : [EP]	Excess Pyruvate
LBP	GLYC ATP NADH NCD	M9 : [PPP] M10 : [AAS]	Pentose Phosphate Pathway Amino acids synthesis
nLBP	GLYC ATP	M4 : [BOX] M8 : [LS]	Beta-Oxidation Lipids synthesis
ATP	LBP nLBP GLYC PHOX	-	-
NADH	AAS KREBS PHOX FERM	M10 : [AAS]	Amino acids synthesis
GLC	-	-	-
O2	PHOX INO2	-	-
NCD	AA KREBS	Amino acids synthesis and Krebs provide the necessary nitrogen and carbon skeletons for anabolism.	
INO2	-	-	-
AA	-	-	-
FA	-	-	-

7.4 Identifying the number of effective states for each variable : Thresholds

After the preliminary identification of the metabolic variables and their list of interactions, the next crucial step is to find the threshold which make each interaction feasible.

The identification of the different thresholds for a given variable is mainly based on the biologist's knowledge of the system and on experimental results. We usually assume the underlying variable is at a basal level (a level insufficient to act on its target) and how increasing its activity influences its targets in a particular incremental order. We describe the thresholds for all variables in this section and we follow the same order as in section 7.3.

1. Glycolysis
   For the catabolism of glucose in the cytosol, both cofactors ADP and NAD+ are needed to produce ATP and NADH respectively. The interdependency between ATP and NADH offers an acceptable explanation for the same threshold of 1 for both of the edges GLYC → NADH and GLYC → ATP. The smooth running of metabolism (normal respiration) occurs in the presence of oxygen and low glucose intake, which explains the threshold of 1 for Krebs (via pyruvate) and PPP.

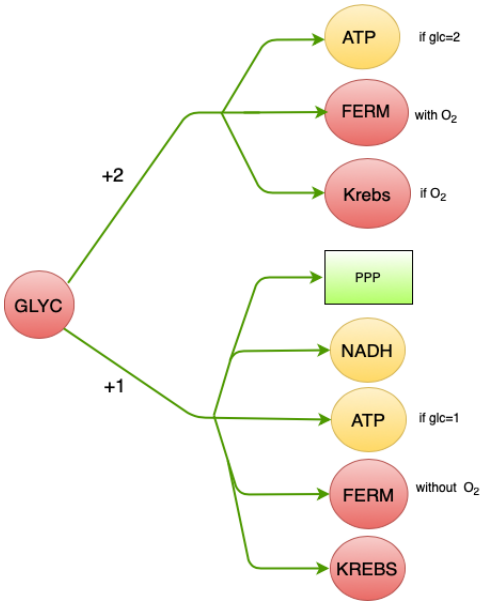


Figure 7.12: Thresholds for glycolysis : Two thresholds justifying the two levels of glucose intake.

   There are two scenarios by which pyruvate can take the fermentation path: first in the absence of oxygen or limited oxygen (known as anaerobic respiration), pyruvate is fermented to lactate in organisms (indicated by the +1 on the edge GLYC → FERM without O_2). The second scenario is when excess pyruvate accumulates in the cytoplasm causing a shift from Krebs to fermentation, even in the presence of oxygen (this explains the threshold of 2 which is a result of high glycolytic activity). An attribution of level 0 represents a glycolysis that does not produce enough intermediates, e.g. pyruvate, useful to other metabolic pathways (such as the Krebs cycle), nor any noticeable ATP.

2. Krebs
   A myriad of enzymatic reactions occurs during the normal oxidative phase of the Krebs cycle producing intermediate compounds NADH, $FADH_2, CO_2$ and ATP. This corroborates with the threshold of 1 for Krebs → NADH at a reasonable level for Oxidation phosphorylation to proceed.

   Classically, in normoxia, Krebs obtains carbon sources for its activities from glucose via glycolysis. In hypoxic condition, Krebs derives its carbon fuel from nitrogen and carbon donors through $\alpha-$Ketoglutarate ($\alpha - KG$). The relative ratio of citrate and other byproducts in this reverse Krebs far exceeds the ratio produced by normal Krebs [140]. This explains the same threshold of 2 for the edges Krebs→ NCD (reductive Krebs) and Krebs → GLYC (over-expression of Krebs using citrate to inhibit glycolysis via PFK1). It is important to highlight that since Krebs supplies NADH for the proper functioning of oxidative phosphorylation, we have preferred to omit the direct link between

Krebs and PHOX (instead Krebs is linked to NADH and NADH to Phox). Krebs also provide the necessary precursors for lipid synthesis. Overall, we conclude that Krebs has two thresholds. A level of 0 means a low flux for Krebs (that is not enough NADH for PHOX).

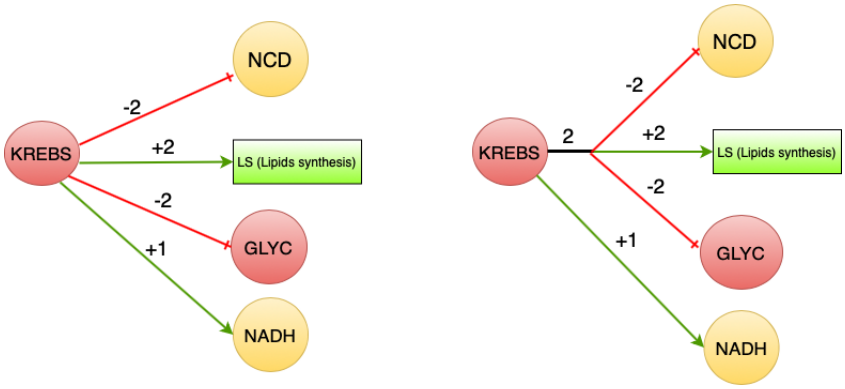


Figure 7.13: Threshold for Krebs. Normal oxidative Krebs occurs at threshold 1 to produce NADH. An alternative reductive role of Krebs is to produce citrate (then acetylCoA) for the production of biomass (LBP). This citrate has an inhibitory effect on glycolysis. The threshold of -2 is the sign of reductive Krebs.

3. Oxidative Phosphorylation
   The omnipresence of oxygen in the mitochondria helps PHOX to reduce NADH (respectively $FADH_2$) to NAD+ (respectively FAD). We are assuming that given the presence of oxygen, this biochemical process will produce ATP and NAD+ at the same time. This explains the same threshold for the edges: PHOX $\rightarrow O_2$, PHOX $\rightarrow$ ATP/ADP, PHOX $\rightarrow$ NADH/NAD+. Only one threshold is available for PHOX. The inhibitory effects of PHOX over NADH and O_2 are grouped together. Note that PHOX below a threshold of 1 is purely an anaerobic condition.

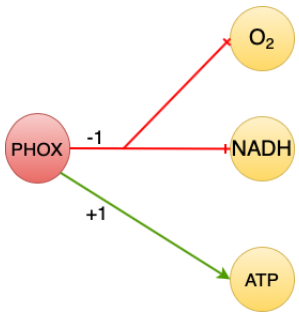


Figure 7.14: Threshold for PHOX : Oxidative phosphorylation consumes NADH and oxygen to produce ATP simultaneously in normal respiration. This explains the same threshold of 1.

4. Fermentation
   In anaerobic conditions, fermentation traps NADH from glycolysis to produce NAD+, which is *de novo* consumed by glycolysis. This justifies its threshold of 1 in the link FERM $\rightarrow$ NADH as FERM has only 1 target (that is FERM is boolean).

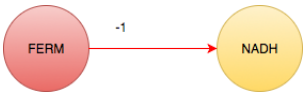


Figure 7.15: Threshold for fermentation : NADH is the only target for fermentation justifying the presence of only one threshold.

Remarks:

- For the time being, we assume that fermentation variable also includes its by-products (in our case lactate).

– We also take into consideration only the presence of glucose (in cancer cells, for example, cell proliferation can occur irrespective of the presence of glucose). In such cases, cells derive part of their energy by consuming the lactate product from neighbouring cells due to low oxygen supply (hypoxic condition) caused by the thickening of the blood vessels.

5. LBP (Lipid biomass production)
LBP is an anabolic process which consumes energy in the form of ATP and is also an activator (it is degraded by BOX) for Krebs. This overall "give and take" occurs at a normal threshold of 1.

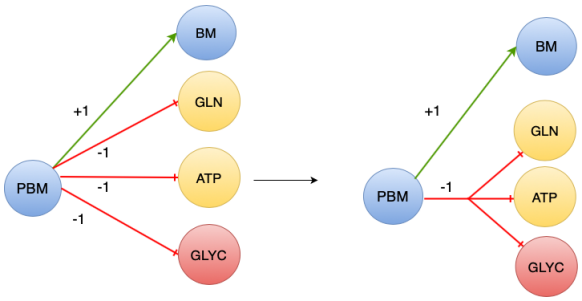


Figure 7.16: Threshold for Lipid biomass production: Necessary minimum level of precursors are supplied to BOX with a meagre depletion of ATP

6. nLBP (non-Lipid biomass production)
There is only one target variable for nLBP which is the depletion of ATP and this explains only 1 threshold for nLBP.

7. ATP/ADP
An accepted level of ATP (energy requirements) is needed for the production of biomass inside cells. Here, we assume the same level is required for lipid and amino acids synthesis (that is a sort of equilibrium). As a reminder, we have grouped the whole cellular ATP in one node (that is we have not differentiated cytoplasmic and mitochondrial ATP separately). We also make a distinction between simple processes (like BOX) which requires low ATP in contrast to the big machinery of PHOX which requires an ATP level of 2. The remaining links share the same threshold of 1 where ATP acts an an activator. All these are summarised in Figure 7.17.

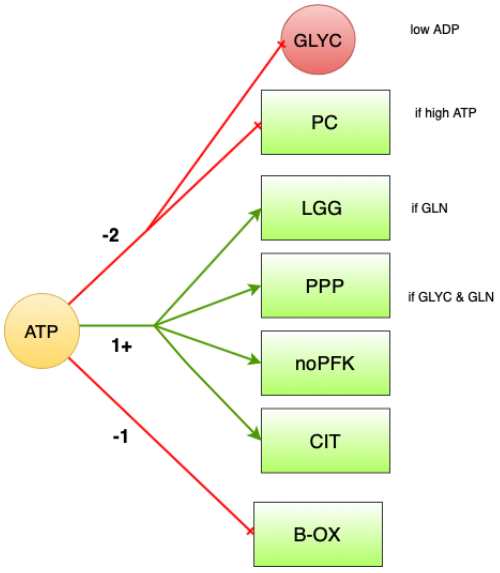


Figure 7.17: Threshold for ATP/ADP: A positive threshold for ATP helping targets and a negative threshold means ADP helping targets

8. NADH/NAD+
We assume equally a balance of the ratio NADH/NAD+ (as well as $FADH_2/FAD$) is present in cytoplasm and mitochondria. NAD+ is consumed by glycolysis to refuel NADH (-1 on the link NADH → GLYC), while NADH is an activator on the other edges. Both FERM and PHOX

consumes NADH to produce NAD+. This offers a feasible explanation of a threshold of 1 for all outgoing edges of NADH. A level of 0 would mean that NADH is not sufficiently strong to act on its targets. We show these interactions in Figure 7.18.

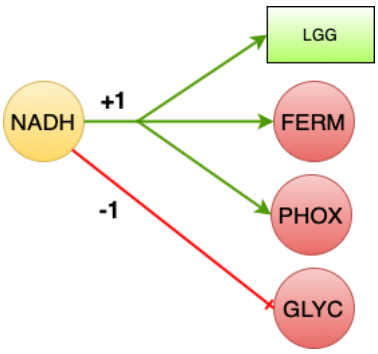


Figure 7.18: Threshold for NADH/NAD+ : NADH is boolean and is consumed (in terms of NAD+) by glycolysis only.

9. Glucose
   The primary source (and crucial element) for running glycolysis is glucose. This is indicated by a + sign on the two edges GLC → GLYC. When there is a mild level of glucose in cells, a normal respiration is observed. However, the whole metabolic pathways are impaired when too much glucose passes through the intracellular membrane. This is typical of the Crabtree and Warburg effects where cells will favour the fermentation pathway despite the high yield of oxidative phosphorylation. This is why we made a distinction by allowing two thresholds to justify that a '1' means low glucose and a value of '2' means high glucose level. This eventually leads to 2 thresholds for glycolysis as well.

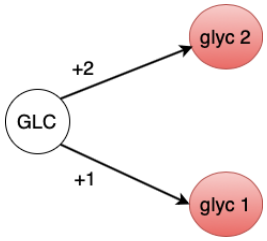


Figure 7.19: Threshold for the input variable glucose : '1' for low glucose and '2' for high glucose levels

10. Oxygen
    We distinguish two levels of oxygen as follows : if oxygen is present in very low amount (that is level 0), cells enter fermentative mode (-1 on EP link). On the other hand, normal oxygen levels (level 1) in the cells favours normal respiration and as such favours Krebs (either via AnO or via reductive Krebs : SAT), and at the same time, it contributes to the normal functioning of the larger PHOX cycle.

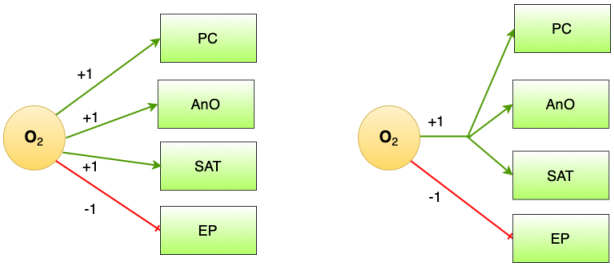


Figure 7.20: Threshold for oxygen : Normal oxygen (value=1) for Krebs to function via SAT or from pyruvate obtained via glycolysis.

11. NCD
    NCD represents the Nitrogen and Carbon Donors, useful to the cell and are derived from amino

acids (AA). These donors are precursors for many anabolic activities : at level 0 NCD action is too low to undergo anabolic processes; at level 1 it can participate to the activation of the PPP; while at level 2, it contributes to intensive anabolic activities like lipid synthesis (LS) and AAS (amino acids synthesis) and also fuels reductive Krebs.

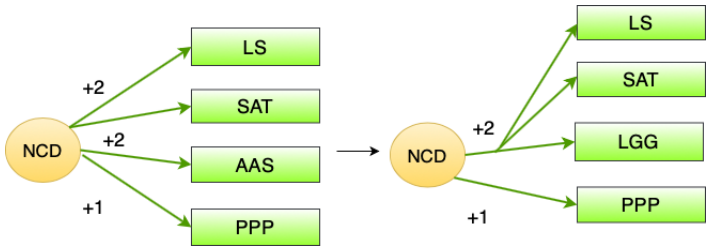


Figure 7.21: Thresholds for NCD : Nitrogen and carbon skeletons are important for almost all anabolic activities. A level of 0 means its inactive state.

12. FA, AA and InO2
The input or environmental variables, namely fatty acids, amino acids and oxygen, refer to the abstract level of nutrients we consider important for metabolic processes, and they play vital roles in the study of Warburg/Crabtree effect. FA and InO2 are both boolean variables simulating different cell's milieu. We differentiate 2 levels for AA since we consider its contribution to lipid and amino acids synthesis varies.

7.5 Logical description of the multiplexes

As a reminder from chapter 5, we distinguished two types of multiplexes : implicit (where there is only one atom and no biological significance in our model) and explicit (where there are at least two atoms influencing the target variables). In this section, we first list all the explicit multiplexes in the same order as described in Table 7.2.

- M1 - [COF] : $\neg(ATP \geq 2) \wedge \neg(NADH \geq 1)$
The multiplex COF, which refers to cofactors, gives the minimal condition necessary to induce normal glycolysis:

  Hypothesis: As soon as there is ADP and NAD+, the glycolysis is working at normal rate.

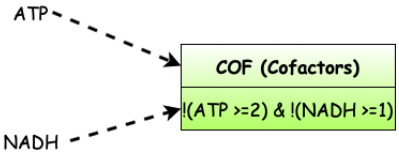


Figure 7.22: ADP (!ATP) and NAD+(!NADH) are both important cofactors required for glycolysis.

- M2 - [GR] : $\neg[(KREBS \geq 2) \wedge (ATP \geq 1)]$
The multiplex GR gives the condition to inhibit glycolysis by ATP (ATP ≥ 1) meaning also the absence of PFK (Phospho-fructo-kinase) and an accumulation of citrate. Moreover, the end product of glycolysis (pyruvate) fuels the TCA cycle and is transformed into citrate, which, if in excess, inhibits glycolysis. So, GLYC is inhibited when both conditions are satisfied as indicated in the multiplex formula.
- M3 - [AnO] : $(GLYC \geq 1) \wedge (O_2 \geq 1)$
A variety of glucose transporters (GLUT1 to GLUT5) are available to facilitate the entry of glucose molecules through the cell membrane [149]. Under normal conditions ($glyc >= 1$), glucose is converted to pyruvate which in turns is converted to acetyl-CoA in the presence of oxygen ($O_2 >= 1$). Pyruvate molecules are then used to continue the Krebs cycle which is the second step of aerobic glycolysis.
- M4 - [BOX] : $(BM \geq 1) \wedge \neg(GLYC \geq 1) \wedge \neg(ATP \geq 1)$
In conditions of glucose deprivation !($glyc >= 1$), cells undergo a catabolic process during which

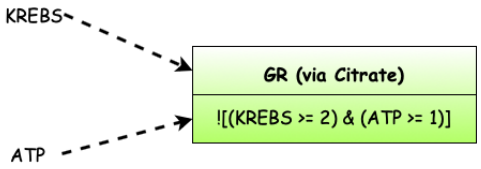


Figure 7.23: Diagrammatic representation of GR. High activity of Krebs produces citrate which inhibits glycolysis through the inhibition of PFK, the pacemaker of glycolysis.

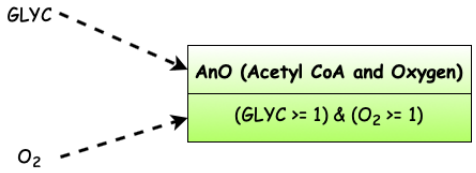


Figure 7.24: Diagrammatic representation of AnO : the conditions necessary for Krebs under normal glycolysis.

fatty acids (storage form of lipid biomass; $LBP >= 1$) are broken down to release a significant amount of acetyl-CoA which enters the mitochondria to be further metabolised in the Krebs cycle. This catabolic activity of fatty acids ensures a continual supply of ATP (ADP is used; $!atp >= 1$). This process is known as $\beta - Oxidation$, hence the name of the multiplex : BOX.

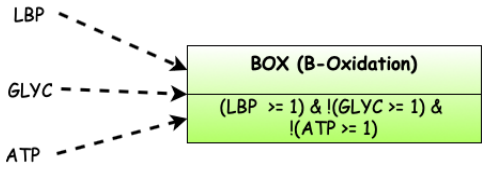


Figure 7.25: Diagrammatic representation of β-Oxidation implementing the degradation of fatty acids in the mitochondria

- M5 - [SAT] : $[((GLYC \geq 1) \wedge (NCD \geq 2)) \vee (GLYC \geq 2)] \wedge (O_2 \geq 1)$
  Biosynthetic precursors, in the form of NCD, act as fuels for the Krebs cycle in conditions of metabolic stress. This stress can take the form of impaired pyruvate transport to the mitochondria or simply an impaired glycolytic pathway. Alpha-ketoglutarate (α-KG) is a well-known intermediate of the Krebs cycle and hence one of the important candidates in the role of cellular metabolism [141]. Metabolic changes have been noticed in cancer cells where aerobic glycolysis is tightly related to the update of glutamine, for example, for fatty acid synthesis [142]. Overall, SAT or α-KG can occur in this stress condition or in normal glycolytic pathway in the presence of high glucose level ($glyc >= 2$). Both steps require oxygen ($O_2 >= 1$).

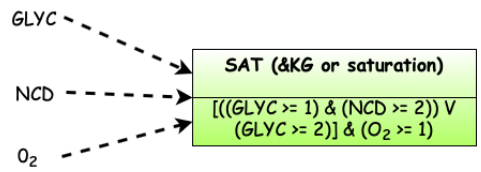


Figure 7.26: Diagrammatic representation of α-KetoGlutarate either in the presence in low glucose or high glucose levels

- M6 - [PC] : $NADH \geq 1 \wedge O_2 \geq 2 \wedge \neg(ATP \geq 2)$
  The multiplex PC gives the condition of normal rate pyruvate respiration.

  Hypothesis: This multiplex defines the respiration of normal or high rate of pyruvate which is made through oxidative branch of the Krebs Cycle and oxidative Phosphorylation. It is a very efficient pathway to producing ATP and renewing the oxidized form NAD+ needed for Glycolysis (30 ATP produced in practice per molecule of glucose). However, it is slow compared to high rate aerobic glycolysis (fermentation pathway under high glycolysis which produce even more ATP and

building blocks per unit of time (Experimental evidence: cells proliferate faster under fermentation than respiration).

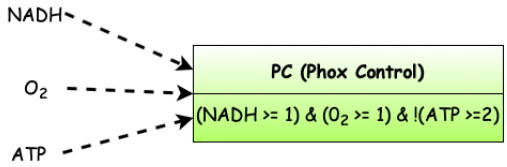


Figure 7.27: Diagrammatic representation of Phox-Control which occurs in the presence of reasonable level of oxygen to convert NADH to NAD+ and ADP to ATP.

- M7 - [EP] : $[((GLYC \geq 1) \wedge \neg(O_2 \geq 1)) \vee (GLYC \geq 2)] \wedge NADH \geq 1$
  The multiplex EP (Excess Pyruvate) gives the condition necessary to induce FERM in the absence of oxygen. For this to occur, we also need NADH with glycolysis as conditions.

  This condition for fermentation corresponds to the one observed by Otto Warburg in cancer cells i.e. independent of the presence of oxygen and under high glucose uptake. Under this metabolic regime, the production of biomass is maximum not because of the ATP production yield is high, in fact it is an inefficient metabolism (2 ATP per molecule of glucose) but because the flux of ATP production per unit of time is high under high rate of fermentation.

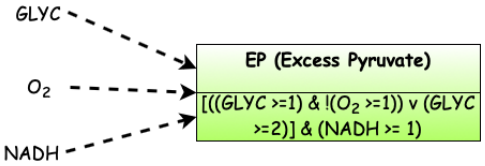


Figure 7.28: Diagrammatic representation of Excess Pyruvate (EP) in both low (absence of oxygen) and high glucose (either low or high oxygen) milieu.

- M8 - [LS] : $((KREBS \geq 2) \vee (NCD \geq 2) \vee (FA \geq 1)) \wedge (ATP \geq 1)$
  Krebs, when over-expressed, is a major provider of precursors (ex acetyl-CoA) needed for lipid synthesis with NCD and FA as donors of the necessary carbon and nitrogen skeletons. This anabolic activity requires a fair amount of ATP for building lipidic biomass.

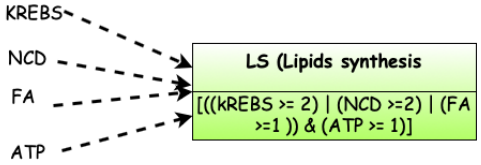


Figure 7.29: Diagrammatic representation of citrate showing its contribution in lipid synthesis (biomass) via AcetylCoA

- M9 - [PPP] : $(GLYC \geq 1) \wedge (ATP \geq 1) \wedge (NCD \geq 1)$
  The multiplex PPP governs the induction of Pentose Phosphate Pathway which takes place at an early stage of glycolysis which therefore needs ATP. Here we make the hypothesis that a sufficient amount of ATP is needed to run PPP. Glycolysis and NCD provides the necessary precursors for PPP.

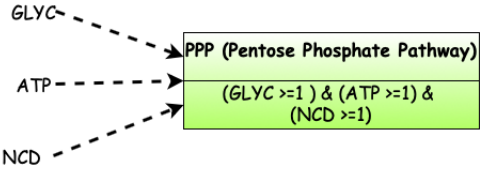


Figure 7.30: Diagrammatic representation of Pentose Phosphate Pathway. We assume a normal contribution of all precursors in terms of energy, nitrogen and carbon skeletons.

- M10 - [AAS] : $(NCD \geq 2) \wedge (ATP \geq 1) \wedge (NADH \geq 1)$
  The multiplex AAS summarises the necessary elements to produce new amino acids, such as nitrogen and carbon given off by the products of degradation of amino acids outside the cell (NCD $\geq$ 2), a large amount of NADH (NADH $\geq$ 1), and ATP at least for some of the amino acid synthesis reactions (ATP $\geq$ 1).

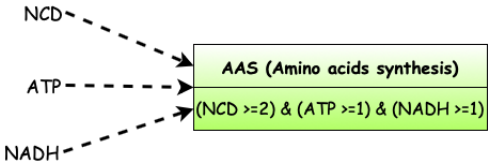


Figure 7.31: Multiplex AAS (Amino acids synthesis) : AAS obtains most of its nitrogen and carbon elements from NCD

7.6 Conclusion : The metabolic graph

In this chapter, we have seen an overview of the main building blocks of the metabolic network. The interconnections between each building block has been displayed which mirror the equivalent biological representation of energy and biomass metabolism. This has given rise to an abstract view of the biological metabolic graph focusing on the regulations rather than enzymatic or molecular reactions. By considering the main metabolic processes as main variables and compacting their interconnections using multiplexes, we have been able to showcase the discrete representation of the energy metabolism at a coarse-grained level. We conclude this chapter with a discrete and formal representation of the metabolic graph which is static, and which will be useful in the next chapter to determine the K parameters; a prerequisite for modelling the dynamics of the network.

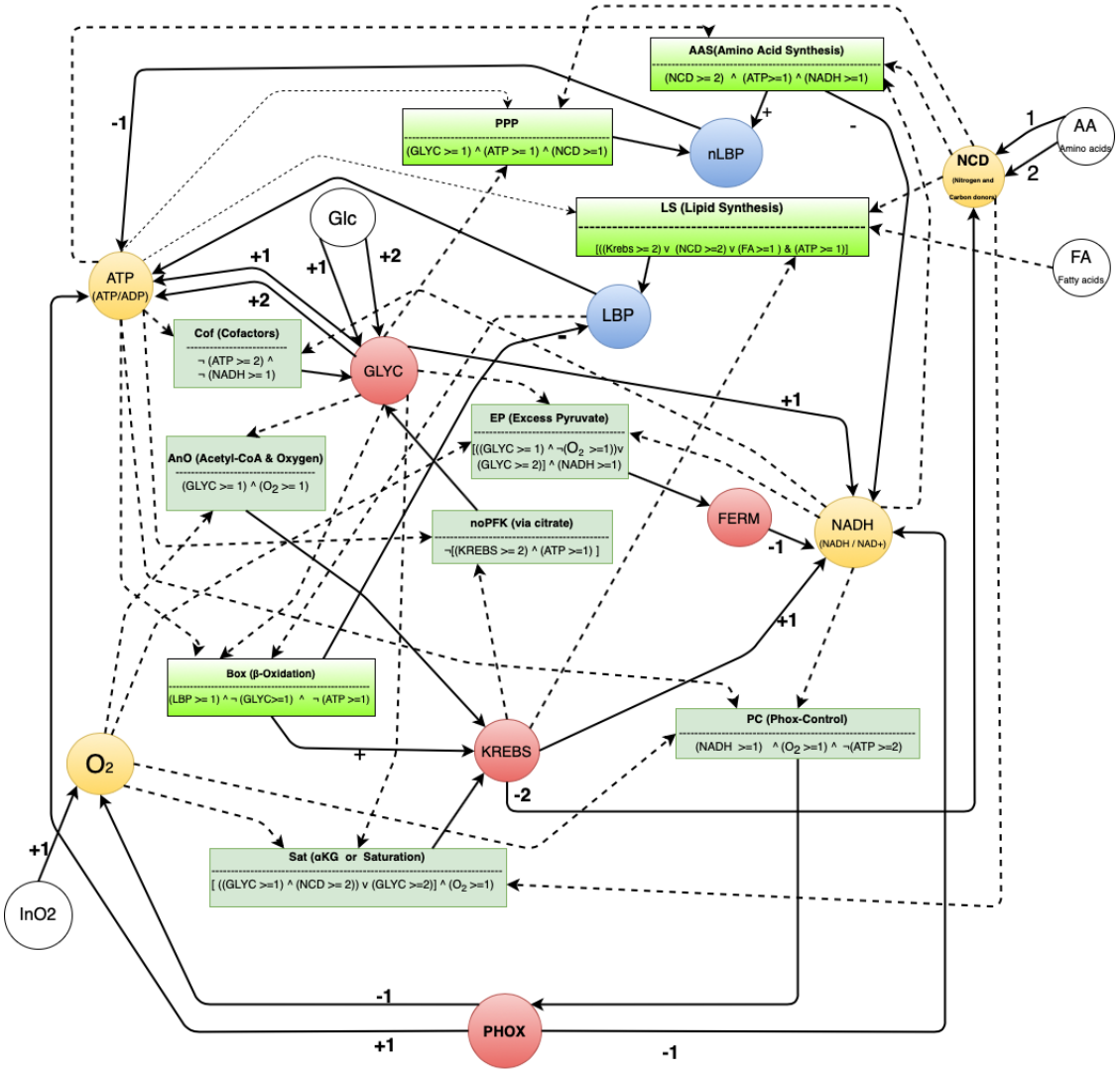


Figure 7.32: The proposed metabolic graph with *14* variables and *10* meaningful multiplexes. Implicit multiplexes are not mentioned.

CHAPTER 8

RELATIVE FORCES BETWEEN BIOLOGICAL REGULATIONS : k-PARAMETERS

8.1 Introduction

The purpose of this chapter is to identify the kinetic parameters for each variable with biological explanation according to each possible set of resources and following the methodology defined in Chapter 5 (Section 5). To better understand regulations and consequently the dynamics of a biological network, one first needs to do an inventory of all the active regulations for each variable in the network and the challenge is to get the value towards which each variable tends to evolve, based on the input signals (or active regulations) it receives. This value is influenced by the activation of the regulators or more precisely the multiplexes (i.e set of multiplexes) acting on the said variable. A multiplex is active when its formula is true or inactive if false. Remind that the influencers on a variable can be activators and inhibitors : an inhibitor is active when its formula is false, and as such the formula for an inhibitor is preceded by a $\neg$ sign (negation). Finally, if there are n input arrows to a given target variable, there will be 2^n possible combination of active resources.
It is a small chapter but it contains a considerable amount of interdisciplinary works between modellers and biologists or biochemists.

For sake of clarity, we will present the K parameters in always the same three steps:

a) eliminate those kinetic parameters where we have contradictions. This can occur in two main cases : first, a variable which passes threshold n must also pass all lower thresholds, and second, a multiplex containing the logical formula of another multiplex cannot occur without the latter multiplex. This helps us to eliminate those parameters which are *useless* because there is no state of the system where they apply.

b) list all kinetic parameters which have 0 values based on the availability of resources.

c) list all kinetic parameters which have values other than 0

We do not apply these steps to variables that have only one resource as it is logical that in the absence of the resource, the value will be 0; otherwise, the value of the K parameter will have the maximum threshold value of the variable : else, it would mean that the interaction graph contains interactions that are never functional.

8.2 Identification of the K parameters

Most of the K parameters have obvious value due to simple facts such as "no resource, no product". The extraction of these K parameters is based on biological literature or parameter values from public databases (e.g. the K_m of an enzyme inhibitor).
Nevertheless, new discovery or unknown experimental facts from us might lead to reconsider certain of these parameter values (but currently, the model is correct with respect to our knowledge).

In this chapter, the template used in the identification of parameters are : on the left we have solid

arrows to indicate the set of resources and on the right we put light arrows to get an indication on the targets. This facilitates the dialogues between the modeller and the biologist in this inventory process.

8.2.1 K-parameters for Glycolysis

Glycolysis acts on two sets of target variables. The first set is activated when glycolysis passes its first threshold and the second set when glycolysis reaches the second threshold as shown in Figure 8.1. Glycolysis has 4 resources: nutrients (2 levels : GLC level 1 and level 2), cofactors (NAD+ and ADP) and the absence of an inhibitor (Glucose regulation via citrate) which inhibits PFK. All possible combinations of the four input variables (GLC1, GLC2, COF, GR) will be considered here to determine whether Glycolysis is able to activate the first set of target variables (K parameter = 1) or the second set of target variables as well (K parameter = 2). Overall, there are 2^4 parameters which will determine when glycolysis is above threshold 1 or 2. For example a higher input of glucose will cause glycolysis to reach its maximum threshold. Let us explain the value for each K parameter and if there are any useless K parameter.

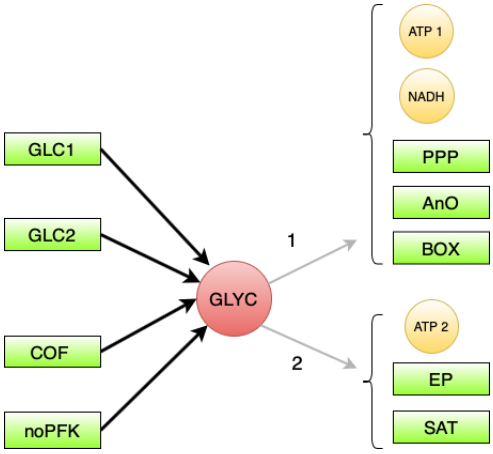


Figure 8.1: Resources for glycolysis : Two activators COF (necessary cofactors ATP & NADH) and noPFK (via escaped citrate from Krebs) and one input variable, GLC (at two levels : 1 or 2).

a) Contradictions indicating useless parameters
Note that $GLC \geq 2$ cannot occur without $GLC \geq 1$. So, the following K parameters where contradictory conditions appears are eliminated as there are inconsistent resources. The following parameters are useless :

$K_{GLYC+\{glc_2\}}$, $K_{GLYC+\{glc_2,noPFK\}}$, $K_{GLYC+\{COF,glc_2\}}$,$K_{GLYC+\{COF,glc_2,noPFK\}}$
(the glc_2 resource implies that glc_1 must also appear).

b) No resources : no glycolysis
Glucose and cofactors are prerequisites for glycolysis: if one is missing in the set of resources, glycolysis does not work and the corresponding K parameters are attracted towards 0. Consequently :

$K_{GLYC+\{\}}$=0
$K_{GLYC+\{COF\}}$=0
$K_{GLYC+\{glc_1\}}$=0
$K_{GLYC+\{glc_1,glc_2\}}$=0
$K_{GLYC+\{noPFK\}}$=0
$K_{GLYC+\{glc_1,noPFK\}}$=0
$K_{GLYC+\{glc_1,glc_2,noPFK\}}$=0
$K_{GLYC+\{COF,noPFK\}}$=0

c) Resources and no inhibition
If the cofactors and glucose are present, the glycolysis is functioning linearly with the increase of glucose. When GLC=1, glycolysis is too weak to act on EP and SAT. When GLC=2, glycolysis is strong enough to activate EP even if O_2=1 and NADH $\geq$ 1, and SAT even if $O_2 \leq$1. Note that for the next two K paramaters, the PFK of glycolysis is functioning as it is not inhibited by noPFK (the inhibitor is a resource, i.e. it is absent): all in all, it implies that the K parameter value is

equal to the value of available glucose.

$K_{GLYC+\{COF,glc_1,noPFK\}}$=1	All resources are present at level 1 and no inhibition
$K_{GLYC+\{COF,glc_1,glc_2,noPFK\}}$=2	All resources are present at level 2 and no inhibition

d) Resources with inhibition
Finally, the last two parameters concern the relative force of the inhibitors with respect to the intake rate of glucose (GLC=1 or GLC= 2). We stipulate this based on K_m constant of citrate on PFK that citrate is not a strong inhibitor, that is, it has the role of lowering glycolysis by one unit : when GLC=1, the K parameter is set to 0, and when GLC=2, the K parameter is set to 1. This is the first "delicate" hypothesis of the model, that is, a hypothesis that can be challenged with future experimental cell-based or in vivo evidence.

$K_{GLYC+\{COF,glc_1\}}$=0	Mild glucose, cofactor and inhibitor are present
$K_{GLYC+\{COF,glc_1,glc_2\}}$=1	High glucose, cofactor and inhibitor are present

We adopt the same steps of reasoning for the other variables, and we do assumptions on difficult parameters with respect to our generic eukaryote metabolism. As such, these parameters (and the set of resources) may be revisited for different research purposes, to adapt to new biological questions or to focus on specific cell types.

8.2.2 K-parameters for NADH/NAD+

NADH/NAD+ plays the role of a reservoir to accept electron and protons extracted from glycolysis and Krebs cycle to released them back either to the respiratory chain to create the proton motrice force or to anabolic pathways for biomass synthesis. As we suppose that the sum NADH and NAD+ is constant and the variable "NADH" stands in fact for the ratio NADH/NAD+ it follows that if NADH=1 (NADH is dominant) then NAD+=0 (in low concentration within the cell) and vice versa.

There are four targets variables for NADH : the "Cofactor" Multiplex COF (NADH/NAD+ is a cofactor of various metabolic reactions), the "Excess Pyruvate" Multiplex EP (NADH is a substrate of FERM to reduce pyruvate into lactic acid), The Oxidative Phosphorylation Control Multiplex PC (NADH provides electron and protons for respiratory chain) and the Amino Acid Synthesis Multiplex AAS (NADH symbolized also NADPH which is used for anabolic pathways). The four target variables of NADH are all activated at the same threshold level of NADH (NADH=1).

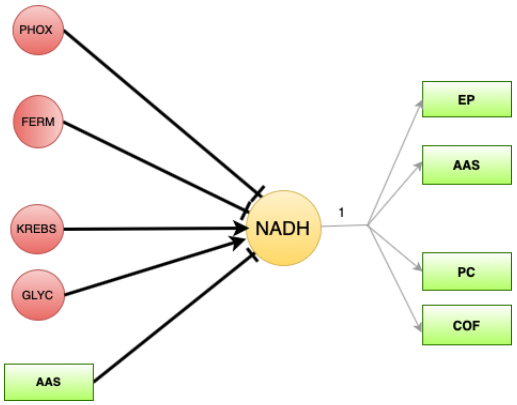


Figure 8.2: Resource and Target variables for NADH with associated thresholds. The resources is constituted of 2 direct activators (KREBS and GLYC) and 3 inhibitors (AAS, PHOX and FERM).

NADH has five resources variables : 3 inhibitors (PHOX, FERM, AAS) and 2 activators (GLYC, KREBS). There are therefore 2^5= 32 K-parameters for NADH. As there is only one threshold, the NADH variable is a Boolean variable ($K_{NADH} \in \{0,1\}$).The resource and target variables of NADH are shown in Figure 8.2.

The determination of the K-parameters relies on hypotheses that stipulates relative force between contradictory signals, i.e. inhibiting and activating signals. These hypotheses correspond in fact to status of the cell (e.g. proliferative versus quiescence mode). The hypotheses are therefore adapted to the type of cell we want to consider in the model. The hypotheses below correspond to a normal cell without the glycolytic phenotype.

Hypothesis 1: PHOX consumption of NADH balance the NADH production of KREBS (normal oxidative direction of KREBS). When such oxidative KREBS is functioning, it include NADH produced by glycolysis in low or medium mode, i.e. when GLYC $\leq$1. PHOX therefore does not balance KREBS + GLYC when GLYC is at high level (glycolytic phenotype). So when KREBS is present with GLYC, then GLYC is supposed to be weak and PHOX consumes all NADH produced by KREBS and GLYC.

Hypothesis 2: when KREBS and GLYC are present, KREBS produces more NADH than GLYC which is suppose to be weak in the respiratory metabolism. So the consideration of these relative forces relies not on time but on yield (number of NADH produced per molecule of glucose consumed).

Hypothesis 3: Inhibition of NADH by PHOX is stronger than inhibition by FERM. This is an hypothesis which is difficult to check experimentally from a metabolic flux point of view. Instead it can be checked more easily through indirect incidence of phenotype (see validation techniques of cell phenotypes in the next chapter).

Hypothesis 4: In a proliferative mode whether in respiratory or fermentative metabolism, cell will tend to produce biomass and the anabolic processes (AAS multiplex) may be considered as a big consumer of NADH. (This might not be the case for quiescent cells). We suppose therefore that AAS is a stronger consumer of NADH than FERM. AAS is therefore equivalent to PHOX in terms of NADH consumption.

We identify all parameters starting with the most obvious considerations, i.e. absence of providers followed by the presence of providers only. The following 21 descriptors are further identified into two steps depending on whether anabolic pathways (AAS) are taken into account or not.

a) No providers
There are 8 K parameters falling in this case. There are all set to the value 0.

$K_{NADH+\{\}}$=0	(no resources)
$K_{NADH+\{PHOX\}}$=0	(PHOX consumer is absent)
$K_{NADH+\{FERM\}}$=0	(FERM consumer is absent)
$K_{NADH+\{AAS\}}$=0	(AAS consumer is absent)
$K_{NADH+\{FERM,PHOX\}}$=0	(PHOX & FERM consumers are absent)
$K_{NADH+\{FERM,AAS\}}$=0	(FERM & AAS consumers are absent)
$K_{NADH+\{PHOX,AAS\}}$=0	(PHOX & AAS consumers are absent)
$K_{NADH+\{FERM,PHOX,AAS\}}$=0	(PHOX & AAS consumers are absent)

We are left with 32 - 8 = 24 parameters to find.

b) No consumers - one provider at least
The second subset of parameters concerns those for which there is no consumer and at least one provider in the combination of resources. There are 3 K parameters in this case, all set to the maximum value 1.

$K_{NADH+\{GLYC,FERM,AAS,PHOX\}}$=1	(no consumers and one provider : GLYC)
$K_{NADH+\{FERM,AAS,KREBS,PHOX\}}$=1	(no consumers and one provider : KREBS)
$K_{NADH+\{GLYC,FERM,AAS,KREBS,PHOX\}}$=1	(all producers, no consumer)

c) Tight between consumers and providers without the presence of AAS (amino-acid synthesis)
There are 9 parameters in this case which are the following with the associated biological explanations.

$K_{NADH+\{GLYC,AAS\}}$=0	(Whether GLYC is low or high PHOX and FERM consumes all NADH produced by GLYC)
$K_{NADH+\{GLYC,PHOX,AAS\}}$=0	(FERM consumes all NADH produced by GLYC even if GLYC is high)
$K_{NADH+\{GLYC,FERM,AAS\}}$=0	(PHOX consumes all NADH produced by GLYC which is weak in respiratory mode)
$K_{NADH+\{KREBS,AAS\}}$=0	(PHOX and FERM consumes all NADH produced by KREBS)
$K_{NADH+\{KREBS,FERM,AAS\}}$=0	(PHOX consumes all NADH produced by KREBS)
$K_{NADH+\{KREBS,PHOX,AAS\}}$=1	(FERM cannot consumes all NADH produced by KREBS)
$K_{NADH+\{GLYC,KREBS,AAS\}}$=0	(PHOX and FERM consumes all NADH produced by GLYC & KREBS)
$K_{NADH+\{GLYC,KREBS,FERM,AAS\}}$=0	(PHOX consumes all NADH produced by GLYC & KREBS)
$K_{NADH+\{GLYC,KREBS,PHOX,AAS\}}$=1	(FERM cannot consume all NADH produced by GLYC & KREBS since GLYC is weak in respiratory metabolism as presence of KREBS suggests)

d) Tight between consumers and providers with amino-acid synthesis
The amino-acid synthesis is not activated (AAS inhibition is function, i.e. absence of the resources). There are 12 parameters in this case. This achieves the total number of parameters: 32= 8+3+9+12

$K_{NADH+\{GLYC\}}$=0	(all consumers consumes NADH produced by GLYC even if GLYC >= 2)
$K_{NADH+\{GLYC,PHOX\}}$=0	(FERM & AAS consumes NADH produced by GLYC)
$K_{NADH+\{GLYC,FERM\}}$=0	(PHOX & AAS consumes NADH produced by GLYC)
$K_{NADH+\{KREBS\}}$=0	(all consumers consumes NADH produced by KREBS)
$K_{NADH+\{KREBS,FERM\}}$=0	(PHOX & AAS consumes NADH produced by KREBS)
$K_{NADH+\{KREBS,PHOX\}}$=0	(FERM & AAS consumes all NADH produced by KREBS)
$K_{NADH+\{GLYC,KREBS\}}$=0	(PHOX and FERM consumes all NADH produced by GLYC & KREBS)
$K_{NADH+\{GLYC,KREBS,FERM\}}$=0	(PHOX & AAS consumes all NADH produced by GLYC & KREBS)
$K_{NADH+\{GLYC,KREBS,PHOX\}}$=1	(FERM & AAS cannot consume all NADH produced by GLYC & KREBS as FERM is supposed to be a weaker consumer of NADH compare to KREBS)
$K_{NADH+\{KREBS,FERM,PHOX\}}$=0	(AAS can consumes all NADH produced by KREBS by Hypothesis 4)
$K_{NADH+\{FERM,GLYC,KREBS,PHOX\}}$=0	(AAS consumes all NADH produced by GLYC & KREBS)
$K_{NADH+\{FERM,GLYC,PHOX\}}$=0	(AAS can consumes all NADH produced by GLYC even with GLYC>=2)

8.2.3 K-parameters for ATP/ADP

Beside other functions, ATP is the "energetic money" of the cell that is created through oxidation of organic matter such as sugar and lipids. It is produced in the cytoplasm (glycolysis) and in the mitochondria through ATP-synthase molecular complex of the respiratory chain. Krebs Cycle and β-oxidation are the two substrate providers in mitochondria for ATP synthesis. The "ATP" variable symbolically represents the ATP/ADP ratio. As for "NADH/NAD+" ratio, when ATP is maximal (highest concentration), the other is minimal (lowest concentration).

The ATP variable has two groups of target variables activated at two different thresholds and has therefore 3 activating states {0,1,2}. In terms of resources, there are three ATP providers : Glycolysis at level 1 (GLYC1), Glycolysis at level 2 (GLYC2) and oxidative phosphorylation (PHOX). There are instead two ATP consumers corresponding to anabolic synthetic pathways whether for lipids 'bio'-production (LBP) or for non-lipids production (nLBP). There are therefore $2^5 = 32$ K parameters associated for this ATP variable.

We use the following hypothesis to resolve the tights between consumers and provider :

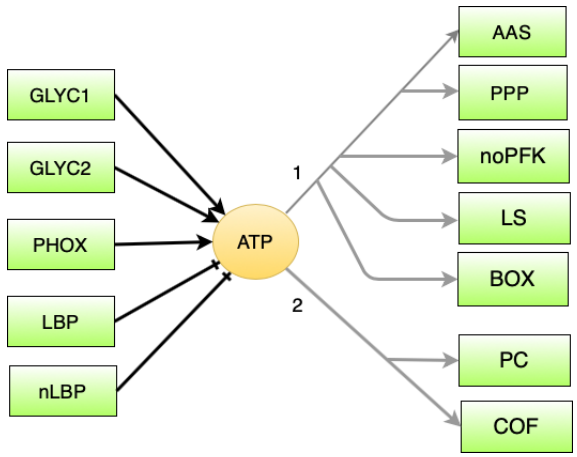


Figure 8.3: Resources for ATP : Both PHOX and GLYC produce ATP while the production of biomass (nLBP and LBP) uses ATP. Remark that the absence of ATP is an activator of BOX.

Hypothesis 1: Lipids (LBP) and non-lipids (nLBP) synthesis require the same amount of ATP. So the inhibitory effect of LBP and nLBP is equivalent.

Hypothesis 2: Oxidative phosphorylation (PHOX) creates more ATP than glycolysis when at medium level (GLYC1) but is equivalent to the ATP production of glycolysis when it is at level 2 (GLYC2).

We start by enumerating the inconsistent situations: as said earlier, the highest level of Glycolysis (GLYC2) implies that its lower levels are also valid, i.e. when GLYC2 is in the set of resources, GLYC1 has to be part as well. As a consequence, all K-parameters where GLYC2 is present alone without GLYC1 are invalid :

$K_{ATP+\{GLYC2\}}$=0
$K_{ATP+\{GLYC2,PHOX\}}$=0
$K_{ATP+\{GLYC2,LBP\}}$=0
$K_{ATP+\{GLYC2,nLBP\}}$=0
$K_{ATP+\{GLYC2,PHOX,LBP\}}$=0
$K_{ATP+\{GLYC2,PHOX,nLBP\}}$=0
$K_{ATP+\{GLYC2,LBP,nLBP\}}$=0
$K_{ATP+\{GLYC2,PHOX,LBP,nLBP\}}$=0

We then investigate the parameters which do not contain any resource providers (value is set to 0).

$K_{ATP+\{\}}$=0
$K_{ATP+\{LBP\}}$=0
$K_{ATP+\{nLBP\}}$=0
$K_{ATP+\{LBP,nLBP\}}$=0

On the contrary, the absence of consumers and the presence of at least one resource provider (activator) will lead the value of the K parameter to its highest level:

$K_{ATP+\{PHOX,LBP,nLBP\}}$=2
$K_{ATP+\{GLYC1,LBP,nLBP\}}$=2
$K_{ATP+\{GLYC1,PHOX,LBP,nLBP\}}$=2
$K_{ATP+\{GLYC1,GLYC2,LBP,nLBP\}}$=2
$K_{ATP+\{GLYC1,GLYC2,PHOX,LBP,nLBP\}}$=2

We now investigate the cases where there is a tight between resources providers and consumers.

1. LBP & nLBP consumes ATP (not part of the resource)

$K_{ATP+\{GLYC1\}}$=0 (all consumers consume ATP produced by GLYC1 : the ATP cannot reach level 1 to activate PPP)

$K_{ATP+\{PHOX\}}$=1 (all consumers cannot consume all ATP produced by PHOX : ATP can reach level 1 to activates PPP)

$K_{ATP+\{GLYC1,GLYC2\}}$=1 (all consumers cannot consume all ATP produced by GLYC2 : ATP can reach level 1 to activates PPP)

$K_{ATP+\{GLYC1,GLYC2,PHOX\}}$=2 (all consumers cannot consume all ATP produced by GLYC2 & PHOX: They both produce sufficiently ATP to inhibit COF or BOX)

$K_{ATP+\{GLYC1,PHOX\}}$=1 (all consumers cannot consume all ATP produced by GLYC2 & PHOX: However ATP is not at a sufficient level to inhibit COF or BOX)

2. LBP alone consumes ATP (not part of the resource)

$K_{ATP+\{PHOX,nLBP\}}$=1 (LBP cannot consume all ATP produced by PHOX: ATP is not at a sufficient level to inhibit COF or BOX

$K_{ATP+\{GLYC1,PHOX,nLBP\}}$=1 (LBP cannot consume all ATP produced by PHOX & GLYC1: ATP is not at a sufficient level to inhibit COF or BOX)

$K_{ATP+\{GLYC1,GLYC2,nLBP\}}$=1 (LBP cannot consume all ATP produced by GLYC1 & GLYC2: ATP is not at a sufficient level to inhibit COF or BOX)

$K_{ATP+\{GLYC1,GLYC2,PHOX,nLBP\}}$=2 (LBP cannot consume all ATP produced by GLYC1 & GLYC2: ATP is at a sufficient level to inhibit COF or BOX)

$K_{ATP+\{GLYC1,nLBP\}}$=0 (LBP consumes all ATP produced by GLYC1 : the ATP cannot reach level 1 to activate PPP)

3. nLBP alone consumes ATP (not part of the resources)

$K_{ATP+\{GLYC1,LBP\}}$=0 (nLBP consumes all ATP produced by GLYC1 : the ATP cannot reach level 1 to activate PPP)

$K_{ATP+\{PHOX,LBP\}}$=1 (nLBP cannot consume all ATP produced by PHOX: ATP is not at a sufficient level to inhibit COF or BOX)

$K_{ATP+\{GLYC1,GLYC2,LBP\}}$=1 (nLBP cannot consume all ATP produced by GLYC1 & GLYC2: ATP is not at a sufficient level to inhibit COF or BOX)

$K_{ATP+\{GLYC1,PHOX,LBP\}}$=1 (nLBP cannot consume all ATP produced by PHOX & GLYC1: ATP is not at a sufficient level to inhibit COF or BOX)

$K_{ATP+\{GLYC1,GLYC2,PHOX,LBP\}}$=2 (nLBP cannot consume all ATP produced by GLYC1 & GLYC2: ATP is at a sufficient level to inhibit COF or BOX)

8.2.4 K-parameters for Krebs

Krebs has a dual role depending on the cell's milieu : oxidative and reductive forms. In its oxidative form (that is at level 1), KREBS is a provider of NADH to PHOX. In its reductive form (that is at level 2), it is an inhibitor of glycolysis via the multiplex noPFK (inhibition of PFK) to lower the expression of the TCA cycle, it depletes nitrogen and carbon reservoirs via NCD (Nitrogen and Carbon donors) to sustain its activities, and finally it is an activator for the production of biomass (a provider of Lipid Synthesis). In Figure 8.4, KREBS has three resources : β-oxydation (BOX through fatty acid degradation), Saturation through (glycolysis and/or glutaminolysis) or just the normal fate of pyruvate after glycolysis (through AnO as Acetyl-CoA is derived from pyruvate, that is in normoxic condition). Note that the Saturation multiplex (SAT) correspond to oxidative saturation, that is glutaminolysis producing α-KG to be oxidized through the KREBS cycle.
Therefore, we have 2^3 parameters to identify.

BOX and AnO provide minimum forces in terms of glycolytic input for Krebs to function at the level 1. The shift from level 1 to 2 occurs when there is either a high glycolytic input or additional nitrogen and carbon elements obtained from NCD. Let us review if there are any kinetic parameters that could be eliminated.

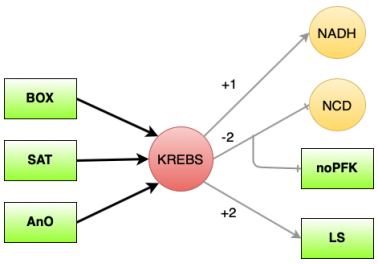


Figure 8.4: K parameters for Krebs : On the left, a combination of three activators for the proper running of Krebs. A level of 1 is sufficient to allow Krebs to produce enough NADH for PHOX. At level 2, Krebs has two roles : either activates lipid synthesis or regulates glucolysis.

To do this exercise, we will need to go a step further this time by analysing the logical formula of all three multiplexes. The logical formula of AnO ($(GLYC \geq 1) \wedge (O_2 \geq 1)$) is already incorporated in the multiplex SAT ([((GLYC ≥ 1) $\wedge$ (NCD ≥ 2)) $\vee$ (GLYC ≥ 2)] $\wedge O_2 \geq 1$) ; both multiplexes are driven by glycolysis. This means that SAT cannot participate without AnO. Moreover, the BOX formula includes $GLYC = 0$. So, this contradicts the other two latter multiplexes. Using this logical reasoning, all K parameters where we have BOX present with at least one of them, will be useless. This results in the elimination of the following parameters : $K_{KREBS+\{SAT\}}$,$K_{KREBS+\{BOX,SAT\}}$,$K_{KREBS+\{AnO,BOX,SAT\}}$ and $K_{KREBS+\{AnO,BOX\}}$.

We can directly infer that $K_{KREBS+\{\}}$=0 as there are no resources contributing to the relative forces of KREBS. On the contrary, when it gets the full support of both SAT and AnO, its level reaches the maximum value of 2. This triggers both the catabolic roles of KREBS either as glycolysis or degradation of amino acids intake, and the anabolic role for synthesis of lipids. This allows us to assign $K_{KREBS+\{AnO,SAT\}}$ the value 2.

The next question is what happens if either AnO or BOX is alone as resource and does not get the support of SAT. In such condition, we assume a normal cell activity where KREBS produces NADH. This further helps us to identify $K_{KREBS+\{AnO\}}$ and $K_{KREBS+\{BOX\}}$ as having value 1.

To summarize the 8 parameters of KREBS are :

$K_{KREBS+\{\}}$=0	(no resources)
$K_{KREBS+\{SAT\}}$	(Invalid; SAT cannot occur without AnO)
$K_{KREBS+\{BOX,SAT\}}$	(Invalid; SAT cannot occur without AnO)
$K_{KREBS+\{AnO,BOX,SAT\}}$	(Invalid; BOX with either AnO or SAT is not feasible)
$K_{KREBS+\{AnO,BOX\}}$	(Invalid; BOX with either AnO or SAT is not feasible)
$K_{KREBS+\{AnO\}}$=1	No SAT support and a normal NADH production
$K_{KREBS+\{BOX\}}$=1	No SAT support and a normal NADH production
$K_{KREBS+\{AnO,SAT\}}$=2	Support of SAT yields maximum NADH output

8.2.5 K-parameters for Oxidative Phosphorylation

Oxidative Phosphorylation (PHOX) is a provider of ATP and O2, and a consumer of NADH. PHOX needs two input molecules to run: NADH and oxygen, which are encapsulated in the Phox-Control multiplex (PC) as shown in Figure 8.5. If one of NADH or oxygen or both are not present (the PC multiplex is false), PHOX does not work.

Since PHOX is boolean, we have only two K parameters as follows:

$K_{PHOX,\{\}}$=0	No resources
$K_{PHOX+\{PC\}}$=1	All conditions are present

8.2.6 K-parameters for Fermentation

Fermentative pathways (FERM) reduce pyruvate by oxidating NADH into NAD+. So, FERM consumes or "inhibits" NADH at a unique threshold 1. The unique resource of FERM is encapsulated in the Excess Pyruvate pathway (EP). As a reminder, the "Excess Pyruvate" multiplex encapsulates the respiration-fermentative shift from respiration to fermentation either in absence of oxygen or in the presence of high

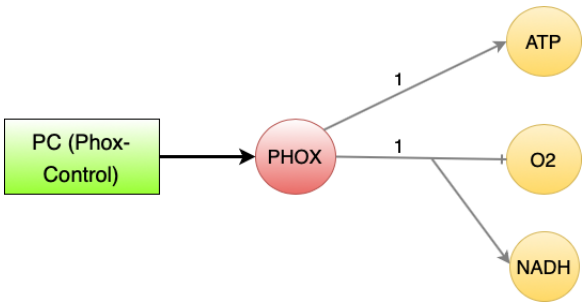


Figure 8.5: Resources for Oxidative Phosphorylation : Only 1 resource which encapsulates NADH and oxygen.

glucose intake. These are shown in Figure 8.6.

Figure 8.6: Resources for Fermentation : Pyruvate is a resource for fermentation

Since FERM is boolean, we have only two K parameters as follows:
$K_{FERM,\{\}}$=0 No resource is present
$K_{FERM,\{EP\}}$=1 Resource is present

8.2.7 K-parameters for NCD

NCD (Nitrogen-Carbon donors) provides the necessary nitrogen and carbon skeletons for the synthesis of amino acids, fatty acids and nucleotides (for the PPP). NCD is a ressource for its 4 targets (see Figure 8.7), at level 1 for Pentose Phosphate Pathway (PPP) and at level 2 for AAS (amino acid synthesis), LS (Lipid Synthesis) and SAT (Saturating Krebs) through anaplerotic reactions. NCD has three resources : KREBS2 (Krebs at level 2), AA1 (Amino acids supply at level 1 and AA2 (Amino acids at level 2). Krebs is an inhibitor (needs nitrogen donors in conditions of stress to fuel the α KG pathway) and AA does the reverse by filling the reservoir of nitrogen and carbon elements. In our model, we assume AA has two levels thus giving rise to 3 resources for NCD. Overall, we have to identify 2^3 parameters. NCD works at two levels : at level 1, it provides the necessary elements for PPP in terms of nucleotides and DNA. At level 2, its contribution to biomass is more consequent : it allows to create αKG via glutaminolysis, as well as amino acids via the amino acids synthesis pathway AAS, and also lipid synthesis LS by providing nitrogen and carbon.

Let us first eliminate invalid parameters : AA2 implies AA1. This will eliminate the following K parameters : $K_{NCD,\{AA2\}}$ and $K_{NCD,\{AA2,KREBS\}}$. The next simpler case is when there are no inhibitors : that is when KREBS is a resource. We will be able to deduce that the level of AA will determine the value of K. For instance, $K_{NCD,\{KREBS\}}$ =0 since there are no donors of nitrogen and carbon elements (that is AA is absent). In the same vein, $K_{NCD,\{\}}$=0. If AA is present at level 1, then this will simulate the production of nucleotide and DNA via PPP; that is $K_{NCD,\{AA1,KREBS\}}$=1. We assume this level of 1 is insufficient to activate either LS or AAS. Lastly, if AA is at level 2, then the corresponding $K_{NCD,\{AA1,AA2,KREBS\}}$=2 allowing the production of biomass via LS and AAS.

More generally, with respect to the thresholds of NCD to be active on PPP, αKG, AAS and LS, we consider that the inhibition of KREBS will lower down the production speed of nitrogen and carbon, but this will not decrease the value toward which KREBS asymptotically tends (technically because there is no degradation of nitrogen and carbon as such). So, $K_{NCD,\{AA1\}} = K_{NCD,\{AA1,AA2\}}$ =1.

To summarize, the 8 parameters of NCD are as follows:

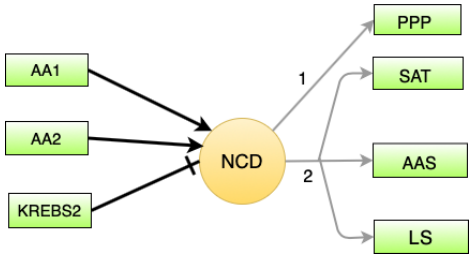


Figure 8.7: Resources for NCD : One activator (input of AA which is a control variable at two levels 1 and 2) and KREBS as the only inhibitor at level 2

$K_{NCD+\{\}}$=0	(no resources)
$K_{NCD+\{AA2\}}$	(Invalid; AA2 cannot occur without AA1)
$K_{NCD+\{AA2,KREBS\}}$	(Invalid; AA2 cannot occur without AA1)
$K_{NCD+\{AA1\}}$=1	(Low AA1 means low level of NCD)
$K_{NCD+\{AA1,KREBS\}}$=1	(Low AA1 means low level of NCD)
$K_{NCD+\{KREBS\}}$=0	(Absence of Krebs as inhibitor)
$K_{NCD+\{AA1,AA2\}}$=1	(Both AA1 and AA2 are present; producing a high level of NCD)
$K_{NCD+\{AA1,AA2,KREBS\}}$=2	(Both AA1 and AA2 are present; producing a high level of NCD)

8.2.8 K-parameters for non lipidic biomass production (nLBP)

nLBP (Non-Lipidic Biomass) refers to the production of only amino acids and related products for the synthesis of proteins. From Figure 8.8, the nLBP variable has only 1 target which is ATP (it needs energy to sustain its activities). It has two resource providers : PPP and AAS; both provide the necessary elements for non-lipidic biomass production. The two activators are independent of each other which means we have 2^2=4 K parameters to identify. If neither activator is present, it is logical that the corresponding K parameter has value 0 : $K_{nLBP,\{\}}$. If at least one activator is present, this allows nLBP to act on ATP setting all remaining parameters to the value 1 : $K_{nLBP,\{PPP\}}$,$K_{nLBP,\{AAS\}}$ and $K_{nLBP,\{PPP,AAS\}}$.

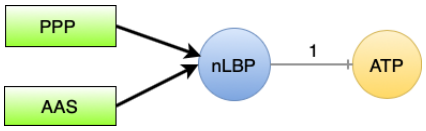


Figure 8.8: Resources for non-lipidic biomass production : 2 activators and only 1 target.

To summarize, the 4 parameters of nLBP are as follows:

$K_{nLBP+\{\}}$=0	(no resources)
$K_{nLBP+\{PPP\}}$ =1	(At least one resource is present)
$K_{nLBP+\{AAS\}}$=1	(At least one resource is present)
$K_{nLBP+\{PPP,AAS\}}$=1	(At least one resource is present)

8.2.9 K-parameters for LBP

LBP (Lipidic Biomass) refers to the production of only lipidic biomass (i.e fatty acids) excluding protein-related production. LBP is boolean and has two targets : ATP and BOX. LBP consumes ATP for lipid synthesis and is a ressource of lipid for beta-oxidation (BOX) in the mitochondria. It has two resources : Lipid Synthesis (LS) and beta-Oxidation (BOX) as shown in Figure 8.9.

All combinations of LS and BOX are satisfiable, so that the 4 parameters are useful. Without lipidic synthesis LBP lacks resources, thus $K_{nLBP,\{\}} = K_{nLBP,\{BOX\}} = 0$. Conversely, with lipidic synthesis, even if BOX inhibits LBP, lipid biomass as precursor activates its degradation and consumes ATP, thus $K_{nLBP,\{LS\}}$=1 and a fortiori $K_{nLBP,\{LS,BOX\}}$=1.

To summarize, the 4 parameters of nLBP are as follows:

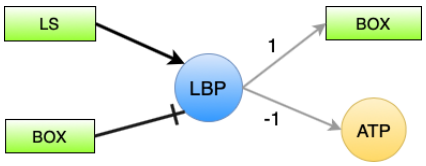


Figure 8.9: Resources for Lipidic Biomass Production : 2 resources and 2 targets

$K_{nLBP+\{\}}$=0	(no resources)
$K_{nLBP+\{LS\}}$ =1	(Lipid synthesis favours non-lipids production)
$K_{nLBP+\{BOX\}}$=0	(Absence of precursors for non-lipids production)
$K_{nLBP+\{LS,BOX\}}$=1	(The absence of BOX does not affect non-lipids production)

8.2.10 K-parameters for Oxygen

Oxygen is a boolean variable (present / absent) with only one activation threshold. If present, it regulates all targets multiplexes either as an activator (PC, AnO, SAT) or as an inhibitor (EP). There are two ressource of O2: Input of Oxygen (In_O2) providing oxygen to the cell and oxidative phosphorylation (PHOX) consuming oxygen as an acceptor of electron of the respiratory chain.

There are therefore 2^4=16 parameters to identify. A lack of input oxygen to the cells will not allow O_2 to activate its resources therefore we have $K_{O_2,\{\}}$=$K_{O_2,\{PHOX\}}$=0. When oxygen supply is available, O_2 always acts on its targets; therefore, $K_{O_2,\{IN\_O_2\}}$=$K_{O_2,\{IN\_O_2,PHOX\}}$=1.

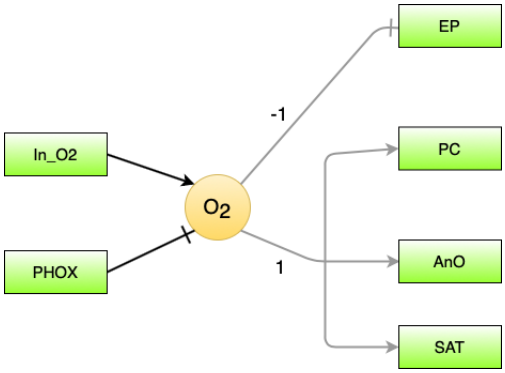


Figure 8.10: Resources for oxygen : 1 activator in the form of input of oxygen and 1 consumer which is oxidative phosphorylation.

To summarize, the 4 parameters of Oxygen are as follows:

$K_{O_2+\{\}}$=0	(no resources)
$K_{O_2+\{\{IN\_O_2\}}$ =1	(Supply of oxygen is available)
$K_{O_2+\{PHOX\}}$=0	(Absence of inhibitor as well as precursor)
$K_{O_2+\{\{IN\_O_2,PHOX\}}$=1	(The absence of PHOX and supply of oxygen)

8.3 Conclusion of the chapter

With the help of a very limited number of function-related hypotheses concerning the relative forces of the ressources on their target variables, common and sometimes details knowledge of central carbon metabolism is leading to a complete determination of the kinetic parameters of the model. Changing the hypothesis might be done if we want to consider different type of cells whether in quiescence or in proliferation as it is the case here. In all types of cells, the model is focusing on the central question: the regulation conditions of the metabolic shift between respiration and fermentation. Biomass production (Lipidic or non-lipidic) is obviously playing an important role in the Warburg effect and hypothesis associated to the Biomass production has a major impact whether we model quiescence or proliferative cells.

In the next chapter, we see how these information will be used as input of DyMBioNet to perform validations of the metabolic network. This procedure will allow us to observe corresponding metabolic phenotypes (if not, it would require debugging of any K parameter which might be misleading).

CHAPTER 9

MODEL VALIDATION

9.1 Introduction

Once the model is constructed, the next crucial step is the validation of the model against biological knowledge on the global behaviour. The goal here is to check whether the model reproduces known phenotypes, i.e. known characteristic of cell growth under various nutrient conditions, or known behaviour or metabolic pathways under the same various input conditions.

We developed essentially two complementary approaches to validate the model:

1. Validation matrix
   The validation matrix (section 9.2) provides a quick overview of the different phenotypic observations of each variable under different environmental contexts. It acts as a bridge between the informal specification in biology and the formal representation of the metabolic model. The validation matrix contains :

   - Environmental context (rows)
   - Variables of the system (columns)
   - And the cells of the matrix contain temporal formulas that formalise the system's properties of each variable under each context

   Some of the properties are observable metabolic properties while some are not directly observable in reality but are considered as valid by the biologists. Empty cells in the matrix simply means we do not have sufficient supportive evidences. To complement this, we use simulations.

2. Simulations
   Simulations are used to check temporal characteristics of variable such as frequency of oscillation or frequency of jumps between states. Simulations are also useful to alert on aberrant or underestimated metabolic characteristics during model construction.

3. Fair path CTL
   As simulation cannot capture phenotype in a long run, formal temporel logic tools as CTL do cope with this. Fair CTL is used to do formal checking on the states of the variables at long run. CTL complements this drawback of simulations by allowing the modeller to express all properties in the validation matrix using fair path CTL. As such, we are able to check all properties by writing CTL formulas.

We explain these three validation techniques in this order.

9.2 Validation Matrix

The given validation matrix lists all known behaviours (phenotypes) about the metabolism in the context of our focus on the Warburg/Crabtree effect, according to different environmental contexts such as nutrient conditions. Each row represents such a context, that is, a setting of input variables. There are 36 possible contexts, so the validation matrix contains 36 lines. Each column represents a biologically interpretable variable, possibly experimentally observable. The intersection of a row and a column represents a metabolic phenotype. An important aspect to retain here is that the validation matrix has been filled *a priori* with known properties from Chapter 2 before the final design of the formal regulatory network.

There are 4 input variables (FA, in_O_2, GLC and AA) which describe the environmental context for our metabolic model and 10 variables of the system which describe metabolic processes. Note that the asymptotic behaviour of variable NCD depends directly of the variable AA, consequently the NCD column would be a copy (up to the "tend()" pattern) to the AA column and therefore is omitted from the validation matrix. Among the input variables, in_O_2 and FA are boolean while AA and GLC have three values (boundary=2). This gives us 36 possibilities (2 x 2 x 3 x 3), which gives rise to 36 rows in the validation matrix : 36 possible biological contexts.

As there are 9 variables, there is a total of 9 x 36 behaviours for all 9 variables whether they oscillate or tend to a stationary state. These two global behaviours of the variable can be coded in term of Fair CTL formula and it is therefore possible to determine whether each variable oscillate or not under the 36 input environmental conditions. There are potentially 9x36 Fair CTL formula constructed for this validation matrix, but some are gray box as already mentioned(see Table 9.1).

The "oscillatory" or "tend towards" behaviour are encoded as follows:

1. **tend** represented by "tendTowards" function which accepts two parameters : a variable and the integer value towards which the variable should go.
   Using Fair CTL, tend(n) for a variable x is simply encoded as $AF(AG(x = n)$. Notice that, contrarily to CTL, the A quantifier does not take into account unfair paths where a possible transition from a given state is ignored while this state is visited an infinite number of times.

2. **osc** meaning an alternance between two extreme values represented by the "oscillate" function. This function accepts the variable three parameters : the variable and the two extreme values between which the variable will oscillate.
   osc(m,n) for a variable x is encoded as follows in Fair CTL : $AF(x = m) \wedge AF(x = n)$ and we adopt a model checking strategy such that we retain the smallest possible value m and the highest possible value n in the model.

Moreover, in Table 9.1, "!" stands for negation. Each test is detailed in the order they appear in the validation matrix of Table 9.1. We regroup the 36 biological contexts into 4 categories defining the boolean combination of lipids and oxygen supply (first 2 columns of the matrix). In some cases, when the phenotypes resemble, we combine the biological context (rows 2 & 3 for example). When no general knowledge about the behaviour of a given variable in a given context is available, the corresponding box remains empty (dark grey). We will try to answer these missing information later on using simulations and CTL.

9.2.1 No lipids and oxygen supply : FA=0 & $In\_O_2 = 0$

We explain rows 1 - 8 with different combinations of glucose and amino acids. If there is no supply of oxygen, it is feasible enough to say that oxygen (O2) will tend to 0 and PHOX also will tend to 0 as it is highly dependent on oxygen.

- ROW 1 : GLC= 0, AA = 0
  Cells fail to receive the necessary nutrient for growth and even survive : each variable should tend to zero.

- ROW 2 & 3 : GLC=0, AA = 1, 2
  Without glucose, cells can survive if the carbon source is represented by amino acids. (There are indeed nutritive milieu without glucose for certain cancer cell lines [157, 158]). Cell energy (ATP) is produced through cataplerotic feeding of amino acids to KREBS cycle which works as an oxidative machinery connected to PHOX to produce ATP. So without O_2, the oxidative process is not possible and all non-gray variables (i.e. O_2) tend to 0.

- ROW 4 : GLC = 1 and AA = 0
  In the absence of oxygen, cells use glucose to ferment due to the delta redox reactions of NADH. A low glucose is sufficient to allow the production of lactate which reduces NADH to NAD+, which is recycled back to glycolysis (NADH will therefore oscillate). Mitochondrial activity is shunted and cytosolic metabolism of glucose follows the unidirectional transformation of glucose molecules to pyruvate and finally to lactate. Following these explanations, we expect the following phenotypic observations: at least fermentation is not down, Krebs and PHOX must maintain a low state, other related cofactors in this glycolysis-fermentation cycle will manifest with time. So, GLYC will

biological context	FA	In_O2	GLC	AA	ATP (0-2)	O2 (0-1)	GLYC (0-2)	nLBP (0-1)	LBP (0-1)	FERM (0-1)	KREBS (0-2)	PHOX (0-1)	NADH (0-1)
Neither lipids nor oxygen supply													
1	**0**	**0**	**0**	**0**	tend(0)	tend(0)	tend(0)	tend(0)	tend(0)	tend(0)	tend(0)	tend(0)	tend(0)
2 & 3	**0**	**0**	**0**	**1 & 2**		tend(0)							
4	**0**	**0**	**1**	**0**	!tend(0)	tend(0)	osc(0-1)			!tend(0)		tend(0)	osc
5	**0**	**0**	**1**	**1**	osc	tend(0)	osc(0-1)	!tend(0)		!tend(0)		tend(0)	osc
6	**0**	**0**	**1**	**2**	osc	tend(0)	osc	!tend(0)	!tend(0)	!tend(0)		tend(0)	osc
7	**0**	**0**	**2**	**0**	!tend(0)	tend(0)	osc			!tend(0)		tend(0)	osc
8	**0**	**0**	**2**	**1**	osc	tend(0)	osc	!tend(0)		!tend(0)		tend(0)	osc
9	**0**	**0**	**2**	**2**	osc	tend(0)	osc	!tend(0)	!tend(0)	!tend(0)		tend(0)	osc
No lipids but oxygen supply													
10	**0**	**1**	**0**	**0**	tend(0)	!tend(0)	tend(0)	tend(0)	tend(0)	tend(0)	tend(0)	tend(0)	tend(0)
11 & 12	**0**	**1**	**0**	**1 & 2**		!tend(0)							
13	**0**	**1**	**1**	**0**	!tend(0)	osc	osc			tend(0)	osc	osc	osc
14	**0**	**1**	**1**	**1**	osc	osc	osc	!tend(0)		tend(0)	osc	osc	osc
15	**0**	**1**	**1**	**2**	osc	osc	osc	!tend(0)	!tend(0)	tend(0)	osc	osc	osc
16	**0**	**1**	**2**	**0**	!tend(0)	!tend(0)	osc			!tend(0)		!tend(1)	osc
17 & 18	**0**	**1**	**2**	**1&2**	osc	!tend(0)	osc	!tend(0)	!tend(0)	!tend(0)		!tend(1)	osc
Lipids but no oxygen supply													
19	**1**	**0**	**0**	**0**	tend(0)	tend(0)	tend(0)	tend(0)	tend(0)	tend(0)	tend(0)	tend(0)	tend(0)
20 & 21	**1**	**0**	**0**	**1 & 2**		tend(0)							
22	**1**	**0**	**1**	**0**	osc	tend(0)	osc(0-1)		!tend(0)	!tend(0)		tend(0)	osc
23	**1**	**0**	**1**	**1**	osc	tend(0)	osc(0-1)	!tend(0)	!tend(0)	!tend(0)		tend(0)	osc
24	**1**	**0**	**1**	**2**	osc	tend(0)	osc	!tend(0)	!tend(0)	!tend(0)		tend(0)	osc
25	**1**	**0**	**2**	**0**	osc	tend(0)	osc		!tend(0)	!tend(0)		tend(0)	osc
26 & 27	**1**	**0**	**2**	**1 & 2**	osc	tend(0)	osc	!tend(0)	!tend(0)	!tend(0)		tend(0)	osc
Lipids and oxygen supply													
28	**1**	**1**	**0**	**0**	tend(0)	!tend(0)	tend(0)	tend(0)	tend(0)	tend(0)	tend(0)	tend(0)	tend(0)
29 & 30	**1**	**1**	**0**	**1 & 2**		!tend(0)							
31	**1**	**1**	**1**	**0**	osc	osc	osc		!tend(0)	tend(0)	osc	osc	osc
32	**1**	**1**	**1**	**1**	osc	osc	osc	!tend(0)	!tend(0)	tend(0)	osc	osc	osc
33	**1**	**1**	**1**	**2**	osc	osc	osc	!tend(0)	!tend(0)		osc	osc	osc
34	**1**	**1**	**2**	**0**	osc	!tend(0)	osc		!tend(0)	!tend(0)		!tend(1)	osc
35 & 36	**1**	**1**	**2**	**1 & 2**	osc	!tend(0)	osc	!tend(0)	!tend(0)	!tend(0)		!tend(1)	osc

Table 9.1: Validation matrix for the cell metabolism regulation model. Each row (resp. column) represents an experimental condition (resp. an observable systemic variable). Thus, each cell of the table formalises the known behaviour of that observable variable in that experimental condition. "osc" means oscillation with either osc(0,1) to indicate that the variable oscillates between the value 0 and 1; or osc(0,2) which means an oscillation between 0 and 1, and also between 1 and 2.

oscillate between 0 and 1 (reflects GLC=1). Even if ATP is consumed for cell maintenance (and at the same time, a mild level is produce during glycolysis), it at least does not tend to zero as cells can survive with only glucose.

- ROW 5 : GLC=1 and AA=1
  Along with a production of ATP through glycolysis and a consumption of ATP by the production of non-lipidic biomass, and with additional support from AA : nLBP does not tend toward 0 and ATP should oscillate. And, as the previous context, the cell is in anaerobic process, and cytosolic metabolism and mitochondrial activity act similarly on the other markers.

- ROW 6 : GLC=1 and AA=2
  No oxygen supply means oxidative Krebs is OFF while a huge supply on amino acids favours the reductive phase of Krebs to provide precursors for lipid synthesis, so LBP does not tend toward 0. The metabolic processes are the same as 5, except that a large intake of amino acids activates glutaminolysis. It creates α-ketoglutarate that can be converted with the reductive Krebs cycle into pyruvate. This accumulation of pyruvate could also be due to a high activity of GLYC, therefore GLYC could sometimes reach its highest level. Thus, we prefer to relax its oscillatory behaviour ("osc" without knowledge of the boundaries instead of "osc(0,1)").

- ROW 7 : GLC=2 and AA=0
  GLC2 trigger the glycolytic phenotype, i.e. high rate fermentation (as GLC level 2 implies GLYC level 2). GLC at level 2 implies GLYC at level 2. This means we will expect glycolysis to reach its maximum threshold and will affect other related processes and cofactors production.

With high glucose intake, glycolysis can sometimes reach its highest level. So, GLYC could possibly oscillate from its lowest to its highest level ("osc" is equivalent here to "osc(0,1)" or "osc(0,2)"). Moreover, the same processes as 4 are impacted, and thus the behaviour of all other markers remain identical.

- ROW 8 : GLC=2 and AA=1
  Here, the catabolic activity (glycolysis, fermentation, oxidative respiration and Krebs cycle) is similar to (GLC=2 and AA=0), so that NADH behaves similarly. The more precise knowledge comes from the endergonic production of non lipidic biomass, so that nLBP does not tend toward 0 and ATP can temporarily decrease to 0, so it oscillates.

- ROW 9 : GLC=2 and AA=2
  For the same reasons as line 6, this context allows for the production of lipid biomass, so at least LBP does not tend toward 0. Other markers behave similarly as in GLC=2, AA=1.

  In the presence of high glucose, glutamine provides the necessary precursors for synthesis of nucleotides via PPP. If glutamine level increases, this favours the reductive phase of Krebs to provide precursors for lipid synthesis (part of krebs is operational which explains it is not 0). Overall, glutamine provides the necessary precursors for the synthesis of biomass. The lack of oxygen milieu means PHOX is not working.

9.2.2 Without lipid intake and with oxygen supply : FA=0 & $In\_O_2 = 1$

In normoxia or in presence of oxygen, the cell can activate its mitochondrial respiratory metabolism in presence of carbon supply. At least O_2 will never tend to 0 as oxygen can diffuse inside the cell and is not consumed.

- ROW 10 : GLC= 0 and AA = 0
  All other markers tend toward 0 because, without glucose and amino acids entries, there is no glucose metabolism, thus no carbon and cofactors sources for anabolism, so we expect no pyruvate available for the Krebs cycle, and thus no oxidative phosphorylation; and lastly this affects also the production of ATP.

- ROW 11 & 12 : GLC=0 and AA = 1,2
  With oxygen the general knowledge does allow us to decide if amino acids intake is sufficient to sustain i.e. under respiratory metabolism.

- ROW 13 : GLC = 1 and AA = 0
  In normoxic condition and with normal intake of glucose without amino acids and lipids, cells can activate respiratory metabolism to produce ATP. As a consequence (presence of producer and consumer) GLYC, KREBS and PHOX oscillate.

  ATP is produced but there is not enough general knowledge about its consumption to assert that ATP tends toward 1 or that it oscillates, so we only assert that it does not tend toward 0. Lastly, according a normal aerobic metabolism, FERM tend toward 0 (as fermentation is less efficient than oxidative phosphorylation).

- ROW 14 : GLC=1 and AA=1
  This line can be considered as the context representing a healthy cell (normal case). It adds amino acid inputs to the previous context, so that non lipid biomass can be produced (nLBP does not tend toward 0). The contribution of amino acids boosts the production of ATP

  Aerobic processes follow the behaviour of 13 but we can be more specific about ATP: there is now an ATP consumption by biomass production, so that ATP oscillates.

- ROW 15 : GLC=1 and AA=2
  This line is similar to the previous context (with AA=1) except that a higher level of amino acids contributes to the production of lipid biomass.

- ROW 16 : GLC=2 and AA=0
  GLYC oscillates as in 14, but the high glucose uptake provokes Warburg/Crabtree phenotype and leads to a high anaerobic glycolysis, even in presence of oxygen. From the general knowledge we only assert that FERM does not tend toward 0. Glycolysis activity is sufficient to regenerate NADH: as explained in 4, NADH oscillates. Similarly to 13 ATP does not tend toward 0. On the opposite, oxidative phosphorylation might be present when the Warburg/Crabtree effect occurs, but for sure not constantly so PHOX does not tend toward 1. Therefore oxygen could be partially

consumed, but O2 does not tend toward 0 because its consumption by oxidative phosphorylation cannot counterbalance the external intake.

- ROW 17 & 18 : GLC=2 and AA=1,2
  The Warburg/Crabtree occurs as in 13. Additionally here, the presence of amino acids intake allows biomass productions, so that nLBP and BLP do not tend toward 0, and thus ATP oscillates.

9.2.3 With lipid intake and no oxygen supply : FA=1 & $In\_O_2 = 0$

- ROW 19 : GLC= 0 and AA = 0
  Lipid intake alone is unable to sustain all carbon dependant metabolic activity of the cell in the absence of oxygen, so, as in 1 and 10 respectively, all markers tend toward 0, except O_2.

- ROW 20 & 21 : GLC=0 and AA = 1,2
  Similarly to lines 2 & 3, there is no general knowledge to assert whether lipid intake alone is able to sustain carbon dependant metabolic activity of the cell: we consider that the future phenotype of the cell is unknown, except hypoxia.

- ROW 22 - 27 : GLC = 1,2 and AA = 0, 1, 2
  With respect to lines 4 to 9, fatty acid intake only sustains lipidic biomass production, and consequently the ATP consumption. Lipids synthesis can be done as soon as ATP is available for the cell even in absence of amino acids and thus LBP does not tend toward 0, and the ATP consumption makes ATP oscillate. Other markers keep the behaviour already described in 4 to 9.

9.2.4 With lipid intake and oxygen supply : FA=1 & $In\_O_2 = 1$

This is the ideal biological context in which the cells benefit from all media necessary for energy and biomass production.

- ROW 28 - 30 : GLC= 0 and AA = 1, 2
  Similarly to lines 11&12, the general knowledge does not allow us to decide if oxygen and lipid intake are sufficient to sustain carbon dependant metabolic activity of the cell. Only normoxia makes no doubt.

- ROW 31 : GLC= 1 and AA = 0
  Compared to line 13, the same reasoning as 22-27 applies.

- ROW 32 : GLC= 1 and AA = 1
  Compared to line 14, fatty acid intake only sustains lipidic biomass production, thus LBP does not tend toward 0 and the other markers keep the same behaviour.

- ROW 33 : GLC= 1 and AA = 2
  With respect to line 32, glutaminolysis feeds Krebs (anaplerotic reactions) and this can fuel lipid synthesis through citrate export from mitochondria. This excess of citrate opens the possibility to fuel fermentation in addition to lipid production. Thus, as a precaution, we leave the behaviour of FERM unknown.

- ROW 34 - 36 : GLC = 2 and AA = 0, 1, 2
  Here, the same reasoning as lines 22-27 applies: LBP does not tend toward 0, and ATP oscillates.

9.3 Simulations

Computer simulations are often the methods of choice to explore an agreement between a model and experimental data in systems biology [156]. In this text, we refer to two types of models: the mathematical model and the biological model. In the mathematical model, we can perform proofs and simulations to validate system's properties. It is a way of getting a thorough understanding of the biological model, propose credible experiments based on observations and trace causalities among the objects in the biological model. These biological observations can be expressed in logical formulas linking the two models. But, we reiterate the fact that the validation matrix is filled from biological knowledge and is independent of the simulation exercise. Using simulations, we are able to identify complementary modelling errors from paths that are not relevant in the biological context under investigations. In non-deterministic cases, the simulation can be carried out at *Ad vitam aeternam* while gaining empirical knowledge of the model. Moreover, simulations that produce the same phenotypic results can be regrouped.

In this section, we will perform simulations to demonstrate three well-known phenotypic characteristics of metabolism : respiration, fermentation, and the CrabTree effect in fermenting yeast. In these three different phenotypes, we will have the chance to see how simulations are helpful and how we can also mimic the cellular environment based on availability of nutrients.

9.3.1 Environmental context of Row 13 : FA=0 & In_O_2 = 1, GLC = 1 and AA = 0

As a reminder, respiration takes place when cells have sufficient amount of oxygen to degrade glucose, and Krebs and PHOX are functioning normally. During this respiration process, the ultimate goal of the cells is to provide sufficient energy for maintenance and growth. As a result of this glucose metabolism, the following key oscillations occur in the cells and for simplicity, we put the curve colour within bracket as shown in Figure 9.1.

- NADH/NAD+ (grenat)
  Oscillation of NADH/NAD+ occurs as NAD+ is reduced to NADH during glycolysis and in the mitochondria, NADH is oxidised back to NAD+. So, overall, the concentration of NADH/NAD+ needs to remain homeostatic which justifies this oscillatory behaviour.
- ATP/ADP (red)
  The production of ATP is mostly likely to increase as soon as the mitochondria activity starts: this is visible in the graph as KREBS and PHOX becomes active, that is they switch from 0 to 1. ATP also changes from a threshold of 0 to 1, then slowly from 1 to 2.
- GLYC (yellow)
  Assuming a constant supply of glucose, the process of glycolysis oscillates as it consumes NAD+ to produce NADH and recovers it back from the mitochondria. This balance of NADH/NAD+ is maintained by glycolysis and Krebs in normoxic conditions. This shows the existence of a certain kind of feedback control which is important to catalyse intermediate reactions.
- KREBS (black)
  Krebs also is likely to fluctuate depending on the availability of its input sources: mostly pyruvate and in condition of stress, acetyl CoA from degradation of fatty acids. When glycolysis oscillates, KREBS also will oscillate as it is dependent on the end products of glycolysis.
- PHOX (green)
  The dependency of oxidative phosphorylation on Krebs for cofactors NADH and FADH accounts for its fluctuations and this dependency is visible in the graph; PHOX appears after KREBS with time.

9.3.2 Environmental context of Row 18 : FA=0 & In_O_2 = 1, GLC=2 and AA=2

This is the case of normal respiration where we have excess of both amino acids and glucose. Excess of glucose can favours the fermentation process even in the presence of oxygen (FERM oscillates in the graph 9.2). Similarly, these variables do not tend to 0 but rather oscillate: GLYC, ATP, NADH and NCD.

9.3.3 Environmental context of Row 20 & 21 : FA=0 & In_O_2 = 1, GLC=0 and AA = 1,2

The prime nutrients (glucose and oxygen) are absent. Fatty acids and amino acids are the only nutrients for the cells, which contribute to the nitrogen and carbon elements for future use. From Figure 9.3, stable state is reached both in the case of Row 20 & 21. This means that all variables will maintain their original values and will not change in such conditions.

9.3.4 Environmental context of Row 29 : FA=1 & In_O_2 = 1, GLC=0 and AA = 1

Similar to the previous cases, here also stable state is reached quickly. From Figure 9.4, we can see that both O2 and NCD tend to 1. Other variables will normally stay at their assigned values.

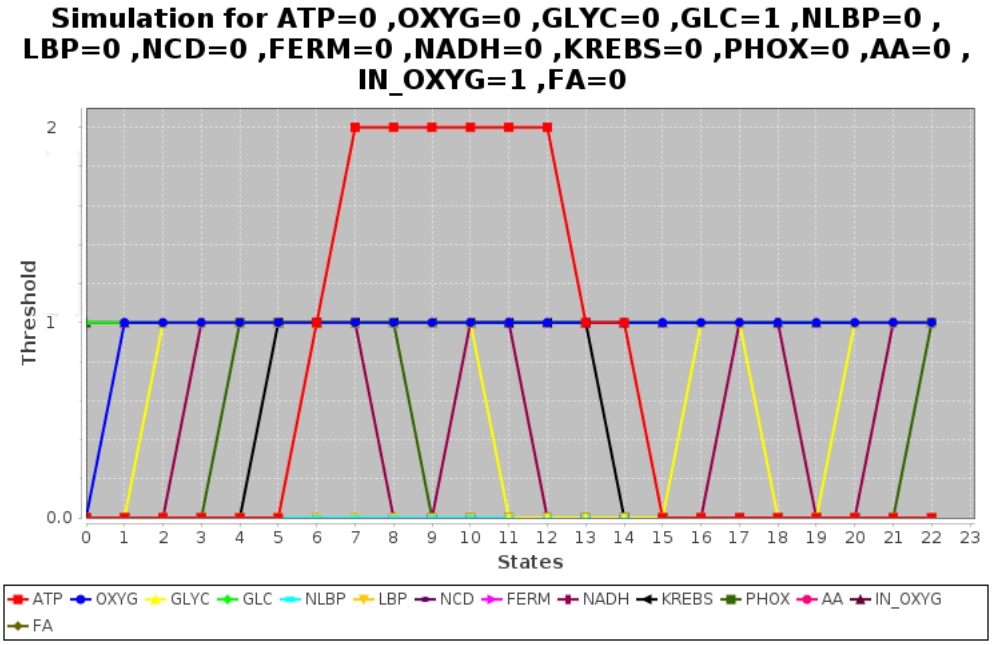


Figure 9.1: Normal Respiration with only input of glucose and oxygen.

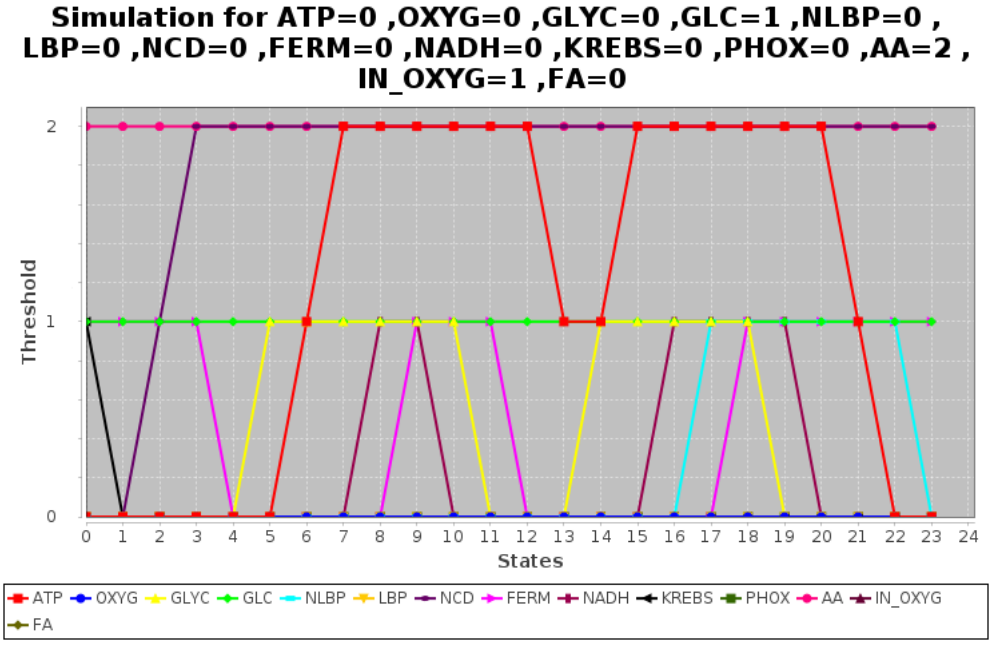


Figure 9.2: Respiration with normal glucose and excess amino acids

9.3.5 Environmental context of Row 35 : FA=1 & In_O_2 = 1, GLC=2 and AA = 1

From Figure 9.5, we can see that the majority of the variables will have the tendency to change from the value 0 to another value. In these conditions, the status of the KREBS shift from 0 and oscillates. This allows us to complete the missing interpretation at row 35 in the validation matrix.

Many biological systems exhibit non-deterministic behaviour. When simulating a dynamical model, it may happen (as it is the case more often) that certain trajectories appear after a very long time due to the non-deterministic behaviour and randomness of certain primary components of the system. Contrarily, it may also happen that some unexpected properties are observed during simulations but this does not readily means that we have refuted the model. Again, due to this non-deterministic feature, some trajectories have less probability of being chosen and their turn may happen after a long time.

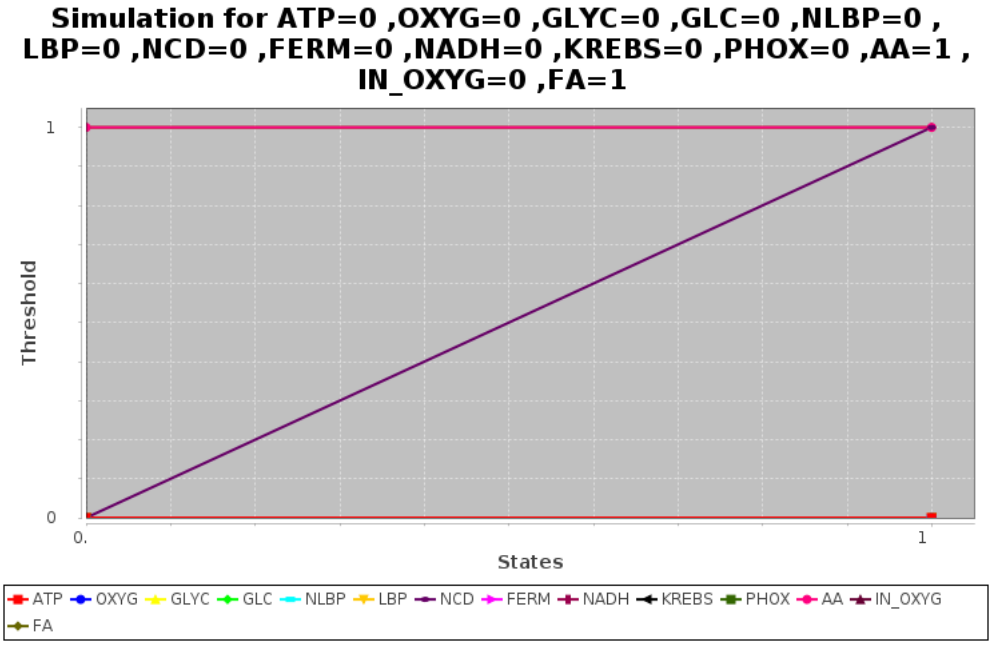


Figure 9.3: Inactive respiration with the presence of only fatty acids and amino acids.

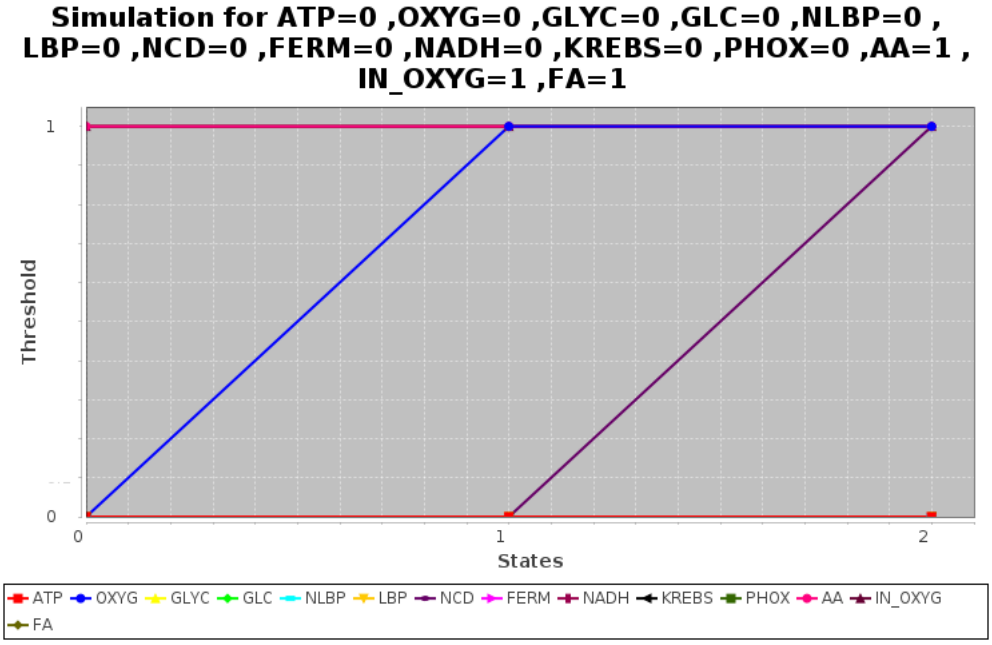


Figure 9.4: Respiration with absence of glucose milieu.

Consequently, simulations offer limited validation capabilities. They have the advantage to coherently exhibit many aspects of the formal model, thus facilitating the discovery of the main modelling errors. But, they are unable to *establish* a property of the model if it involves possibly infinite traces. This leads to using temporal logic in order to fully exploit the validation matrix.

9.4 Fair path CTL

In this section, we re-iterate the use and importance of fair path CTL already described in Chapter 5 (Section 5.7) and details provided in Annex 11.6. Due to the non-deterministic property of the metabolic network and its complexity, the use of classical CTL did not contribute much in the evaluation of certain biological property even in the presence of the favourable conditions.

Several fair path CTL formulas are implemented in terms of macros in our tool, DyMBioNet. For

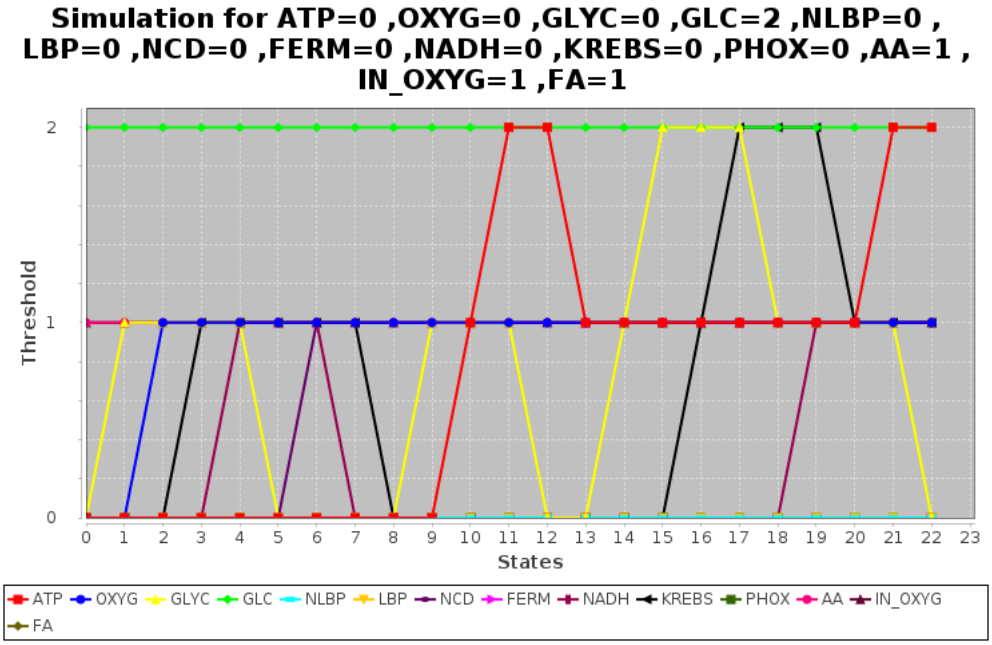


Figure 9.5: Respiration with high glucose and all other nutrients present.

every fair path property that are verified, they are transformed to their equivalent CTL forms (see Section 5.7 for the translations). These CTL formulas are then processed by SMBioNet which is integrated in DyMBioNet.

9.4.1 Useful CTL macros

Here, we formalise three scenarios which are common characteristics of the variables in the proposed abstract model: boolean oscillations, multivalued oscillations and tendency of variables to reach a fixed threshold. Theoretically, variables can show oscillatory sign during which they alternate between two thresholds 0 and 1; this is a typical boolean attitude. However, if a variable has more than 2 thresholds, then another possibility is many oscillations between the different thresholds. Finally, some variables may tend towards a particular value (for example the variables tend to 0 due to lack of resources). First, we will show how each of them are formalised properly, then give an example for each.

9.4.1.1 Oscillate(x)

Boolean variables can oscillate between 0 and 1. For example NADH and FERM are boolean variables that oscillate between 0 and 1 (due to the loop between glycolysis and fermentation). The implementation

```
/**
 * This method takes a variable and return the CTL equivalent of its
 * oscillation between 0 and 1
 *
 * @param variable
 * @return the normal CTL equivalent for the Fair CTL for oscillation
 */
public String oscillate(String variable) {
    return "AG (!(E [(" + variable + " < 1) U (AG(" + variable + " < 1))] | E[(" + variable + " >= 1) U (AG("
            + variable + " >= 1))]))";
}
```

Figure 9.6: Java implementation of fair path CTL for oscillation between 0 and 1

is shown in Figure 9.6.

9.4.1.2 OscillatePlus(x,low,high)

For variables having thresholds higher than 1, they may oscillate between *low* and *high*, where $low \neq 0$. For example, a variable x with boundary 2, can oscillate between 0 and 1, and also between 1 and 2. This fair path conversion is shown in Figure 9.7.

For example ATP has a threshold of 2. In normal respiration, we have observed that ATP oscillates between 0 and 1 as well as between 1 and 2. Here for ATP, low=0, med=1 and high =2 and it is written as oscillate(ATP,0,2).

```java
/**
 * This method takes a variable and return the CTL equivalent of its
 * oscillation between 0 and n
 *
 * @param variable
 * @param low
 * @param high
 * @return the normal CTL equivalent for the Fair CTL for oscillation++
 */
public String oscillatePlusPlus(String variable, int low, int high) {
    return "AG (!(E [(" + variable + " < " + high + ") U (AG(" + variable + " < " + high + "))] | E[(" + variable
            + " > " + low + ") U (AG(" + variable + " > " + low + "))]))";
}
```

Figure 9.7: Java implementation of fair path CTL for oscillation between 0 and boundary, b

9.4.1.3 tendTowards(x,n)

Under certain cellular conditions, a variable x can tend towards a particular value n.

```java
/**
 * This method builds the CTL formula for a variable showing the tendency to
 * go towards a particular bound
 *
 * @param variable
 *            to be checked
 * @param value
 *            towards which the variable is attracted
 * @return CTL formula for tending towards a particular value
 */
public String tendTowards(String variable, int value) {
    return "!(E[EF(!(" + variable + "=" + value + ")) U (AG(EF(!(" + variable + "=" + value + "))))])";
}
```

Figure 9.8: Java implementation of fair path CTL for a variable tending towards a particular value

9.5 Conclusion of the chapter

To summarise, the validation matrix has been instrumental for validating metabolic phenotypes where knowledge was available; given the multiple scenarios based on the large number of variables. This allowed for a fast validation of the model with the biologists. Unidentified phenotypes (blank cells) were completed using Fair CTL.

In software engineering, verification and validation form an integral part in the software development life cycle; similarly validation is fundamental to the modelling of complex networks, independent of the formalism used. This is the equivalent of the testing phase to make sure the end product has passed the set of tests (functional and structural). In this chapter, we cross-check the final abstract model to make sure the mathematical model reproduces at least the phenotypes of interest from the biological model. The verification of the model followed two complementary steps: simulations and model checking with fair path CTL.

As a key concept, we applied another tool inspired from software engineering which proved valuable as a side tool: the *validation matrix* which is constructed meticulously. The validation matrix acted as an inventory system for the model and as mentioned has been tabulated even *before* the completion of the model. The interdisciplinary supervising of this PhD has facilitated this step. It may seem obvious and surprising that a large majority of K parameters have been found without the use of CTL or any other related techniques. This is truly exceptional but we are thankful to the huge knowledge available on the metabolism. This is a more than one century old field of science with extensive literature of which about 100 articles were consulted to help the parameter identification of the model. Arguably, this shows that the choices we made initially on the variables and their categorisation, the set of multiplexes, and the abstraction of several processes and metabolites, have been coherently done to finally get a valid model. We did the simulations using DyMBioNet and model checking using fair path CTL.

Simulations allowed us to get a quick grasp of the energy metabolic network and its regulations, and at the same time showed preliminary metabolic traits and detect any anomalies in terms of unobserved phenotypes. Model checking technique with fair path CTL helped us to find those unobserved phenotypes and at the same time serve as a tool to validate those correct simulations. Fair CTL has been a pivotal tool in the validation process helping us to describe observable metabolic properties to have a mutual understanding both in terms of biological questions and mathematical formalism. To track the non-determinism of the model and do fairness to all possible trajectories, we sought the help of fair path CTL.

Finally, our mathematical model is a formal model of the metabolic regulation in eukaryotes. As the metabolic network is a central machinery for cell proliferation, our proposed model can be integrated in more complex and large networks (more parametrisation will be required) including, in particular, cell division cycle. These models are of interest for studying the control of cell proliferation (for example cancer, parasite related diseases, etc). We give some fruitful applications where our model is being used in the next final chapter.

CHAPTER 10

CONCLUSION

This manuscript demonstrated the interdisciplinary work needed for the logical modelling of the regulation of the metabolic pathways : a contribution brought together by computer scientists and biochemists. The main contributions of the thesis are reminded in this chapter; we then show how our model could be used as input for other projects and discuss the main possible avenues for future works.

10.1 Contributions of the thesis

We have achieved a rather big and intricated model of central carbon metabolism to understand the regulation mechanisms that control the metabolic shift between *respiration* and *fermentation*. This model includes several novative abstractions, without which it would not be possible to manage the modelling task, due to a large number of interdependent variables. As a result, we obtain a fully consistent abstract model that offers a "global view" never offered up to now within a fully mathematically defined model with dynamics. This finds many interesting applications as this model is pluggable in metabolic related research (for example in cancer therapy).

The choice of a *discrete* model has been decisive in the success of our modelling objective. This choice also is somehow "non-standard" in a domain (metabolism) where all models are based on quantitative flux modelling. Owing to this choice, we have been able to provide a coarse-grained model able to mimic the metabolic network behaviour. The model uses sufficiently global components of energetic metabolism in order to identify parameters through global understanding of these components (metabolic pathways). As a result, it has been possible to determine all regulation kinetic parameters K which basically inform the cellular component or pathway which action to make according to input signals (sometimes a combination of activation and inhibitory signals).

We have put in place a well-defined methodology to tackle the degree of complexity of the metabolic network. This comprises of two parts : first, the K parameters have been determined using a set of "thought experiments" supported by a large number of biochemical knowledge, supplemented secondly by using a "validation matrix" which exposes a systemic view of the expected global behaviour of the model.

The model has been validated using proof checking techniques from software engineering that were applied to the R. Thomas formalism. Validation of experimental phenotype has been possible using Computation Tree Logic (more precisely Fair path CTL) which assesses whether an experimental phenotype listed in a validation matrix is reachable by the model or not at a certain time in the future.

We developed the software platform DyMBioNet according to the aforementioned methodology. DyMBioNet was essential to put in place a software environment to easily manage all the *14* variables (and 100 K parameters) and also to observe the overall dynamics of the system. Thus, we constructed the DyMBioNet platform for simulation purposes as well as to check a host of Fair CTL properties.

10.1.1 Contribution to theoretical biology

Due to the high complexity of biological networks, one often needs to abstract from the structural specifics of a mechanism and represents it in a skeletal, coarse-grained manner. There is a lot of biological researches that have been successful for the study of gene and protein networks by applying a certain degree of abstraction [159]. In doing so, we lost information on molecular details but however, we gained sufficient precision on the phenotypic behaviour of the different metabolic pathways and their regulations.

This central notion of abstraction presents powerful strategies where the causal relationships can be identified. By using this mindset, we have successfully been able to abstract the pathways regulating the production of energy and biomass: two pivotal elements for cell survival and proliferations. We show this abstraction as follows:

- the ability to regroup biological entities in different cell compartiments. For example, we have abstracted two main cofactors ATP and NADH present in cytosol and mitochondria.
- the ratio of ATP/ADP and NADH/NAD+ have been regrouped in only one variable ATP and NADH respectively. ADP is represented as $\neg$ATP and NAD as $\neg NADH$. At the same time, ATP (respectively NADH) also abstract their derivatives GTP (respectively NADPH).

On this understanding of abstraction, this has allowed us to reduce drastically the number of variables and build a minimised version of the metabolic regulatory graph. Modelling the dynamic behaviour of the metabolic network could have taken two forms either using classical continuous frameworks or using a discrete approach. We opted for the discrete dynamical approach and we give our justifications in the next sub-section.

10.1.2 Modelling strategy

For a long time, differential equations have been the classical choice for the study of biological systems. However, differential equations present a major difficulty: The set of parameter values must be determined from curve fitting that ask for many experiments with precise measurements. The number of real parameters is comparable to the number of discrete parameters of our approach but they can take an infinity number of real values, making their identification far more complex.

We illustrate this by assuming we have the in-degree (number of variables acting on it) of a variable set to n, which in differential equation would lead to $2n + 2$ parameters. In our model, most of the in-degrees n lies between 2 and 3, and as we have seen (in chapter 4), the use of multiplexes greatly reduces the number of in-degree for each variable. If we were to use differential equations, the number of kinetic parameters for n would therefore lies between 6 and 8. In the discrete logical approach, on average, n is between 4 and 8. The difference in numbers of parameters is not significant in the two approaches. But, the discrete formal approach only requires finding discrete finite values (0 to 2) whereas finding continuous values in differential equation is far more difficult with a larger domain. Finding these values from learning techniques based on heuristics depend heavily on the amount of data as input and more often, most of these precise data is unknown. Therefore, this allows for only a partial identification of parameters with differential equations. On the contrary, by selecting an abstract level of intervals, our logical approach allows for an exhaustive search in the identification of K parameters.

We used Thomas' framework, as a large majority of interactions are of regulatory nature. We thought the identification of integer parameters would be easier than any continuous approach as we would benefit from the use of model checking tools. Even better, after completing the model, we realised that not only the boundary for almost all variables lies between 0 and 2 but also the in-degree for almost all variables is less than 3. This gives rise to a fairly smaller number of kinetic parameters to identify, compared to differential equations, and this reinforces *a posteriori* our choice for a discrete approach. As the boundary for some variables exceeds a boolean value, we opted for the multivalued Thomas alternative. Model checking technique using Fair path CTL has allowed us to easily verify known metabolic traits, and confirmed our K parameter values.

10.1.3 Methodology

Putting in place a strict methodology was pivotal for the smooth and effective progress of the thesis due to the large complex size of the network, and also to avoid missing any regulatory paths during the coarse-grained construction of the metabolic network. On one hand, the interdisciplinary "though experiment" guided us to the determination of the 100 K-parameters with the help of the biochemist thank to the available rich bibliography. On the other hand, the validation matrix (inspired from software engineering) was useful for depicting the global behaviour of the model. Metabolism is a well-studied metabolic network, most of the known phenotypic behaviours were listed in the validation matrix. Unknown cellular behaviour were queried using our formal checking tools.

This methodology is comparable to classic software engineering : the interaction graph and the values of the K parameters determined from biochemical transitions play a similar role as a computer

program with its detailed stepwise instructions, whereas the validation matrix is comparable to a formal specification of a program since it formalises the expected global behaviour of the model. Then, model checking for CTL is the tool to formally prove correctness.

Just like in software engineering practices, it is crucial to keep the independence between (1) the writing of the specification and the final testing of the product and (2) the development of the detailed program. In the same way, the independence of the K parameters identification using biochemistry knowledge and the validation matrix by phenotypic analysis in biology, was crucial for a good quality validation of the metabolic model proposed. Compared to the thesis plan, the validation matrix was written well before the identification of the K parameters (therefore before testing the global behaviour of the model) by our platform DyMBioNet.

To end, it is important to highlight that it is the first time, to our knowledge, that this type of systemic methodology is being put in place for modelling in systems biology due to the size of the model, among the largest, using R. Thomas theory.

10.1.4 Verification of proposed abstract model

We implemented two complementary ways of verifying the correctness of our model and all the underlying regulations in the metabolic network : graphic simulations and model checking with CTL formulas.

Graphic simulations using DyMBioNet helped us for checking the model for basic regulations and well-known phenotypes (for example oscillations in certain environmental contexts) in a specific timeframe. This approach enabled us to proceed relatively fast with the model and gave us some relative guarantee that the K parameters were good estimations. Furthermore, these simulations gave us sufficient grounds to get a base model which is easily configurable. Unfortunately, due to the large number of variables, simulations were slow and it was almost impossible to detect the behaviour of the variables in the long run.

For unknown environmental contexts and for an infinite timeframe, we revert to model checking where unknown biological properties were verified using CTL. Due to the complexity of the network, the huge number of variables and the high number of retroactions, classical CTL fails to check certain properties (certain paths were never visited which means wrong interpretations of certain phenotypes). To ensure equity in all the paths, biological traces were handled using *fair path* CTL. This guaranteed to ignore spurious unfair paths where a given transition is neglected although its initial state is reached an infinity number of times. that all paths would at least be visited once an infinite number of times.

In between, we highlighted the importance of the *validation matrix* that allows us to cross-check dynamic properties of the model with known biological observations. Simulations have been instrumental to get well acquainted with the mathematical model and have been carried out with our new tool, DyMBioNet. Each simulation result was verified against the cells for a given context. For long term verifications of variables, complex properties in the normal CTL were translated and formalised using Fair path CTL.

Finally, we have a generic, complete and fully validated abstract model of the regulation of energy and biomass production of metabolism in eukaryotes. This generic model can serve as a blueprint for the design of more complicated biological systems and some of these systems are discussed in the next section.

10.1.5 DyMBioNet : A modelling platform for Thomas' models

In the context of complex biological regulation networks, we have designed a configurable software architecture to deal with the dynamics of the networks. The DyMBioNet platform is a modular tool which is extensible allowing future users to easily incorporate other functionalities.

The set of existing options bundled with DyMBioNet include : a visual GUI to design a biological network from scratch, customise K parameters, threshold values and variable colors, a charting interface for simulation purposes, and the possibility to visualise a 3D representation of state transition diagram of small biological networks (≤ 3 variables). The tool has been instrumental for verifying known biological phenotypes and to go further, users can benefit from checking these same phenotypic behaviours (and unknowns as well) using Fair path CTL. Also, a reporting feature is available to generate global evolutions of all variables under certain environmental conditions set by the user.

10.2 Future directions

The model we have developed using the R.Thomas framework allows to implement the behaviour of different types of cells. We are able to model normal cells (quiescience and / or proliferation) or to add certain properties which are unique to cancerous cells. If our model is based only on normal cells which are proliferative in nature with the classic respiration-fermentation shift, the different hypotheses for other types of cells can be realised by modifying certain K parameters.
With the implementation of Fair path CTL for path equity in the model, there is the possibility to do certain phenotypic screening of cells to identify conditions necessary for the activation or inhibition of phenotypes of interest (like fermentation). More interesting, this can enable us to find important combination of actions necessary to reverse the Warburg effect alongside the main metabolic pathways of central carbon metabolism. These combinations (which can be encapsulated in multiplexes) can be translated into an action in poly-pharmacology (a combination of drugs) for reversing the Warburg effect and suggest novel approaches for cancer metabolism. The choice of each drug activating or inhibiting a certain metabolic pathway cannot predicted by the model. This expertise is left to the pharmacologist.
Integrating our "turnkey" system into more elaborated complex models will not necessarily imply adding many new variables and neither exploding many existing variables into subunits. Nevertheless, the bibliographical knowledge acquired from the 100 kinetic parameters can be foundational for further specific contexts. This can take two twists:

- the model is controlled by other variables (on top and above what we have considered for our context) which act on the existing input variables. For example, if we have an oncogene maintaining the value of glucose to 1, this will reduce the number of parameters and will also have a cascading effect on the model as a whole: all multiplexes using glucose greater than 1 can be simplified, possibly bringing new constraints on some target variables, which in turn, can allow further simplifications.
- the model is merged into a more complex system where it serves as a "black box" input to other subsystems. For example, incorporating the model with the cell cycle or the circadian clock.

We may also imagine a hybrid version of this metabolic regulation by incorporating the notion of time: that is how much time we spend in a given discrete state. But, due to the degree of abstraction, this is hardly achievable as many details are hidden in the abstracted variables. So, we do not include this as a future work, at least before a notably long term.

This section is devoted to the list of future works entailing the regulation of energy and biomass regulation in eukaryotes. First, we give a brief explanation of an ongoing project which has already started using our generic model and second we present other potential metabolically-engineered disciplines.

10.2.1 The project "PAIR Pancreas"

This project involves 5 french research teams including I3S, INSERM Marseille, INSERM Toulouse, INSERM Lyon and INSERM Lille.

Cell to cell communication occurs via signalling pathways to accomplish the transmission of important biological information. This intercellular communication is complex, forming a network of networks where the output of one cell becomes the input of other cells. In such complexity, feedback loops can arise and there is possibility of recursivity. In cancer progression, this communication is altered where cancer cells persuade normal cells to give unique growth signals for proliferation. These growth signals are under the control of a family proteins known as *Ras* proteins (including HRas, NRas and KRas). But it is well-known in cancer biology that, among the Ras genes, *KRas* is the most mutated and active gene. K-Ras orchestrates (via the energy and biomass production) ROS production which is harmful to cells. Persistent ROS generation can damage DNA and can activate p53 to promote full-blown apoptosis [166].

As such, the objectives of the PAIR pancreas project are to elucidate the communication between different types of cells with more emphasis on KRas protein and ROS. The workflow for this project is to better understand cell-to-cell communication as follows: normal cells and cancer cells, normal cells and aggressive cells, and finally between normal cancer cells and aggressive cancer cells.

This study of information interchange between normal cells and cancer cells can open up new therapeutic windows to pancreas cancer treatment.

10.2.2 A rather natural continuation : Interplay between the circadian cycle, cell cycle and metabolic regulations

Cells have built-in mechanisms to sustain their activities (growth and repair) by sensing the organism's demand, which are controlled in a timely manner. This is to limit damage and maintain homeostasis: both important for cell's survival and the smooth running of the organism as a whole. The state of the organism is orchestrated by three main biological regulations: metabolism to give energy and biomass, cell cycle which integrates cell division procedures and the circadian clock which imposes a rhythm for each process to optimise the succession of biological processes (and at the same time to optimise resource usage).

However, highly proliferative cells impose their own rules and bypass these setups. Cancer, for example, is characterised by an uncontrolled cell growth supported by metabolic reprogramming. They do not follow the usual pathways of normal cells and more often they display abnormal cellular capacities that allow them to become aggressive and dominate neighbouring cells. On top of that, they become resistant to the body immune systems.

This motivates to focus researches on the crossroad between the study of the three aforementioned cycles and how their inter-communications can provide insights for chrono-therapeutics approaches for many diseases. It would be of outmost interest to understand the dynamics between the following networks:

1. Metabolism and cell cycle
   The metabolic requirements of dividing cells differ largely from that of resting cells: they need to replicate their DNA and need more biomass [176, 177]. This means that cell cycle progression is highly dependent on metabolism which itself relies on substantial nutrients supply. Evidence is emerging in support of the coordinated temporal regulation of metabolism directly by the cell cycle modulators [175].

2. Cell cycle and Circadian cycle
   Recent research has shown that a disruption of the circadian timing system in mice causes increased tumour development [174]. The formal regulation of the circadian cycle on the cell cycle implemented using a mathematical model can provide a precise understanding of this intuitive mechanics. This can open doors for precise medicines to revert the uncontrolled proliferative properties of cancer cells. For example, there is a possibility to target specific times when cancer patients can be administrated medicines for sustaining health.

3. Circadian cycle and metabolism
   Circadian rhythms and cellular metabolism are intimately linked [171]. Multiple systemic and molecular mechanisms exist that connect the circadian clock with metabolism at all levels, from cellular organelles to the whole organism, and deregulation of this circadian-metabolic crosstalk can lead to various pathologies [172].

10.2.3 Research opportunities other than diseases

Many micro organisms and plants display metabolic properties that offer potential for commercial applications [160, 161]. We discuss two emerging metabolic-engineered processes as follows:

- Biodegradation
  There is a global concern on the accumulation of all forms of plastics as environmental wastes. Many strategies are being adopted worldwide to ban plastics and use paper as alternatives. Synthetic plastics, which are widely present in materials of everyday use, are ubiquitous and slowly-degrading polymers in environmental wastes [162]. Some species of Pseudomonas have shown great efficiency for degrading polluants including plastics, oils and other types of polymers. These kind of bioremediations through metabolic engineering are useful for a sustainable environment and should be used at a larger scale. A better way of achieving this, is to modulate the existing generic metabolic model to identify ways of increasing the biomass yield in a short duration.

- Biofuels
  The efficient production of biofuels like bioethanol, biohydrogen and biodiesel are safer alternatives for a greener environment as they have shown cleaner burning characteristics. They can significantly reduce the emissions of greenhouse gases and are considered as replacements for many petroleums products. Recently, microorganisms are being metabolically engineered to convert carbon elements (example carbon dioxide) to effective biochemicals [164, 165]. In these processes, many parameters

can be tuned to enhance fuel production.

In short, we believe that energy and biomass form the left and right hands of the cell: both are needed to carry out all the activities for continual existence of the cell. In this study, we have modelled part of the whole mesh of networks in metabolism: the regulation of the energy and biomass production. The study of the metabolism is complex, yet exciting. It has just started and we hardly know how many applications and discoveries will be unraveled in the future.

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CHAPTER 11

ANNEX

11.1 Classical Logics

Logic is at the basis of all formal approaches. In this context, we give a panoramic view of what constitutes general logics.

11.1.1 General Logics

Nowadays, many modellers of complex systems are embracing logic as a useful tool to solve problems in biology, and bioinformatics among others. Logic, which for decades, has been used in software engineering for validating or invalidating programs, is progressively paving its way in other areas like medicine, drug design and the like. A general overview of the main aspects of logic is shown in Figure 11.1 with a brief introduction of the different parts.

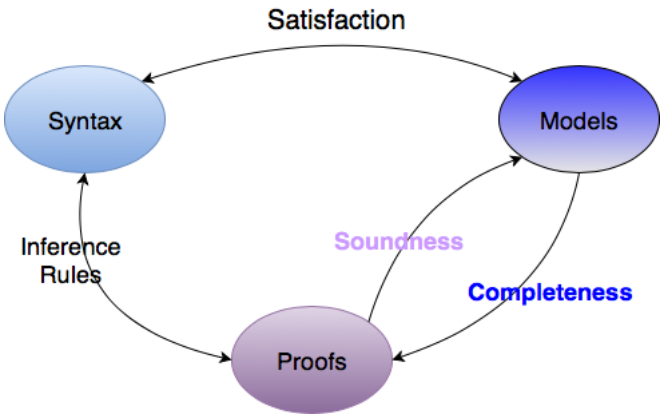

Figure 11.1: General logics : *Syntax* defines all the allowable sets of propositions formulas that can be stated about models. Possible *Models* are mathematically defined as sets where the formulas can be interpreted, allowing to define if a model *satisfies* a formula or not. *Proofs* are syntactic transformations allowing to derive new formulas from a given set of formulas hypotheses via *inference rules*. Finally, one requires these newly inferred formulas to be satisfied in all models satisfying the hypotheses (*soundness*) , and where the expressive power if weak enough, the reverse property, *completeness*, may be ensured.

According to [**?**], a general logic is defined by 5 notions (sections 11.1.1.1 to 11.1.1.5) that are linked by important properties (section 11.1.1.6):

11.1.1.1 Signatures

The first object that defines general logics is a set of possible signatures. A signature (denoted as Σ) is in practice a set of symbols that are specific to a given studied question. We can attach static information to each symbol and this information will establish how each symbol can be used in formulas. So, the signature is not only the set of symbols but can also impose the arity of each symbol or typing rules for example. In our study, for example, the following two symbols are used among others: FERM and GLYC (representing fermentation and glycolysis respectively). Then, in our case, the corresponding static information for these two symbols are : "FERM" is boolean and "GLYC" can take a maximum value of 2.

11.1.1.2 Well-formed formulas

This is the second object of general logics which defines the set of well-formed formulas for each signature, Σ denoted For(Σ), and additional connectives such as $\wedge$, $\vee$, $\neg$ and so on. In practice, formulas are recursively defined from the symbols of Σ. For example "(FERM = 1) $\wedge$ (GLYC $\geq$ 1)" will be a well-formed formula for our study but "(FERM = 2)" is not because, FERM being boolean, it can only be compared to 0 or 1. Symbols such as connectives, quantifiers, modalities, etc. are common to the logic under consideration. They do not depend on the specific question, contrarily to signatures.

11.1.1.3 Models

The third object of general logics is the definition of the set of models that provide the semantics of a given signature denoted as Mod(Σ). A model is usually a set together with a collection of functions or relations that are considered as "realisations" of all symbols in Σ. For example in our study, a state of the model[1] which associates to each symbol in the signature a value which is less than its corresponding boundary, can constitute a model in the signature.

11.1.1.4 Satisfaction relation

This is the definition of a set of couple (M, ϕ) $\leftarrow$ Mod(Σ) x For(Σ) such that M satisfies the formula ϕ , denoted M $\models \phi$. In practice, it defines inductively a set of theoretic interpretation of the system. For example, the formula (FERM = 1) $\wedge$ (GLYC $\geq$ 1) is satisfied in the model whose state contains FERM=1 and GLYC=2 but is not satisfied in any model where one of FERM or GLYC has the value 0, for instance.

11.1.1.5 Inference relation

The inference relation is an object in general logics which represents the ability to represent formal proofs.

Inference rules
Formally, proof systems refer to the existence of an inference tree that is used to derive new formulas from existing ones. The primary goal of this system is to prove that some deductions are valid in all models, in a way that allows us to understand the reasoning that sustains the formula. For example :

$$\frac{\psi_1, \psi_2, ..., \psi_3}{\psi}$$

where the denominator is often referred to as the conclusion and the numerator as the set of premises. Here, this can be explained that to prove ψ, it is sufficient to prove the formulas ψ_1 to ψ_n, and once they are proved, ψ also is proved.
We can summarise inference as follows : for $\Phi \subset$ For(Σ) and for all $\phi \in$ For(Σ), $\Phi \vdash \phi$ means that there exists a proof tree whose leaves belong to Φ, Φ is the root, and internal nodes are inference rules. A detailed overview of natural deduction is annexed in 11.3.

For illustration purposes, let us assume the following inference rules :

$$\frac{}{x \leq x}\ (1) \qquad \frac{x \leq y \qquad y \leq z}{x \leq z}(2) \qquad \frac{x \leq y}{x \leq y+1}(3)$$

$$\frac{\psi \qquad \phi}{\psi \wedge \phi}(4) \qquad \frac{x \leq y \qquad y \leq x}{x = y}\ (5) \qquad \frac{\psi \qquad x = y}{\psi[x \leftarrow y]}(6)$$

Now, suppose Φ = {FERM=1, GLYC=2, GLC=0} and ψ = FERM $\leq$ GLYC $\wedge$ GLC $\leq$ GLYC, the question that can be asked is whether $\Phi \vdash \psi$?. The whole inference validation steps are shown in Figure 11.2.

Remark:
When applying (3), e.g in $\frac{1 \leq 1}{1 \leq 2}$ (3), we have made implicit the knowledge about addition. In fact, one should read 1 as $s(0)$ and 2 as $s(s(0))$ (where s is the successor symbol). Then, we should use the

[1] According to this approach, each state represents a model, and we will see later that temporal logic allows us to implement this (Kripke structure)

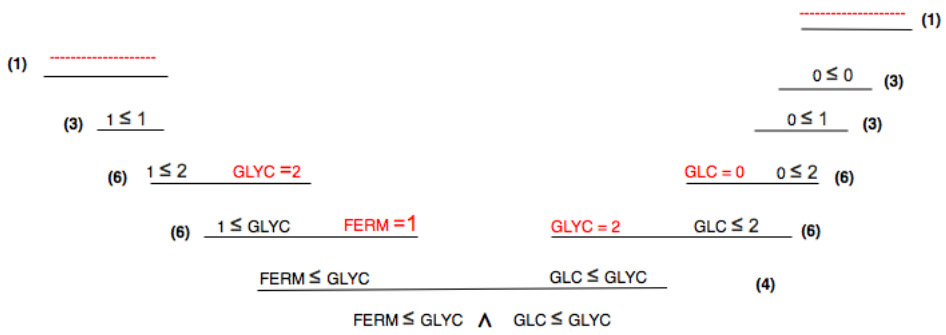

Figure 11.2: Proof tree proving FERM ≤ GLYC using inference rules. Dotted lines and red text refer to the *leaves* of the proof tree.

inference rule (3) under the more detailed (but less intuitive) form $\frac{x \leq y}{x \leq s(y)}$.

Note that proof trees are not the only way to define "$\vdash$". For example, if the set of models is finite and if there exists a model checking algorithm able to decide if $M \models \psi$ whatever M and ψ, then $\Phi \vdash \psi$ can be defined by checking all models.

11.1.1.6 Important logic properties

There are few properties that allow to evaluate the pragmatic usefulness of a logic:

- Soundness is always required
  An inference relation is called *sound*, if all what we can prove is valid. More precisely : for all signatures Σ, all subsets $\Phi \subset for(\Sigma)$ and all formulas $\psi \in for(\Sigma)$, we have:
  - if $\Phi \vdash \phi$ then for all models $M \in Mod(\Sigma)$, if $M \models \Phi$ then $M \models \psi$ (where $M \models \Phi$ means : $\forall \phi \in \Phi, M \models \psi$)
- Completeness is the reverse property
  For all signatures Σ, all subsets $\Phi \subset \text{For}(\Sigma)$ and all formulas $\phi \in \text{For}(\Sigma)$, we have:
  - If for all models $M \in Mod(\Sigma) : (M \models \Phi) \implies (M \models \phi)$, then $\Phi \vdash \phi$
    Completeness is difficult to obtain and there are several interesting logics that are incomplete. In fact, due to the Gödel theorem, a logic with recursivity is likely to be incomplete.
- Decidability
  This simply means that there exists an algorithm which always finds the proof tree if it exists, or else fails, in *finite* time.

In the next section, we give an insight on propositional logic, which is mostly the basic case of almost all logics.

11.1.2 Propositional Logic

In the past, diverse logics have been studied by philosophers, mathematicians and computer scientists. The simplest one is propositional logic. It is a logic which consists of a finite set of statements (called atoms) which constitute the signature and can be either true or false within a model. One can construct more complex statements using logical connectives.

11.1.2.1 Syntax

The language of propositional logic is inductively defined as follows:

- There is a finite set $P = \{p_1, .., p_n\}$ of propositions where p_i is an atomic proposition formula (P is the signature)
- If ϕ is a propositional formula, then $\neg\phi$ (the negation of ϕ) is also a propositional formula.

- If ϕ and ψ are proportional formulas, then $(\phi \wedge \psi)$ is also a propositional formula (the conjunction of ϕ and ψ).

This defines inductively For(P).

Generally, an atomic proposition describes a simple event. For example: "Glucose is present" is simplified using an abbreviation such as glc (so that $glc \in P$). Conventionally, $(\phi \vee \psi)$ is used as an abbreviation for $\neg(\neg\phi \wedge \neg\psi)$ which is the disjunction of ϕ and ψ. Similarly, $\phi \Rightarrow \psi$ is an abbreviation for $\neg\psi \vee \phi$. For example, if $glc \in P$, one can write the following propositional formulas:

– glucose is present : glc

– glucose is absent: $\neg glc$

– glucose is present and absent: $glc \wedge \neg glc$

Finally, the overall set of connectives used in propositional logic are listed in Table 11.1:

Symbol	Name	Example
$\neg$	Negation(NOT)	Glucose is not present ($\neg glc$)
$\vee$	Disjunction(OR)	glucose or glutamine is present ($glc \vee glut$)
$\wedge$	Conjunction(AND)	Both glucose and glutamine are present ($glc \wedge glut$)
$\Rightarrow$	Conditional(IMPLICATION)	If oxygen is present, then oxidative phosphorylation is present($oxyg \Rightarrow oxph$)

Table 11.1: Propositional Connectives : Apart from $\neg$ which is a unary connective, the other connectives are binary

Remark that (glc $\wedge\neg$ glc) is a well formed formula although it means that glucose is both present and absent. The truth of a formula is a question that is independent of its well-formedness.

11.1.2.2 Semantics

Evaluating the truth value of any well-formed formula is precisely the role of the semantics. Logically, with n atomic propositions in P, we will have 2^n possible models in Mod(P) since we are dealing with truth values (True or False). Mathematically, this will be written using the following: $M : P \rightarrow \{True, False\}$. M is a model mapping all propositional atoms $(p_i..p_n)$ to either a True or False value. When the truth values of the atoms of a formula are known, the truth value of the formula is determined using a truth table. The list of truth table for all connectives is found in Annex 11.2.

Once the truth values for all propositional atoms of P have been given, the truth value of the whole formula can be automatically deduced by using its expression tree. The evaluation takes a bottom-up approach by starting from the leaves (first atoms at the bottom).

11.2 Truth Tables

Similarly, the truth table for logical OR, NOT, and IMPLICATION connectives are shown in Table 11.2 , 11.3 and 11.4 respectively.

glucose(glc)	oxygen(oxyg)	$glc \vee oxyg$
False	False	False
False	True	True
True	False	True
True	True	False

Table 11.2: Logical Connective OR

glucose(glc)	oxygen(oxyg)	$glc \Rightarrow oxyg$
False	False	True
False	True	True
True	False	False
True	True	True

Table 11.3: Logical Connective IMPLICATION

glucose(glc)	$\neg\ glc$
False	True
True	False

Table 11.4: Logical Connective NOT

Inference rules	Description
$\frac{\gamma \vdash p \qquad \gamma \vdash q}{\gamma \vdash p \wedge q}$	(i) AND introduction If we have proved on one hand p and on the other hand, we have proved q, then we have proved $p \wedge q$
$\frac{\gamma \vdash p \wedge q}{\gamma \vdash p} \qquad \frac{\gamma \vdash p \wedge q}{\gamma \vdash q}$	(ii) AND introduction. If we have proved p and q, therefore we have proved p. And similarly, if we have proved p and q, then we have proved q
$\frac{\gamma \vdash p}{\gamma \vdash p \vee q} \qquad \frac{\gamma \vdash q}{\gamma \vdash p \vee q}$	(iii) OR introduction. If we have proved p, then we have either p or q. And similarly if we have proved q, then we have proved p or q
$\frac{\gamma \vdash p \vee q \quad \gamma_{,p} \vdash r \quad \gamma_{,q} \vdash r}{\gamma \vdash r}$	(iv) If under the hypothesis p, we have proved r and under the hypothesis q, we have equally proved r, then if either the hypothesis p or q $(p \vee q)$ is true, r will be true.
$\frac{\gamma_{,p} \vdash q}{\gamma \vdash p \Rightarrow q}$	(v) if under a certain set of axioms and the hypothesis p , we have proved q, then we have proved that p implies q. This is called the deduction rule and is not true in all logics.
$\frac{\gamma \vdash p \qquad \gamma \vdash p \Rightarrow q}{\gamma \vdash q}$	(vi) This is called the Modus Ponens inference rule. If from γ, we have proved p and at the same time we have also proved that $p \Rightarrow q$, then we have proved from γ, q will hold.
$\frac{\gamma \vdash p \qquad \gamma \vdash \neg p}{\gamma \vdash \bot}$	(vii) If under γ, we have proved that both p and $\neg p$ are true, then under the same conditions, we have proved that γ is incoherent as we cannot say that all are true and all are false at the same time.
$\frac{\gamma \vdash \bot}{\gamma \vdash p}$	(viii) If we can prove that under *gamma*, that it is incoherent, then we can prove anything (p, $\neg p$,...)

Table 11.5: Natural deduction rules

11.3 Natural deduction

Natural deduction is a way to prove the validity of a sequent.Other alternatives are available.

Example $\Sigma : \{a, b, c, d\}$
$\gamma : \{c \Rightarrow d, b \Rightarrow d, a \Rightarrow b \vee c\}$
We want to prove $a \Rightarrow d$ written as $\gamma \vdash a \Rightarrow d$

The steps for constructing the proof tree is shown in Table 11.6 and each step is justified as follows:

(a) To prove $\gamma \vdash a \Rightarrow d$, the only rule that applies is rule (v) from which we get $\gamma_{,a} \vdash d$. Now, to prove $\gamma_{,a} \vdash d$, we apply rule (iv) to get (b).

(b) This gives rise to further three sets of rules to prove: given a and by adding b to the set of hypothesis, we assume that we will deduce d and similarly given a and by adding c, we will be able to deduce d. This means that by adding either b or c to the set of hypothesis, we will utlimatey deduce d.

(c) From $\gamma_{,a} \vdash b \vee c$, we get $\gamma_a \vdash a$ and $\gamma_{,a} \vdash a \Rightarrow b \vee c$ using rule (vi),where we substitute r as $b \vee c$. We apply the same rule for both $\gamma, a, b \vdash d$ (to get $\gamma, a, b \vdash b$ and $\gamma_{a,b} \vdash b \Rightarrow d$) and $\gamma_{,a,c} \vdash d$ (to get $\gamma_{,a,c} \vdash c$ and $\gamma_{,a,c} \vdash c \Rightarrow d$)

(d) For $\gamma_{,a} \vdash a$, we already have a in the set of hypothesis, so this is true. $a \Rightarrow b \vee c$ is already in γ, given above, so it is true. Next, we have b already in the set of hypothesis, so $\gamma_{,a,b} \vdash b$ is true. The same procedures are applied to $\gamma_{a,b} \vdash b \Rightarrow d$, $\gamma_{,a,c} \vdash c$ and $\gamma_{a,c} \vdash c \Rightarrow d$. Hence , we have proved $\gamma \vdash a \Rightarrow d$ is true in γ. This simple example can give rise to a proof tree with 11 nodes

(c)

$$\frac{\gamma_{,a} \vdash a \quad \gamma_{,a} \vdash a \Rightarrow b \vee c \qquad \gamma_{,a,b} \vdash b \quad \gamma_{,a,b} \vdash b \Rightarrow d \qquad \gamma_{,a,c} \vdash c \quad \gamma_{,a,c} \vdash c \Rightarrow d}{}$$

(b)

$$\frac{\gamma_{,a} \vdash b \vee c \qquad \gamma_{,a}, b \vdash d \qquad \gamma_{,a,c} \vdash d}{}$$

(a)

$$\frac{\gamma, a \vdash d}{\gamma \vdash a \Rightarrow d}$$

Table 11.6: Example of a proof tree

11.4 CTL semantics

The semantics for the remaining CTL formulas are listed here in this order : AXp, A[p u q], EFp, EGp, EXp and E[p u q].

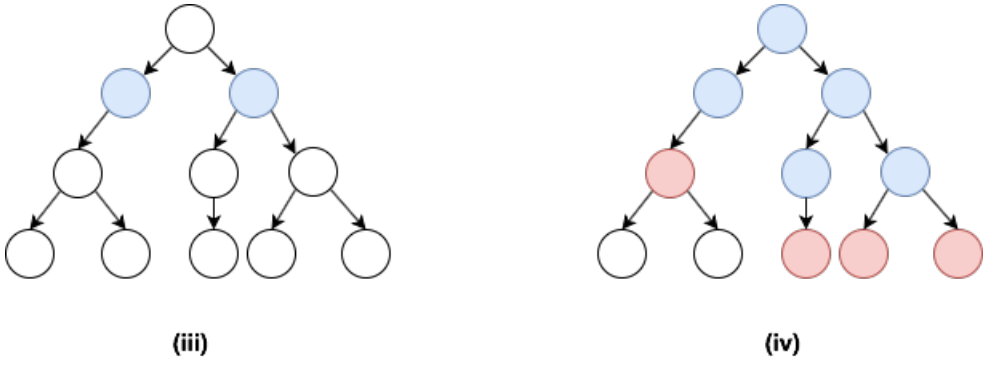

Figure 11.3: Temporal Semantics. (i) AX(p) : in general, p holds in all next states of a given previous state(ii) A [p u q] : p holds until q for all cases in the tree

11.5 Model Checking as a Kripke structure

Definition : Given a graph $\mathcal{G}$, we say that state v models ψ ,written as $\mathcal{G}$, $v \models \psi$. This can increase our confidence in the correctness of the model. We can also study counterexamples which can allow us to pinpoint the source of the error, correct the model, and try again. Counterexamples are paths from the initial state to a state where certain properties fail. In the study of our metabolic network, we use CTL to express our specifications which will be validated by a model checker.

We again take our example from Figure , but this time we assume there are some properties true in each state x and y. Our model checking can be represented as Kripke structure over a set of atomic propositions P with a four-tuple: $G = (\mathcal{V}, v_0, \mathcal{E}, \mathcal{L})$ where :

- $\mathcal{V}\{\mathcal{G}\}$ is a finite set of states.
- $v_0 \subseteq \mathcal{V}\{\mathcal{G}\}$ is the set of initial states.
- $\mathcal{E}\{\mathcal{G}\}$ is a transition relation representing the edges.
- $\mathcal{L} : \mathcal{V} \to 2^P$ is a function that labels each state with the set of atomic propositions true in this state.

We illustrate the above definitions with our graph from Figure

- $\mathcal{V}\{\mathcal{G}\} = (x, y)$
- x is the initial state
- $\mathcal{E}\{\mathcal{G}\} = (\{x, y\}, \{y, y\}, \{x, y\})$
- Assuming a list of properties are true in $\mathcal{L}\{\mathcal{G}\}$ such that $\mathcal{L}\{x\} = p, q, \mathcal{L}\{y\} = q$. This means that both p and q are true in state x and q is true in state y.

11.6 CTL fairness for transition paths

Fair paths in CTL

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1 Introduction

We consider a Kripke structure (V, E, L) and focus on its fair paths. Such a path p is an infinite path of (V, E) verifying : if a vertex v occurs infinitely often in p, then all the edges of E starting from v occur infinitely often in p. It is very natural to consider this paths when (V, E) is seen as an undeterministic discrete dynamical system whose set of states is V and such that : if the system is in state u at time t then the probabilities for the system to be in state v at time $t + 1$ is > 0 if $(u, v) \in E$, and 0 otherwise. Indeed, a path then describes a possible evolution of the system if and only if it is a fair path.

In this note, we show that all the temporal operators of the Computational Tree Logic can be interpreted on fair paths without leave this logic. This allows to use the powerful computational tools related to CTL in order to check properties on fair paths.

2 Preliminaries

Throughout this note, $G = (V, E)$ denotes a finite directed graph in which each vertex has at least one successor : for each $u \in V$ there exists $v \in V$ such that $(u, v) \in E$.

A *path* of is a map p from $\mathbb{N}$ to V such that $(p_i, p_{i+1}) \in E$ for all $i \in \mathbb{N}$ (the image of i by p is denoted p_i). The set of images of p is denoted $p(\mathbb{N})$ and is called the *set of vertices of* p. A path *from* u is a path p such that $p_0 = u$. A path from u *to* v is a path p from u such that v is a vertex of p. More generally, a path from u to $A \subseteq V$ is a path p from u such that p has at least one vertex in A.

In the following, we need the notions of trap domain and attractors. An *trap domain* is a non-empty set $A \subseteq V$ such that, for all $(u, v) \in E$, if

$u \in A$ then $v \in A$. An *attractor* is a smallest trap domain with respect to the inclusion relation.

Proposition 1 *If u and v belong to a same attractor, then there exists a path from u to v.*

proof. Suppose that u and v belong to a same attractor A. Let T be the set of w such that there exists a path from u to w. Clearly, T is a trap domain and is included in A. Since there is no trap strictly included in A, we have $A = T$. So $v \in T$: there is path from u to v. □

Proposition 2 *For each vertex u, there exists a path from u to an attractor.*

proof. Let $u \in V$ and let T be the set of v such that there exists a path from u to v. Clearly, T is a trap domain, so there exists at least one attractor included in T. □

3 Fair paths

Definition 1 *A* fair path *is a path p verifying : for all $(u, v) \in E$, if the number of i such that $p_i = u$ is infinite, then the number of i such that $p_i = u$ and $p_{i+1} = v$ is also infinite.*

Note that, from each vertex, there exists at least one fair path. Not also that if p is a fair path, and if p' is a path such that there exists j such that $p'_{i+j} = p_i$ for all $i \in \mathbb{N}$, then p' is a fair path.

Remark 1 *In order to see why this notion of fair path is interesting, let us see G is seen as a discrete dynamical system : the vertex set V corresponds to the set of possible states for the system, and E corresponds to the set of possible transitions between states : when the system is in state u at time $t+1$ then it is in a state v such that $(u, v) \in E$ at time $t+1$. This usual dynamical interpretation can be slightly specify by stating that, when the system is in state u at time t, the probability for the system to be in state v at time $t+1$ is, for all state v such that $(u, v) \in E$, strictly greater than zero. A path then describes a possible evolution of the system if and only if it is a fair path.*

Proposition 3 *Let p be a fair path, let u be a vertex of p, and let p' be a path from u. If the number of i such that $p_i = u$ is infinite then, for all $j \in \mathbb{N}$, the number of i such that $p_i = p'_j$ is infinite.*

Proof. We proceed by induction on j. If $j = 0$ then $p'_j = u$ and so, following the conditions of the proposition, the number of i such that $p_i = p'_j = u$ is infinite. If $j > 0$ then, by induction hypothesis, the number of i such that $p_i = p'_{j-1}$ is infinite. Since p is fair, and since $(p'_{j-1}, p'_j) \in E$, we deduce that the number of i such that $p_i = p'_{j-1}$ and $p_i = p'_j$ is infinite. It is then obvious that the number of i such that $p_i = p'_j$ is infinite. □

Proposition 4 *A path p is fair if and only if there exists an attractor A such that, for all $(u, v) \in E$ with $u \in A$, the number of i such that $p_i = u$ and $p_{i+1} = v$ is infinite.*

Proof. ($\Leftarrow$) Let p be a path and suppose that there exists A verifying the condition of the proposition. Then there exists j such that $p_j \in A$, and since A is a trap domain, we have $p_k \in A$ for all $k \geq j$. Now, let $(u, v) \in E$ and suppose that the number of i such that $p_i = u$ is infinite. Then, there exists $k \geq j$ such that $p_k = u$ and we deduce that $u \in A$. By hypothesis, the number of i such that $p_i = u$ and $p_{i+1} = v$ is infinite : p is a fair path.

($\Rightarrow$) Let p be a fair path. Since $p(\mathbb{N})$ is finite, there exists at least one vertex w for which the number of i such that $p_i = w$ is infinite. Then, following Proposition 2, there exists an attractor A and a vertex $x \in A$ such that there is a path from w to x, and following Proposition 3, the number of i such that $p_i = x$ is infinite. Now, let (u, v) be any edge of E with $u \in A$. Following Proposition 1, there exists a path from x to u, and we deduce that the number of i such that $p_i = u$ is infinite. Since p is fair, we deduce that the number of i such that $p_i = u$ and $p_{i+1} = v$ is infinite. □

4 Handling fair paths in CTL

Let $\mathcal{A}$ be a set of *atomic* propositions, and let L be a map from V to the set of subsets of $\mathcal{A}$. The triple (V, E, L) is usually called a *Kripke structure*. The Computational Tree Logic (CTL) allows the expression of a great number of properties on such a structure.

The set of CTL *formulas* over $\mathcal{A}$ is inductively defined by : the atomic propositions of $\mathcal{A}$ are CTL formulas ; if ϕ and ψ are CTL formulas, then $\neg\phi$, $\phi \vee \psi$, $\phi \wedge \psi$, and

$$\text{EX}(\phi),\ \text{EF}(\phi),\ \text{EG}(\phi),\ \text{E}(\phi\,\text{U}\,\psi),\ \text{AX}(\phi),\ \text{AF}(\phi),\ \text{AG}(\phi),\ \text{A}(\phi\,\text{U}\,\psi),$$

are CTL formulas. The Kripke structure (V, E, L) satisfies a formula if all the vertices of V satisfy this formula ; and a vertex v satisfies this formula according to L and the set of paths starting from v, that we denote $P(v)$. More precisely, the satisfactory relation $\models$ between vertices and formulas is

inductively defined by : for all $a \in \mathcal{A}$ and for all CTL formula ϕ and ψ,

$$\begin{array}{rcl}
v \models a & \iff & a \in L(v). \\
v \models \neg\phi & \iff & v \not\models \phi. \\
v \models \phi \wedge \psi & \iff & v \models \phi \ \wedge \ v \models \psi. \\
v \models \phi \vee \psi & \iff & v \models \phi \ \vee \ v \models \psi. \\
v \models \mathrm{EX}(\phi) & \iff & \exists p \in P(v),\ p_1 \models \phi. \\
v \models \mathrm{AX}(\phi) & \iff & \forall p \in P(v),\ p_1 \models \phi. \\
v \models \mathrm{EF}(\phi) & \iff & \exists p \in P(v), \exists i \in \mathbb{N},\ p_i \models \phi. \\
v \models \mathrm{AF}(\phi) & \iff & \forall p \in P(v), \exists i \in \mathbb{N},\ p_i \models \phi. \\
v \models \mathrm{EG}(\phi) & \iff & \exists p \in P(v), \forall i \in \mathbb{N},\ p_i \models \phi. \\
v \models \mathrm{AG}(\phi) & \iff & \forall p \in P(v), \forall i \in \mathbb{N},\ p_i \models \phi. \\
v \models \mathrm{E}(\phi \,\mathrm{U}\, \psi) & \iff & \exists p \in P(v), \exists j \in \mathbb{N},\ p_j \models \psi,\ \forall i < j,\ p_i \models \phi. \\
v \models \mathrm{A}(\phi \,\mathrm{U}\, \psi) & \iff & \forall p \in P(v), \exists j \in \mathbb{N},\ p_j \models \psi,\ \forall i < j,\ p_i \models \phi.
\end{array}$$

When (V, E) is seen as an undeterministic discrete dynamical system as in Remark 1, the set possible evolutions of the system from an initial vertex v is given by the set of fair paths starting from v, that we denote by $P'(v)$, and not by $P(v)$. The CTL is then not satisfactory since a formula can be false at v because of the paths present $P(v) \setminus P'(v)$ which do not correspond to possible evolutions of the system. Typically, we can have $v \not\models \mathrm{AF}(\phi)$ whereas all the possible evolutions of the system from v lead to a state verifying ϕ.

This lead us to consider a variant of CTL, denoted CTL$'$, allowing the expression of formulas interpreted according to the fair paths. More precisely, the syntax CTL$'$ is obtained from the one of CTL by adding the following six temporal operators

$$\mathrm{E}'\mathrm{F}(\phi),\ \mathrm{E}'\mathrm{G}(\phi),\ \mathrm{E}'(\phi \,\mathrm{U}\, \psi),\ \mathrm{A}'\mathrm{F}(\phi),\ \mathrm{A}'\mathrm{G}(\phi),\ \mathrm{A}'(\phi \,\mathrm{U}\, \psi).$$

The semantic of CTL$'$ is then obtained by adding the following equivalences to the ones given above :

$$\begin{array}{rcl}
v \models \mathrm{E}'\mathrm{F}(\phi) & \iff & \exists p \in P'(v), \exists i \in \mathbb{N},\ p_i \models \phi. \\
v \models \mathrm{A}'\mathrm{F}(\phi) & \iff & \forall p \in P'(v), \exists i \in \mathbb{N},\ p_i \models \phi. \\
v \models \mathrm{E}'\mathrm{G}(\phi) & \iff & \exists p \in P'(v), \forall i \in \mathbb{N},\ p_i \models \phi. \\
v \models \mathrm{A}'\mathrm{G}(\phi) & \iff & \forall p \in P'(v), \forall i \in \mathbb{N},\ p_i \models \phi. \\
v \models \mathrm{E}'(\phi \,\mathrm{U}\, \psi) & \iff & \exists p \in P'(v), \exists j \in \mathbb{N},\ p_j \models \psi,\ \forall i < j,\ p_i \models \phi. \\
v \models \mathrm{A}'(\phi \,\mathrm{U}\, \psi) & \iff & \forall p \in P'(v), \exists j \in \mathbb{N},\ p_j \models \psi,\ \forall i < j,\ p_i \models \phi.
\end{array}$$

Proposition 5 *We have the following equivalences :*

$$v \models \mathrm{E}'\mathrm{F}(\phi) \iff v \models \mathrm{EF}(\phi) \quad (1)$$
$$v \models \mathrm{A}'\mathrm{F}(\phi) \iff v \models \neg\mathrm{E}'\mathrm{G}(\neg\phi) \quad (2)$$
$$v \models \mathrm{E}'\mathrm{G}(\phi) \iff v \models \mathrm{E}(\phi \,\mathrm{U}\, \mathrm{AG}(\phi)) \quad (3)$$
$$v \models \mathrm{A}'\mathrm{G}(\phi) \iff v \models \neg\mathrm{E}'\mathrm{F}(\neg\phi) \quad (4)$$
$$v \models \mathrm{E}'(\phi \,\mathrm{U}\, \psi) \iff v \models \mathrm{E}(\phi \,\mathrm{U}\, \psi) \quad (5)$$
$$v \models \mathrm{A}'(\phi \,\mathrm{U}\, \psi) \iff v \models \mathrm{A}'\mathrm{F}(\psi) \wedge \neg\mathrm{E}'(\neg\psi \,\mathrm{U}\, \neg\phi \wedge \neg\psi) \quad (6)$$

Proof. The non-obvious equivalences are the equivalences (3) and (6).

To prove (3), suppose first that $v \models \mathrm{E}'\mathrm{G}(\phi)$, and let $p \in P'(v)$ be such that $p_i \models \phi$ for all i. Following Proposition 4, there exists an attractor A such that $A \subseteq p(\mathbb{N})$, and we deduce that $u \models \phi$ for all $u \in A$. Let j be such that $p_j \in A$. Since A is an attractor, for all $p' \in P(p_j)$, we have $p'(\mathbb{N}) \subseteq A$. So $p_j \models \mathrm{AG}(\phi)$, and since $p_i \models \phi$ for all i, we deduce that $v \models \mathrm{E}(\phi \,\mathrm{U}\, \mathrm{AG}(\phi))$.

Now, suppose that $v \models \mathrm{E}(\phi \,\mathrm{U}\, \mathrm{AG}(\phi))$: there exists $p \in P(v)$ and $j \in N$ such that $p_j \models \mathrm{AG}(\phi)$ and $p_i \models \phi$ for all $i < j$. So, given any fair path $p' \in P'(p_j)$ we have $p'_i \models \phi$ for all $i \in \mathbb{N}$. Consider the path p'' that we obtain by concatenating the prefix of p of length $(j-1)$ and p' : for all $i \in \mathbb{N}$, $p''_i = p_i$ if $i < j$, $p''_i = p'_{i-j}$ otherwise. Clearly, $p''_i \models \phi$ for all i, and $p''_0 = v$. Furthermore, since p' is a fair path, p'' is a fair path, and we deduce that $v \models \mathrm{E}'\mathrm{G}(\phi)$.

To prove (6) suppose first that $v \models \mathrm{A}'(\phi \,\mathrm{U}\, \psi)$. This obviously implies $v \models \mathrm{A}'\mathrm{F}(\psi)$. Now, suppose, by contradiction, that $v \models \mathrm{E}'(\neg\psi \,\mathrm{U}\, \neg\phi \wedge \neg\psi)$. Then, there exists $p \in P'(v)$ and j such that $p_j \models \neg\phi$ and $p_i \models \neg\psi$ for all $i \leq j$. So, if $p_k \models \psi$ then $k > j$, and since $p_j \models \neg\phi$, we deduce that $v \models \neg\mathrm{A}'(\phi \,\mathrm{U}\, \psi)$, a contradiction.

Now, suppose that $v \models \mathrm{A}'\mathrm{F}(\psi) \wedge \neg\mathrm{E}'(\neg\psi \,\mathrm{U}\, \neg\phi \wedge \neg\psi)$. Let $p \in P'(v)$. Since $v \models \mathrm{A}'\mathrm{F}(\psi)$ there exists a smallest j such that $p_j \models \psi$. Now, suppose, by contradiction, that there exists $i < j$ with $p_i \models \neg\phi$. By the choice of j, we have $p_k \models \neg\psi$ for all $k \leq i$, and we deduce that $v \models \mathrm{E}(\neg\psi \,\mathrm{U}\, \neg\phi \wedge \neg\psi)$, a contradiction. □

According to this proposition, any formula of ϕ of CTL′ can be translated into a formula ψ in CTL which is equivalent to ϕ, that is, such that $v \models \psi \iff v \models \psi$. Furthermore, the proposition gives a constructive way to achieve this translation. This means that CTL′ formulas can be handled in practice by the powerful verification tools related to CTL.

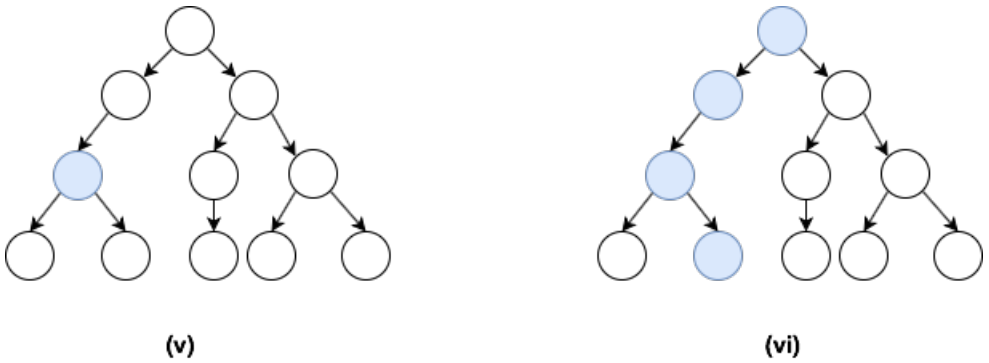

Figure 11.4: Temporal Semantics. (i) EF(p) : there exists a state in the future where p holds (ii) EG(p) : if p holds in a state, then in general p holds in all successive states.

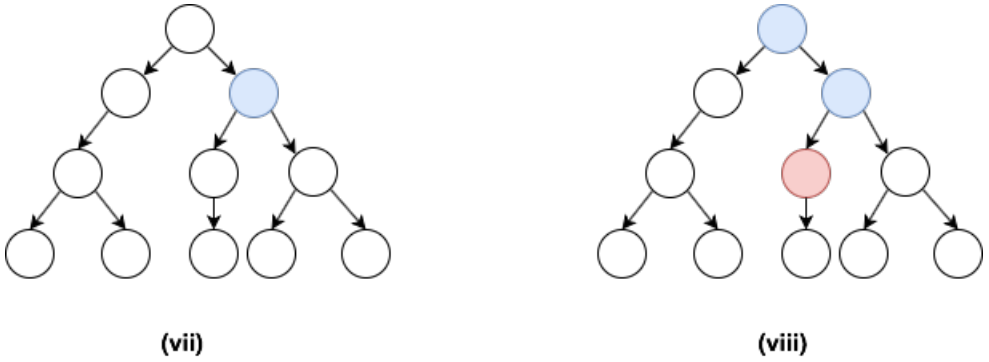

Figure 11.5: Temporal Semantics. (i) EF(p) : for a given state, there exists a next state where p holds (ii) E[p u q] : there exists a trajectory where p holds until a state where q holds

11.7 SMBioNet

11.7.1 SMBioNet file

This section shows the complete SMBioNet file of the energy metabolism network with all the regulation details.

VAR

```
glc=0 2;
glyc= 0 2;
ferm= 0 1 ;
krebs = 0 2 ;
phox = 0 1;
gln = 0 3;
prod_biom = 0 1;
oxyg = 0 1;
nadh = 0 1;
atp = 0 2;
cons = 0 1;
in_gln = 0 1;
in_oxyg = 0 1;
biom = 0 1;
```

REG

```
nad [!(atp >= 2) & !(nadh >= 1)] => glyc;
pyr [(glyc >= 1) & (oxyg >= 1)] => krebs;
vpp [(glyc >= 1) & (atp >= 1) & (gln >=1)] => prod_biom;
lipids [(gln >= 3) & (atp>=1)] => prod_biom;
```

```
gpdh [(glyc >=1)] => nadh;
#cit [!((krebs >= 2) & !(prod_biom >=1))] => glyc;
#cit [((krebs >= 2) & !(prod_biom >=1))] => prod_biom;
cit1 [!(krebs >= 2)] => glyc;
cit2 [(krebs >= 2)] => prod_biom;
nut [!(prod_biom >= 1)] => gln;
ex_py [(((glyc >= 1) & !(oxyg >=1)) | (glyc >= 2)) & (nadh >=1)] => ferm;
pc [(nadh >=1) & (oxyg >=1) & !(atp >=2)] => phox;
b_ox1 [(biom >= 1) & !(glyc >= 1) & !(atp >= 1)] => krebs;
sat [(((glyc >=1) & (gln >= 2)) | (glyc >= 2)) & (oxyg >= 1)] => krebs;
glyc2 [(glyc >=2)] => atp;
glyc1 [(glyc >=1)] => atp;
consa [!(cons >=1)] => atp;
oxygi [(in_oxyg >=1)] => oxyg;
phoxo [!(phox >=1)] => oxyg;
glu1 [(glc >= 1)] => glyc;
glu2 [(glc >= 2)] => glyc;
prodbb [(prod_biom >= 1)] => biom;
glni [(in_gln >=1)] => gln;
krebsg [!(krebs >=2)] => gln;
krebsn [(krebs >=1)] => nadh;
phoxn [!(phox >=1)] => nadh;
fermn [!(ferm >=1)] => nadh;
phoxa [(phox >=1)] => atp;
prob_bioma [!(prod_biom >=1)] => atp;
b_ox2 [!((biom >= 1) & !(glyc >= 1) & !(atp >= 1))] => biom;
```

PARA

```
# Parameters for glc

K_glc = 2 ;


# Parameters for glyc

K_glyc = 0 ;
K_glyc+cit1 = 0 ;
K_glyc+glu1 = 0 ;
K_glyc+nad = 0 ;
K_glyc+cit1+glu1 = 0;
K_glyc+glu1+glu2 = 0 ;
K_glyc+glu1+nad = 1 ;
K_glyc+cit1+nad = 0 ;
K_glyc+glu1+glu2+nad = 1 ;
K_glyc+cit1+glu1+nad = 1 ;
K_glyc+cit1+glu1+glu2 = 0 ;
K_glyc+cit1+glu1+glu2+nad = 2 ;

# Parameters for ferm

K_ferm = 0 ;
K_ferm+ex_py = 1 ;

# Parameters for krebs

K_krebs = 0 ;
K_krebs+b_ox1 = 1 ;
K_krebs+pyr = 1 ;
K_krebs+pyr+sat = 2 ;
```

```
# Parameters for phox

K_phox = 0 ;
K_phox+pc = 1 ;

# Parameters for gln

K_gln = 0 ;
K_gln+glni = 3 ;
K_gln+krebsg = 0 ;
K_gln+nut = 0 ;
K_gln+glni+krebsg = 3 ;
K_gln+krebsg+nut = 0 ;
K_gln+glni+nut = 3 ;
K_gln+glni+krebsg+nut = 3 ;

# Parameters for prod_biom

K_prod_biom = 0 ;
K_prod_biom+cit2 = 1 ;
K_prod_biom+lipids = 1 ;
K_prod_biom+vpp = 1 ;
K_prod_biom+cit2+lipids = 1 ;
K_prod_biom+lipids+vpp = 1 ;
K_prod_biom+cit2+vpp = 1 ;
K_prod_biom+cit2+lipids+vpp = 1 ;

# Parameters for oxyg

K_oxyg = 0 ;
K_oxyg+oxygi = 1 ;
K_oxyg+phoxo = 0 ;
K_oxyg+oxygi+phoxo = 1 ;

# Parameters for nadh

K_nadh = 0 ;
K_nadh+fermn = 0 ;
K_nadh+gpdh = 0 ;
K_nadh+krebsn = 0 ;
K_nadh+phoxn = 0 ;
K_nadh+fermn+gpdh = 0 ;
K_nadh+gpdh+krebsn = 0 ;
K_nadh+gpdh+phoxn = 0 ;
K_nadh+fermn+krebsn = 0 ;
K_nadh+krebsn+phoxn = 1 ;
K_nadh+fermn+phoxn = 0 ;
K_nadh+fermn+gpdh+phoxn = 1 ;
K_nadh+fermn+gpdh+krebsn = 1 ;
K_nadh+fermn+krebsn+phoxn = 1 ;
K_nadh+gpdh+krebsn+phoxn = 1 ;
K_nadh+fermn+gpdh+krebsn+phoxn = 1 ;

# Parameters for atp  [why are there 24 params instead of the whole 32 ??]

K_atp = 0 ;
K_atp+consa = 0 ;
K_atp+glyc1 = 0 ;
K_atp+phoxa = 0 ;
```

```
K_atp+prob_bioma = 0 ;
K_atp+consa+glyc1 = 0 ;
K_atp+glyc1+glyc2 = 0 ;
K_atp+glyc1+phoxa = 0 ;
K_atp+glyc1+prob_bioma = 0 ;
K_atp+consa+phoxa = 1 ;
K_atp+phoxa+prob_bioma = 0 ;
K_atp+consa+prob_bioma = 0 ;
K_atp+glyc1+glyc2+prob_bioma = 1 ;
K_atp+consa+phoxa+prob_bioma = 2 ;
K_atp+glyc1+phoxa+prob_bioma = 0 ;
K_atp+consa+glyc1+phoxa = 1 ;
K_atp+consa+glyc1+prob_bioma = 2 ;
K_atp+consa+glyc1+glyc2 = 1 ;
K_atp+glyc1+glyc2+phoxa = 0 ;
K_atp+glyc1+glyc2+phoxa+prob_bioma = 1 ;
K_atp+consa+glyc1+glyc2+phoxa = 1 ;
K_atp+consa+glyc1+phoxa+prob_bioma = 2 ;
K_atp+consa+glyc1+glyc2+prob_bioma = 2 ;
K_atp+consa+glyc1+glyc2+phoxa+prob_bioma = 2 ;

# Parameters for cons

K_cons = 0 ;

# Parameters for in_gln

K_in_gln = 0;

# Parameters for in_oxyg

K_in_oxyg = 1 ;

# Parameters for biom

K_biom = 0 ;
K_biom+prodbb = 1 ;
K_biom+b_ox2 = 0 ;
K_biom+b_ox2+prodbb= 1 ;
```

11.8 Important Java classes

There are three important classes that are central to this modeling software namely : nodes (respectively multiplexes and edges) which are represented by its equivalent Java class , Node.java (respectively Multiplex.java and Edge.java) and are listed in the following subsections. All the elements used to create the interaction graph are all uniquely identified by a name.

11.8.1 Node.java

A node in any graph is represented by a couple (x,y) coordinates and its threshold level to show its evolution (represented ultimately by a color) in the network. These composed the essential attributes of the node.

```
package fr.unice;

/**
 * A class for modelling a given node in the regulatory graph
 *
 * @author rajeev
 *
```

```
 */
public class Node {

        public Node() {
        }

        /**
         * Constructor for creating a node with the following parameters
         *
         * @param name
         * @param xcoor
         * @param ycoor
         * @param threshold
         */
        public Node(String name, int xcoor, int ycoor, int threshold, int defaultLevel)
                super();
                this.name = name;
                this.xcoor = xcoor;
                this.ycoor = ycoor;
                this.threshold = threshold;
                this.defaultLevel = defaultLevel;
        }

        /**
         * @return the name
         */
        public String getName() {
                return name;
        }

        /**
         * @return the xcoor
         */
        public int getXcoor() {
                return xcoor;
        }

        /**
         * @return the ycoor
         */
        public int getYcoor() {
                return ycoor;
        }

        /**
         * @return the threshold
         */
        public int getThreshold() {
                return threshold;
        }

        /**
         * @param name
         * the name to set
         */
        public void setName(String name) {
                this.name = name;
        }

        /**
```

```
         * @param xcoor
         * the xcoor to set
         */
        public void setXcoor(int xcoor) {
                this.xcoor = xcoor;
        }

        /**
         * @param ycoor
         * the ycoor to set
         */
        public void setYcoor(int ycoor) {
                this.ycoor = ycoor;
        }

        /**
         * @param threshold
         * the threshold to set
         */
        public void setThreshold(int threshold) {
                this.threshold = threshold;
        }

        /**
         * set the default level of a given node
         */
        public int getDefaultLevel() {
                return defaultLevel;
        }

        /**
         *
         * @param defaultLevel
         */
        public void setDefaultLevel(int defaultLevel) {
                this.defaultLevel = defaultLevel;
        }

        private String name;
        private int xcoor;
        private int ycoor;
        private int threshold;
        private int defaultLevel;
}
```

11.8.2 Edge.java

There are two types of edges in our system: one linking a node (source node, denoted by a dotted line) to a multiplex and another one linking the multiplex to another node (target node denoted by a solid line).

```
package fr.unice;

/**
 * A class for modelling an edge in the regulatory graph
 *
 * @author rajeev
 *
 */
public class Edge {

        /**
```

```
 * Constructing an edge with the following parameters
 *
 * @param name
 * @param fromNode
 * @param toNode
 */
public Edge(String name, String fromNode, String toNode) {
        super();
        this.name = name;
        this.fromNode = fromNode;
        this.toNode = toNode;
}

/**
 * @return the name
 */
public String getName() {
        return name;
}

/**
 * @return the fromNode
 */
public String getFromNode() {
        return fromNode;
}

/**
 * @return the toNode
 */
public String getToNode() {
        return toNode;
}

/**
 * @param name
 * the name to set
 */
public void setName(String name) {
        this.name = name;
}

/**
 * @param fromNode
 * the fromNode to set
 */
public void setFromNode(String fromNode) {
        this.fromNode = fromNode;
}

/**
 * @param toNode
 * the toNode to set
 */
public void setToNode(String toNode) {
        this.toNode = toNode;
}

private String name;
private String fromNode;
```

```
        private String toNode;
}
```

11.8.3 Multiplex.java

A multiplex is similar to a normal node (name, x and y coordinates) with an additional attribute, formula, which defines the logical contribution of one or more nodes on another node.

```
package fr.unice;

/**
 * A class for modelling a multiplex
 *
 * @author rajeev
 *
 */
public class Multiplex {

        /**
         * Constructor
         *
         * @param name
         * @param xcoor
         * @param ycoor
         * @param formula
         */
        public Multiplex(String name, int xcoor, int ycoor, String formula) {
                super();
                this.name = name;
                this.xcoor = xcoor;
                this.ycoor = ycoor;
                this.formula = formula;
        }

        /**
         * @return the name
         */
        public String getName() {
                return name;
        }

        /**
         * @return the xcoor
         */
        public int getXcoor() {
                return xcoor;
        }

        /**
         * @return the ycoor
         */
        public int getYcoor() {
                return ycoor;
        }

        /**
         * @return the formula
         */
        public String getFormula() {
                return formula;
```

```
        }

        /**
         * @param formula
         * the formula to set
         */
        public void setFormula(String formula) {
                this.formula = formula;
        }

        /**
         * @param name
         * the name to set
         */
        public void setName(String name) {
                this.name = name;
        }

        /**
         * @param xcoor
         * the xcoor to set
         */
        public void setXcoor(int xcoor) {
                this.xcoor = xcoor;
        }

        /**
         * @param ycoor
         * the ycoor to set
         */
        public void setYcoor(int ycoor) {
                this.ycoor = ycoor;
        }

        private String name;
        private int xcoor;
        private int ycoor;
        private String formula;

}
```

11.8.4 Config.xml

The main properties of the model are configured in this file. We needed a way to store previously configured parameters by the user for future use. These include : color of nodes/edges/multiplexes, speed of the simulation, last opened model, color equivalent of thresholds.

```
<?xml version="1.0" encoding="UTF-8" standalone="no"?>
<config>
<speed>1</speed>
<currentFile>genes_xyz.xml</currentFile>
<network_thres bgcolor="0,0,0">
<node_thres_0>153,0,153</node_thres_0>
<node_thres_1>0,255,204</node_thres_1>
<node_thres_2>255,0,204</node_thres_2>
<node_thres_3>255,102,0</node_thres_3>
</network_thres>
<charts bgcolor="255,255,255" title="Simulation for genes X=0, Y=1 and Z=1"
xaxis="States" yaxis="Threshold"/>
<network/>
<multiplex>204,255,51</multiplex>
```

```
<dottedEdge>204,0,255</dottedEdge>
<normalEdge>255,102,102</normalEdge>
</config>
```

11.8.5 Config.java

The Config.java file acts as an interface between the Config.xml file and the metabolic model.

11.8.6 Network.java

The core methods of DyMBioNet are centralized in this class. All interactions (CRUD actions) between the user and the software are implemented in this java class.

```
List of methods

void addCTL(java.lang.String ctl)
This method will add a new CTL or update the existing CTL for a given model

int addEdge(int mode, java.lang.String edgeName, java.lang.String fromNode,
java.lang.String toNode)
THis method enables the user to add an edge in the graph

void addModelFromSMBioNet(java.lang.String fileName, java.util.ArrayList
<Node> nodeArr)
THis method will be used when user wants to run model(s) generated from
SMBioNet (validating a certain CTL formula)

boolean addMultiplex(java.lang.String multName, java.lang.String formula,
java.lang.String xcoor, java.lang.String ycoor)
This method allows the user to add a multiplex with the following parameters:

boolean addNode(java.lang.String nodeName, java.lang.String xCoor, java.lang.String
 yCoor, java.lang.String threshold)
This method is used to add a node to your network file with the following parameters:

void addParameter(java.lang.String nodeId, java.lang.String rowCol, int defaultValue,
 boolean isEnd)
This method is used to add K parameter from Kinetic window to xml file

boolean convertToSmBioNet()
This method is used to generate SmBioNet from DymBioNet xml file

void generateModelFromSmBioNet(java.lang.String filename, java.io.File f)
Generate model from SmBionet file

void generateNetwork(java.lang.String fileName, java.lang.String fileDesc)
This method is used to generate xml file from classic SymBionet file

java.lang.String getCTL()
This method will return CTL formula for this model

int[] getDefaultParamsOfNodes(Node[] nodes)
This method retrieves the default parameters for each node.... simulation

Edge[] getEdges()
This method is used to get list of edges from a given xml file / network

Multiplex[] getMultiplexes()
This method will output the list of multiplexes from the graph
```

```
Multiplex getMultiplexInfoByName(java.lang.String multName)
Get all information on a multiplex by its name

Node getNodeInfoByName(java.lang.String nodeName)
Get all information on a node by its name in the graph

Multiplex[] getPosMults(boolean positive)
This will return the list of activations

int getStartMultiplex()
This method is called from toolbar when creating a multiplex

void readFile(java.lang.String fileName)
THis method is used to initialise a model for reading

void removeEdge(java.lang.String edgeName)
This method is used to remove an edge

void saveFile(java.lang.String fileName)
This method is called each time a network is created, a node, edge or
multiplex is added

void updateFromSMBioNet(int modelNo, java.util.ArrayList<java.lang.String>
 arrNodes, java.util.Map<java.lang.String,java.lang.Integer> valNodes)
Update xml file based on the model chosen by the user from 'Launch SMBioNet'
 window

void updateKParameters(java.lang.String nodeName, int row, int col,
java.lang.String newValue)
This method will be called when user modifies K Parameter in Kinetic table

void updateMultiplexFormula(boolean checked)
This method will change whether multiplex formula mustbe shown or not

boolean updateNodeParams(org.graphstream.graph.Graph g, java.lang.String
nodeType, java.lang.String nodeName, java.lang.String[] newValues)
This method is used to update parameters for a node or multiplex in the xml file

void updateNodePos(int nodeType, java.lang.String nodeName, int x, int y)
This method will be used to update current position of a given node/multiplex
```

11.8.7 Metabolism.xml

The final version of the metabolic model in the XML format.

```
<?xml version="1.0" encoding="UTF-8" standalone="no"?>
<network>
<description>Metabolic network</description>
<nodes>
<node default="0" id="ATP_ADP" kparam="1" thres="2" weight="1" xcoor="-50" ycoor="100">
ATP_ADP<K id="KATP_ADP00000" value="0"/>
<K id="KATP_ADP00001" value="0"/>
<K id="KATP_ADP00010" value="0"/>
<K id="KATP_ADP00011" value="0"/>
<K id="KATP_ADP00100" value="0"/>
<K id="KATP_ADP00101" value="0"/>
<K id="KATP_ADP00110" value="0"/>
<K id="KATP_ADP00111" value="0"/>
<K id="KATP_ADP01000" value="0"/>
<K id="KATP_ADP01001" value="0"/>
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Modélisation discrète de la régulation du métabolisme énergétique des cellules eukaryotes et validation formelle de sa dynamique

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Résumé

Nous présentons une modélisation formelle de la régulation du métabolisme énergétique de la cellule eucaryote. Le choix original de cette modélisation est de considérer explicitement des abstractions des principaux processus cellulaires qui pilotent ce métabolisme, réduisant ainsi considérablement le nombre de variables à prendre en compte dans le modèle. De plus, le formalisme de modélisation introduit par Réné Thomas est particulièrement adapté à une vision qualitative des phénomènes de régulation, de sorte que le modèle repose sur seulement 14 variables et 112 paramètres entiers. En revanche, le modèle possède de nombreux cycles de retroaction fortement intriqués, qui rendent la dynamique du système très complexe. Comme dans toute modèlisation de système complexe, la difficulté majeure est l'identification des valeurs des paramètres de manière cohérente avec les comportements dynamiques connus.

L'identification des paramètres a été effectuée sur la base d'une abondante connaissance biologique moléculaire, et la validation du modèle a été effectuée par model checking sur plus de 160 formules temporelles (incluant les principaux phénotypes connus, en particulier l'effet Warburg). Il s'agit d'un travail minutieux qui n'a pu être mené à son terme qu'en mettant en place une méthode pluridisciplinaire de modélisation et une plateforme logicielle (DyMBioNet), qui constituent également une contribution importante de la thèse. Le modèle achévé a été conçu comme un "noyau formel" réutilisable en connexion avec d'autres réseaux de régulation comme le cycle cellulaire et l'horloge circadienne, par exemple en vue d'application au cancer ou à la chronothéraphie. L'outil DyMBioNet présente plusieurs fonctionnalités incluant la possibilité de faire des preuves en CTL, la simulation ainsi que la visualisation d'un système complexe. Enfin, la méthodologie définie ici et son outillage DyMBioNet pourront être réutilisés directement pour construire d'autres modèles formels de régulation de grande taille.

Mots-clés: Modélisation, Réseaux biologiques, Logique formelles, Métabolisme

Discrete modelling of the energy metabolism regulation of eukaryotic cells and formal validation of its dynamics

Abstract

We present a formal model of the regulation of the energetic metabolism in eukaryotic cells. The main originality of this model is to consider explicitly an abstraction of the main metabolic processes that pilot this metabolism, thereby greatly reducing the number of variables in the model. Moreover, the modelling framework proposed by Réné Thomas is particularly well suited for a qualitative view of regulatory networks resulting in a model with 14 variables and 112 parameters, with integer values. However, the model contains a lot of feedback loops which are intricately linked and which makes the dynamic of the system very complex. As in all complex system modelling, the main difficulty is to identify the value of all parameters in a coherent way with respect to known dynamic behaviours.

The identification of parameters has been smoothed due to a large repertoire of knowledge in molecular biology, and the validation of the proposed model has been done by model checking, in more than 160 temporal logic formulas (including the main metabolic phenotypes, notably the Warburg effect). It has been a meticulate process which has been successful by putting in place a solid and pluridisciplinary method of modelling together with a software platform (DyMBioNet), both pivotal for this thesis. The model has been conceived to be used as a backbone, which can be plugged with other regulatory networks like the cell cycle or the circadian clock, for potential applications to cancer or chronotherapy. The DyMBioNet software is bundled with three main functionalities including verifying system properties with CTL, simulation as well as visualisation of a complex system. Furthermore, this well-defined methodology, and its software platform DyMBioNet, would be useful to directly construct other formal regulatory networks of large size.

Keywords: Modelling, Biological networks, Formal logics, Metabolism