THE BIOCOMPLEXITY PROJECT

A Senior Honors Thesis

by

CHRISTOPHER MEYER SEWELL

Submitted to the Office of Honors Programs
& Academic Scholarships
Texas A&M University
in partial fulfillment of the requirements of the

UNIVERSITY UNDERGRADUATE
RESEARCH FELLOWS

April 2001

Group:
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Paul Lindahl
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ABSTRACT

The Biocomplexity Project. (April 2001)

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The ultimate goal of the Texas A&M Biocomplexity Project is to numerically and visually simulate a complete chemical mechanism for a simplified cell. It will incorporate the rapidly growing knowledge about cellular components, and will highlight some of the emergent properties arising from the interactions among these components, providing a greater understanding of certain cellular processes. This thesis describes the completed work with which I have been involved in this project, including several related subproblems. A complete set of chemical reactions was written to model selected metabolic and cell-cycle processes in the simplest prokaryotes. A computer program was written to read, analyze, and stochastically simulate such mechanisms, and a 3D computer animation was produced to visualize the cell-cycle reactions. In order to better understand the properties of regulation in a cell, six mechanisms were written and analyzed mathematically. The models examined the effects of negative feedback, cooperativity, oligomerization, transcription, and transcription feedback on the sensitivity of the binding of a protein to its gene and on the range of synthesis and degradation "perturbments" the system could tolerate. A data-fitting program was developed using a searching algorithm known as simulated annealing in order to find values of rate constants that best fit data for the desired realistic behavior of the mechanical cell. This algorithm was used to fit experimental data involving the enzyme acetyl CoA synthase (which is present in simple chemoautotrophic prokaryotes) to mechanistic models. Therefore, the groundwork has been laid for the project. An initial mechanism has been written, a program is available to simulate it, regulatory mechanisms are better understood, and a method to fit concentration data has been established. The next step is to divide the system into modules, incorporating the best regulatory mechanisms, and to fit each model to realistic data and link the solved, regulated modules into a complete system.

ACKNOWLEDGMENTS

I would like to thank all of those who have assisted me with this project, including Dr. Paul Lindahl, Dr. Jeff Morgan, Dr. Thomas Ioerger, Dr. Xiangshi Tan, Ernie Maynard, Charles Johnson, and Erik McKee.

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CHAPTER I

INTRODUCTION

Over the last decade, the structure and function of dozens of complex biochemical molecules in the cell have been determined. Some research, such as the Human Genome Project, has made headlines, but significant progress has been made in many aspects of the fields of genomics, proteomics (the study of the function of proteins), transcriptomics (the study of RNA), and metabolomics (the study of cellular nutrients). The Texas A&M Biocomplexity Group was formed in 1998 by Professor Paul Lindahl of the Texas A&M Department of Chemistry, with the goal of using this information to develop a complete chemical mechanism for a simplified but functional simulated cell. The process involves writing a sequence of chemical reactions to model the metabolic, regulatory, and cell-cycle reactions in the cell, finding kinematic rate constants for these equations which produce an autocatalytic (self-reproducing) system with components whose concentrations vary with time in a manner consistent with those of a real cell, and producing animations based on data from the numerical simulations.

SUMMARY OF THE THESIS

Due to the ongoing nature of this long-term project, many results are not yet available for this thesis. However, I have completed work on several sub-problems within the context of the biocomplexity project, and these form the basis for this thesis. Chapter II describes the development of Mechanical Cell Three, the group's first comprehensive cellular chemical mechanism. In addition to a full set of step-by-step reactions, this phase of the project included the coding of a mechanical cell simulating computer program and the creation of a three-dimensional computer animation. Dr. Lindahl was responsible for writing most of the mechanism, with some help from graduate student Erik McKee and myself. Mr. McKee and I wrote the computer programs. Dr. Lindahl, Mr. McKee, undergraduate student Charles Johnson, and I created the animation. A vital component of a cellular mechanism is good regulation. A thorough mathematical analysis was made of several characteristic regulatory mechanisms, and these results are presented in Chapter III. Dr. Lindahl, Professor Jeff Morgan of the Texas A&M Department of Mathematics, and I performed this analysis. A paper based on this study, "Quantitative Analysis of Protein Homeostatic Mechanisms Used in Living Systems," may soon be submitted to *The Journal of Theoretical Biology*. In order to find best-fit rate constants for the desired "choreography" of our mechanical cells, a data-fitting

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This thesis follows the style and format of *The Journal of the American Chemical Society*.

program was developed. However, this program was found to also be very useful for fitting data from two experiments performed by other members of Dr. Lindahl's group. An overview of these experiments, which are related to the biocomplexity project in that they involve the study of an enzyme important in the simple methanogens on which our mechanical cells are based, and an analysis of the fitting procedure are presented in Chapter IV. The experiments were performed by graduate student Ernie Maynard and post-doctorate researcher Dr. Xiangshi Tan. I wrote the program to fit the data, using the Adaptive Simulated Annealing algorithm of Les Ingber. Mr. Maynard's experiment is reported in a paper submitted to the *Journal of the American Chemical Society*, "Kinetic Mechamism of Acetyl-CoA Synthase: Steady-State Synthesis at Variable CO/CO2 Pressures," and Dr. Tan's paper, "Kinetics of the Methyl Group Transfer between Acetyl-Coenzyme A Synthase and the Corrinoid-Iron-Sulfur Protein from Clostridium thermoaceticum," may also soon be submitted to the same journal.

REVIEW OF LITERATURE

A review of the literature is particularly important in several areas of this project. A basic knowledge about others' attempts to create a mechanical cell is necessary. The analytical study of regulatory mechanisms is vital for obtaining a working cell, and is a major part of this thesis, and therefore a review of known cellular regulatory mechanisms and of previous studies of such systems is valuable. Finally, a brief review of the development, abilities, and uses of the simulated annealing searching algorithm is important to use it efficiently in fitting data.

Mechanical Cells. A few other research groups have begun the development of "virtual" cells. Tomita and coworkers at Keio University in Japan have written a computer program called "E-cell" which simulates enzyme activity and genetic processes in simple living systems (Normile 1999). E-cell allows users to control the genes and enzymes in the cell, and numerically simulates the system using differential equations. The consequences of nutrient deficiencies or gene deletions may be explored. E-cell is modeled on *Micoplasma genitalium*. However, a number of important aspects of cellular mechanisms have yet to be simulated. E-cell, for example, is not autocatalytic, does not replicate or partition its genome, and does not change in volume or synthesize most of its metabolites.

Regulatory Mechanisms. Cellular proteins are synthesized by first producing a complementary mRNA strand from a gene on the DNA (transcription), and then binding chains of amino acids with complementary tRNA anticodons to the mRNA at ribosomes (translation) (Wagner 2000). The rate at which the genes are expressed can be controlled in a number of ways.

Transcription in prokaryotes is initiated when RNA polymerase binds to the promoter region of the operon containing the gene. Thus, transcription may be prevented by the binding of repressor proteins to the operator region of the operon, blocking RNA polymerase from binding and traversing the gene (Lewin 1997). The repressors may be proteins synthesized from separate regulatory genes, or they may be the same protein for which the gene encodes. In the second case, regulation is controlled by the concentration of a product of the process being regulated, and is therefore an example of feedback inhibition (Voet & Voet 1995). In negative feedback control regulation, as the concentration of a protein increases, its rate of binding the operon of its own gene increases, shutting down further synthesis of itself and therefore lowering its concentration.

If the first binding of a protein to its gene induces a conformational change which increases the ligand binding affinity, then the protein may bind cooperatively (Voet & Voet, 1995) . This mechanism is described by the induced-fit hypothesis. Cooperative negative feedback control regulation may increase the sensitivity of gene expression to protein concentrations because each successive binding is more rapid than the previous. Multiple proteins may bind together to form oligomers, called dimers or tetramers in the common cases of two proteins or four proteins (Voet & Voet, 1995). Oligomers may be formed and then bind to the gene, rather than multiple proteins binding the gene one at a time. Experiments have shown that this may sharpen feedback sensitivity, especially as associated with nonlinear conditions such as multistability and oscillations (Smolen et. al. 2000). Multiple, non-cooperative bindings can decrease transcription rates without entirely shutting down synthesis (Almagor & Paigen 1987).

Transcription and translation may be viewed as two steps in a cascade, in which the product of one reaction serves as a catalyst in the next reaction. Cascades exhibit more flexibility of control and can provide enormous amplification of changes in reactant concentrations (Voet & Voet 1995). Cyclic cascades, in which an enzyme is converted between more and less active forms by phosphorylation, are common in cells. For example, the allosteric control of glycogen phosphorylase through ATP, G6P, and glucose inhibition and AMP activation is accomplished with a bicyclic cascade. Cascades can provide large response amplification in signal transduction. For example, if the binding of one hormone causes a change in a membrane receptor which activates it to catalyze ten G proteins, each of which catalyzes the production of ten of some other protein, there has been a response of one hundred molecules of a product due to one signal molecule using a two-step cascade. The bleaching of one rhodopsin molecule in a photoreceptor membrane can cause the hyrolysis of one hundred thousand cyclic guanosine monophosphate molecules (Bray 1995). In general, the more levels in the cascade, the greater the amplification. It can be proven that for an idealized simple cascade, the total response is the product of the

response coefficients for each level (Kholodenko et. al. 1997). Shacter-Noiman and colleagues established experimental evidence for cascade amplification in a cyclic-AMP-dependent phosphorylation-dephosphorylation system (Shacter-Noiman, Chock, and Stadtman 1983). In addition to providing amplification, cascades may also be able to convert continuously varying signal concentrations into discrete on/off outputs (Ferrell 1996). James Ferrell studied this switch-like response in a three-level MAP kinase cascade, and hypothesized that the behavior may be due to stoichiometric inhibitors, partial enzyme saturation, or multistep phosphorylations.

Gene expression may also be affected by the stability of the mRNA molecules, which controls whether the product of transcription survives long enough to be translated (Lewin 1997).

Cellular regulation is complicated by the fact that random stanstical variation can be a significant factor when regulatory circuits operate at very low concentrations (McAdams & Arkin 1999). Regulated proteins may be present in small copy numbers, and there may be only one gene for that protein per cell. Multiple gene copies or parallel or interconnected control pathways can introduce redundancy, which may provide a more reliable regulatory network under these conditions. Feedback loops may also be used to provide stability despite the random variations.

A number of people have noted the properties of different types of regulatory mechanisms involved in their research. Douglas Axe and James Bailey developed a method to mathematically compare regulatory mechanisms (Axe & Bailey 1993). Each mechanism is divided into the subsystem, including those reactions directly involved in the synthesis of the protein, and the surrounding system, including all other cellular activities, which is treated as static and independent of the subsystem, according to the Independent Surrounding System approximation. Their Recovery from Intrinsic Displacements procedure is then used to determine a displacement state for the subsystems so that the states for each model correspond to equally probable stochastic deviations. The perturbments are transient departures of small magnitude. All parameters are displaced based on their interdependences. Since the perturbations are mechanism-dependent and are all equally probable, fair comparisons may be made. Using experimental rate constants, the differential equations for the mechanisms may be solved, and the time required for fifty percent recovery from the perturbation may be calculated. They used this method to test the cooperative binding hypothesis for *rpoB*.

Several groups have modeled gene regulatory networks with Boolean circuits. Denis Thieffry and David Romero determined logical parameters which would allow a feedback circuit to generate multistationary or oscillatory behavior (Thieffry & Romero 1998). Since the percent of possible parameter values that

produce a functional circuit decreases geometrically with circuit length, biochemical regulatory systems might be able to be decomposed into reasonably independent feedback-controlled regulatory modules. Harley McAdams and Lucy Shapiro used electrical circuits to model a lysis – lysogeny decision circuit for bacteriophage lambda, using signal time delays to account for protein synthesis and decay rates (McAdams & Shapiro 1995).

Simulated Annealing Search Algorithm. N. Metropolis developed the first Monte Carlo importance-sampling algorithm in order to solve large-dimensional path integrals in statistical physics problems (Metropolis et. al. 1953). S. Kirkpatrick generalized this method to minimize any non-convex cost function (Kirkpatrick 1983). Simulated annealing uses random importance sampling of parameter space instead of using deterministic methods. L. Ingber developed Adaptive Simulated Annealing, which has a temperature schedule that decreases exponentially in annealing time, making it faster than Cauchy annealing algorithms and much faster than Boltzmann annealing techniques (Ingber 1989). It is used to solve a wide variety of large stochastic nonlinear multi-dimensional problems in Gaussian-Markovian space (Ingber 1995).

CHAPTER II

THE MECHANICAL CELL

The recent rapid advances in fields such as genomics and proteomics have vastly increased our knowledge about the function of many individual cellular components. However, the emergent properties arising from the complex interactions among these many components remain largely unknown. The most effective way to gain a thorough understanding of a complicated natural system is to model it. An accurate model may be used to test hypotheses about the consequences of any number of changes or perturbments to the system. Unfortunately, there is not yet enough data available to model any complete living cell. However, it is the goal of the Biocomplexity Group to establish the methodology for the creation of such a model using a somewhat simplified "mechanical" cell, using as much real data as possible but filling in gaps with hypothetical mechanisms. While real cells may be too complex to fully model in the near future, various important properties, such as regulation, specific cell cycle events, and metabolism, may be simulated and studied. The ability to simulate a wide variety of cellular processes numerically and visually (with three-dimensional animation) with high-powered computers is a valuable tool for theory formulation and testing and for educational purposes.

Mechanical Cell Three (MC3) is the Biocomlexity Group's most recent simulated cell. It is based on the simplest known living system, *Micoplasma genitalium*. This 0.2 micrometer-long bacterium has a genome consisting of 580,000 base pairs encoding 480 genes. The complete metabolism of this organism, as well as several others, has been determined, and is available from places such as Pangea Systems. MC3 is a simplification even of this, consisting of twenty-nine genes, but nevertheless maintains the key components of the *Micoplasma* cell-cycle, regulatory, and metabolic pathways.

There were three major aspects to the development of the MC3 model. First, a series of chemical reactions was written to describe all of the cellular processes we wanted to model. Secondly, computer programs had to be created to process, simulate, and animate the model. Finally, the system must be analyzed mathematically and fit to rate constants and initial concentrations that provide the desired behavior and "choreography" of the time-dependent choreographies of the components. The only phase of this aspect for which complete results have been obtained is for the study of regulatory mechanisms (described in Chapter III).

THE CHEMISTRY OF MC3

Reaction Mechanisms. Molecular mechanisms are step-by-step descriptions of how molecules react. Each step is indicated by an elementary chemical reaction of the form A+B $\leftrightarrow$ C+D, and may be unimolecular and/or reversible, but trimolecular reactions are highly unlikely. Such chemical reactions may be easily converted into a system ODEs. For example, the simple set of reactions

$$G + A \xrightarrow{k_1} G + P \tag{2-1}$$

$$P + Q \xrightarrow{k_2} PQ \tag{2-2}$$

describes a system in which a gene G is a catalyst for the synthesis of a protein P from an amino acid A, and the protein binds another protein Q to form PQ. The change in the concentration of each component per unit time is a function of the rate constants and the reactant concentrations in each reaction in which it is involved. If it is a product of the reaction, its concentration increases (positive term); if it is a reactant, its concentration decreases (negative term). For example, the equation for the protein P is

$$\frac{dP}{dt} = +k_1[G][A] - k_2[P][Q] \tag{2-3}$$

These equations can then be solved for a set of rate constants by fitting to experimental data, as is often done in kinematic studies of enzymes (see Chapter IV). Living systems consist of a set of molecular components which react in accordance with a complex mechanism to catalyze the synthesis of another copy of themselves (and waste) from raw materials,

$$\{R_1, R_2 \ldots\} \xrightarrow{\{C_1, C_2, \ldots\}} \{C_1, C_2 \ldots\} + \{W_1, W_2 \ldots\} \tag{2-4}$$

Thus, living systems can be viewed as complicated enzymes that catalyze reactions, the products of which are copies of themselves, and MC3 attempts to model them as such.

MC3 consists of 17,131 individual chemical reactions of the form A+B $\leftrightarrow$ C+D, involving 11,725 components. Reactions were divided into "k-groups", by which the number of parameters to fit was reduced by considering similar reactions to have similar rate constants. In addition, most metabolites were assumed to be present in time-independent constant concentrations, allowing them to be "absorbed"

into modified rate constants. For example, a second-order rate expression k[A][B] simplifies to k'[A] where k' = k[B] if B is unchanging with time. Each reaction was also associated with a "V-group", depending upon the volume (cytoplasm, membrane, inner surface, outer surface, or genome) in which it occurs. Some reactions, referred to as counter reactions, involved the binding of polymers. In this case, the number of components in the polymer was irrelevant to the rate, but was relevant to the mass balance of the system. Counter reactions share the same rate constant (they are in the same k-group), and give the reaction form and range of n, allowing them to be expanded into individual reactions by the simulation program (see below). Logically, the reactions can be classified into metabolic, cell-cycle, and regulatory reactions according to their function.

Metabolism in MC3. MC3 is a methanogen that grows chemoautotrophically in an environment with carbon dioxide, hydrogen, hydrogen sulfide, phosphate ion, nitrogen, and nickel. A general overview of MC's metabolism is illustrated in Figure 1. Methane, ATP, acetyl-CoA, ribose, three types of amino acids, and phospholipids are produced from raw materials in reactions catalyzed by synthases. Four types of nucleotides are produced from the amino acids, ribose, and ATP in a reaction also catalyzed by a synthase. The genome has 174 bases, and twenty-seven of its five-base genes code for proteins. It also includes oriC, the origin of replication, and terM, the terminus of replication. Proteins are tripeptides and are synthesized using a ribosome and ATP.

Cell-Cycle Reactions in MC3. The cell-cycle reactions are responsible for genomic replication and partitioning and for cell division. Due to the complexity and size of the cell-cycle reaction system, attempts have been made to combine reactions into several levels of abstraction. For example, in the highest abstraction level, illustrated in Figure 2, hundreds of reactions are combined in a single step. This reduces the number of rate constants for which to solve. Solutions at a high level may aid in determining the rates of each reaction for which it is an abstraction, since it is the net rate for the pathway.

According to Figure 2, a newborn cell has a MINE protein bound to a genome (indicated by an O) and to a chain of MUKB proteins. This chain is degraded by a MINE from the other side of the cell, which moves across the cell, and then a MUKY protein is inserted between the two MINEs. A ring of FTSZ proteins then begins to form around the circumference of the cell, starting at the left and right faces of the MUKY. When the osmotic pressure inside the cell is high, phospholipids are inserted into the membrane, releasing ADP. The ADP activates DNAA, which causes two POLA molecules to bind the genome. The POLAs then traverse the genome in opposite directions, synthesizing a second copy of the genome. When they come back together at the other end of the circular genome, they cause a WIRC:TRGD:WIRC trimer

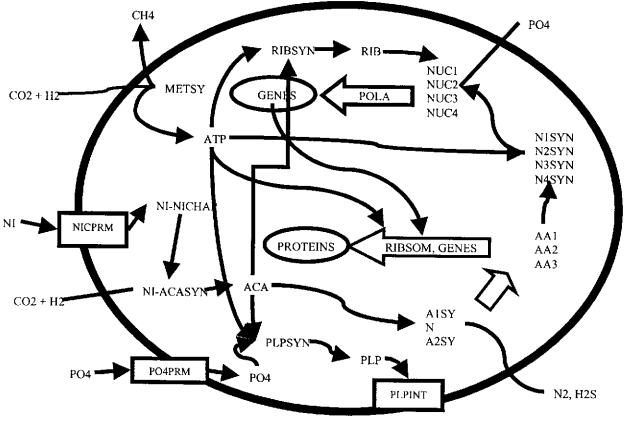


Figure 1. Schematic Metabolism of Mechanical Cell Three

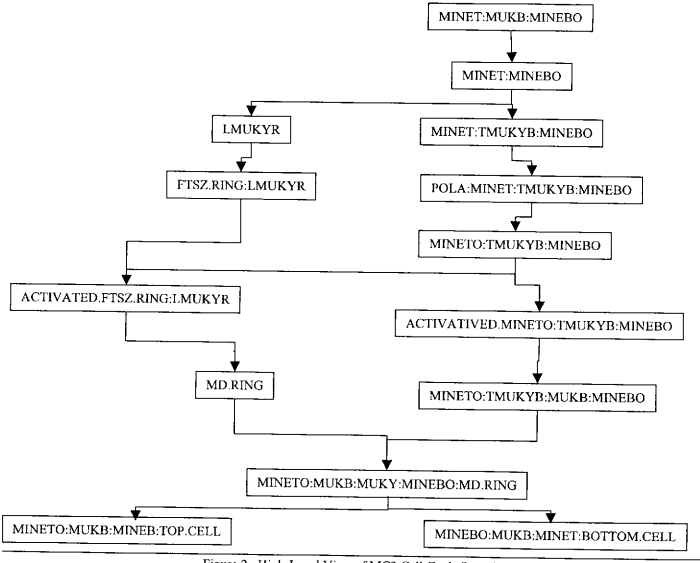


Figure 2. High-Level View of MC3 Cell Cycle Reactions

to dissociate. TRGD then binds MUKY, and a MUKB chain then begins to form from the top and bottom sides of MUKY:TRGD. The two copies of the genome are bound to the two original MUKBs, so as the chain lengthens the copies are partitioned into the two halves of the cell. When the FTSZ ring has formed, the two WIRC molecules can bind the terminal FTSZs, and begin degrading the ring. As the ring gets smaller, the cell constricts from a single sphere eventually into two spheres, connected by a minimum-diameter ring. Two MINE molecules displace the minimum diameter ring, which is released as waste, and the cell is divided into two newborn cells.

Regulation in MC3. Regulatory systems are important in MC3 for several reasons. Overall, hundreds of types of proteins are created and consumed throughout the cell-cycle, but their concentrations must vary minimally. Even unused proteins will decrease in concentration as the volume increases, and must therefore be synthesized. In addition, the most promising method for determining rate constants involves partitioning the reactions into small functional modules which can be solved independently. However, each module will either generate or consume components involved in the reactions of other modules. The main difficulty with linking these separately solved modules will be that the rate constants obtained by simulating two independent modules may be substantially different from those required once the modules are linked. The key to linking is for each module to be independently regulated, because they should then be able to respond to changes in the rates by which shared inputs and outputs are consumed or generated. The first step in the analysis phase of the MC3 model was therefore to examine the properties of regulatory mechanisms. (See Chapter III.)

COMPUTER SIMULATION AND ANIMATION

The second aspect of developing a mechanical cell was to create the computer programs necessary to read in the reactions, rate constants, initial concentrations, and other pertinent information describing the model, and then to perform useful analysis of the system and carry out numerical simulations. Of course the computer programs are independent of the actual description of MC3; changes in the description, or even entirely new mechanisms can be simulated by simply changing the descriptions in the input files. The development of these programs was an important part in the establishment of a methodology to deal with the simulation of cells.

Inputting the Description of a Mechanism. A set of reactions and their associated data may be loaded into the program through input text files which adhere to a precisely defined format. The name, categories, rate constant group, volume group, reactants, products, stoichiometric coefficients, and (optionally) counter parameters of each reaction are read line-by-line and passed to functions which

construct the data structures necessary for efficient manipulation of the data. Reactions with up to two counters are expanded to individual reactions by iteratively replacing occurrences of the counter variable(s) with all numbers in the counters' range(s), performing arithmetic as necessary (i.e., replacing n+1 with 2 when n=1). Space for a reaction struct is dynamically allocated on the memory heap for each reaction as it is parsed. Structs for each new component, category, k-group, and V-group are also created as they are read. All instances of each type of struct are connected to each other by forming linked lists. This abstract data type provides constant-time tail-insertion and allows run-time control of memory allocation and deallocation for variable reaction set sizes. The lists are also highly interconnected. Each reaction struct contains pointers to an entry in the k-group list and an entry in the V-group list, as well as lists of pointers into the category and component lists for all of its categories, reactants, and products. Each component struct contains lists of pointers to all reactions in which it appears as a reactant and to all reactions in which it appears as a product. Another text file containing initial copy-numbers and k-group values is parsed. Read values are passed to functions which update the corresponding components for the appropriate list entries. Initial structural parameters (such as cell volume, membrane thickness, surface area, and the sizeof MUKB, FTSZ, and PLPs) are either specified or calculated from other specifications. From these values, the total number of particles in the cytoplasm is calculated, as an indicator of osmotic pressure. Then, the amount of waste needed in the environment to match that osmotic pressure is calculated and included in the environment.

Stochastic Simulation. The main simulation loop calculates the time at which a reaction will occur as well as which reaction will occur at that time. The method used is essentially the Next Reaction method developed by Gillespie. First, the stochastic "a" value for each reaction is calculated. This equals the product of the k for that reaction multiplied by the current copy number of each reactant, and divided by the current volume raised to the power one less than the number of reactants. The sum of all a-values is called asum. The time at which the next reaction occurs, called tau, equals the current time + $\{1/\text{asum}\}\ln(1/r_1)$, where r_1 is a random number between 0 and 1. Let V = asum*r_2, where r_2 is another random number between 0 and 1, and let W be a running total of a-values from an arbitrarily ordered list of the reactions. The reaction executed at time tau is that which first causes W to exceed V. After each reaction is executed, the copy numbers of each component involved are updated accordingly. The osmotic pressure inside the cell is determined and cell volumes are adjusted until the pressure matches that of the environment. This volume is used to calculate the new size and shape of the membrane.

Monitoring Cellular Conditions. An important aspect of the model is to provide not only the reactions required for the cell to grow and replicate, but also those that could indicate cell death. After each reaction, the program compares the values of numerous parameters to a list of specified conditions which, if

obtained, would indicate that the cell has died. One condition measures the percentage of the membrane volume occupied by PLP. If the percent occupancy is greater than 100%, the cell dies because this is physically impossible. If it is less than 50%, the cell dies because of a "leaky" membrane. If certain counter reactions reach their maximum or minimum values, this is also cause for cell death as it indicates unregulated growth. If any such condition is met, the program terminates and provides an "autopsy" report stating the cause of death and the condition of the cell at that time. If the cell remains viable, the program determines whether the components generated in the last step of the cell cycle have been produced. If so, the simulation is complete; if not, the main loop repeats until these components are generated. After each iteration of the main loop, the current time and the copy numbers of each component are printed to a tab-delimited text file. This file can be opened in a spreadsheet application so that copy numbers may be graphed as a function of time.

Completeness Checks. The large number of reactions involved makes the probability of errors very high. One means by which the set of reactions is analyzed is by determining which components are either made or used, but not both. If the detected "dead-end" components are anything besides starting materials or waste products, the reaction set must contain an error. Another means by which the reaction set is analyzed is by determining whether every reaction could be executed. In this analysis program, raw materials and the bare minimum of newborn cell components are assumed present. Every reaction that could occur under these conditions (i.e., where all components are present at non-zero copy numbers) is executed. Then the products of these reactions are given non-zero copy numbers and any additional reactions that could occur are executed. This process continues until no additional reactions can be executed, thereby revealing any unexecuted reactions in the set. The reaction set can also be organized by functional category as well as by name, allowing easy location of reactions and a means for visual inspection and individual analysis.

Pathway Searches. Another means by which the reaction sets can be analyzed is by identifying pathways from one component to another. When a component reacts to generate products, it may be viewed as the parent and the products can be considered its children. The children may also react, generating its children and the grandchildren of the original component. A pathway is defined as the series of reactions connecting a parent to a particular descendent. These pathways may be traced by first following all of the pointers in the reactants list of the initial component's struct (i.e., the list of pointers to the reactions in which the component is a reactant). Then all of the component pointers in the products list of these reactions must be followed. This identifies all of the components which can be generated from the initial component through a one-step pathway. This process is repeated until the goal component is encountered. Some pathways are quite indirect and lengthy, suggesting that they are kinetically unimportant. Reactions

or components already encountered are not repeated in the pathway; a second encounter is a "dead-end," as is an encounter with any component specified in an input file as unimportant to pathway determinations. Due to the large number of reactions and components in the full system, searching for pathways of more than a few dozen steps becomes intractable. Breadth-first search exhibits exponential growth in memory usage (because the previously visited nodes must be stored in order to print the pathway once the goal component is found), while depth-first search experiences exponential growth in run time. However, this algorithm has been used successfully in some cases, such as determining the pathway by which the "assembly" used for cell cycle processes converts from its beginning to final form.

Generation of ODEs. The kinetics of complex chemical systems are typically solved using numerical methods and by assuming large numbers of species reacting deterministically. Since MC3 involves small numbers of species reacting stochastically, the strategy is to solve our system as though it was deterministic, and then use the resulting deterministic k-values to calculate stochastic a-values. This procedure should be legitimate when the results of a large number of simulations are averaged. Thus, with the reaction set loaded into the simulator, the ODEs corresponding to the time derivatives of each component can be generated. This is done by traversing the component list and following several levels of pointers to obtain the k-groups and components involved in all the reactions in which the current component is a reactant. These equations are outputted to a file. For the 17,131 reactions describing MC3, this corresponded to 11,725 ODE's and 659 k-groups.

Computer Animations. In addition to computer-generated numeric simulations, computer animations are important to the development of a cellular model because the complex three-dimensional interactions among components over time can be very difficult to understand without visualization. A qualitative description of the most important cell-cycle reactions of MC3 was generated using the LightWave graphics program, producing a three-minute animation video. Processes such as genomic replication, MUKB chain formation, the constriction of the FTSZ ring, and cell growth and division were represented. Figure 3 includes several "screen shots" from the animation. In the future, we hope to use the numeric simulation to drive the animation. By modifying the format of the output file from the numerical simulator, this file may be used as input for LightWave, allowing the animation to accurately represent the changes in copy numbers for all components as determined by the stochastic simulation. Additional spatial information may also be tracked by the component structs and similarly outputted to this file.

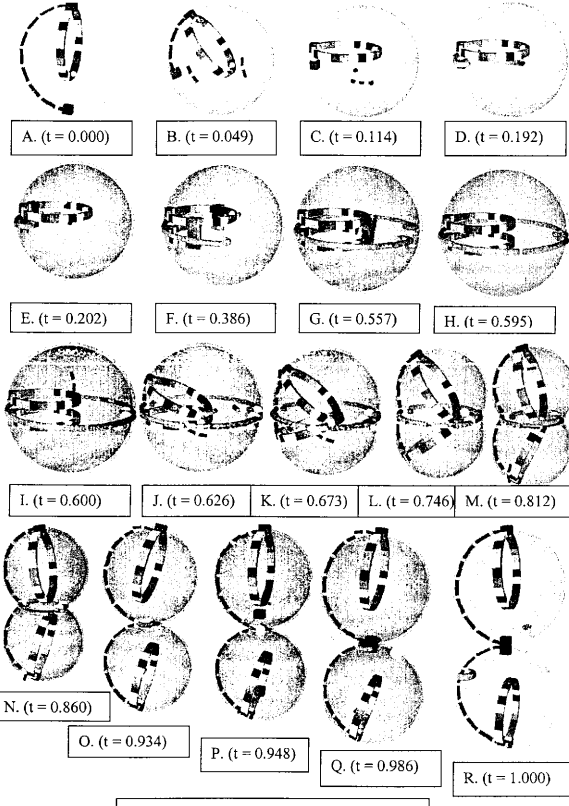


Figure 3: Frames from MC3 Computer Animation

ANALYSIS OF MC3

Much work remains in the analysis of MC3. A proposal for a two-year grant for this purpose has been submitted to the National Institute of Health. Rate constants that provide a realistic "choreography" of concentrations as a function of time for each component have not yet been determined, and therefore there are no results from successful simulation runs to report yet. Results have however been obtained in some subproblems within the analysis of MC3. Chapter III describes the comparative features of various regulatory mechanisms, while Chapter IV discusses the data-fitting algorithm used to find best fit rate constants and how this has been applied to related experimental data.

CHAPTER III

THE REGULATORY MECHANISMS

Protein concentrations in living systems are affected by numerous processes, including transcription, translation, proteolysis, and cell volume changes. Depending on the physiological role of the protein, other processes may also perturb levels. Proteins involved in cell-cycle processes tend to change concentration when they are active (such as when a structure for which they are a component is being built) and then change again in the opposite direction when the activity is complete (such as when the structure disassembles). Proteins involved in metabolism or gene expression change concentration as they bind or unbind substrates, products, or effector molecules, or as they are covalently modified or unmodified.

METHODS OF CELLULAR REGULATION

Negative Feedback Control. Despite these varied and opposing influences, concentrations of most cellular proteins presumably deviate only slightly around a specific value, at least at equivalent stages of the cell cycle. In prokaryotes, the predominant homeostatic mechanism involves negative feedback control of transcription. Autoregulated proteins (P) bind to regions of DNA (G) that control the expression of the genes which encode them, thereby inhibiting transcription at high protein concentrations and stimulating it at low concentrations (where "high" and "low" are defined relative to the value of the binding constant K_{PG} associated with the reaction $P + G \rightleftarrows P{:}G$). Other P's are regulated indirectly through the binding of transcription factor (TF) proteins to the regions of the DNA that control transcription. The binding of TF to G is influenced by effector molecules (ε) whose concentrations are influenced, in turn, by P. For example, ε may be the product of a P-catalyzed reaction. In homeostatic mechanisms, either the binding of εTF to G will repress transcription of P, or the binding of TF to G will stimulate it. Alternatively, if ε is a substrate, either the binding of εTF to G will stimulate transcription or the binding of TF to G will inhibit it.

Cooperativity. Transcription factors tend to exist in a monomer - oligomer equilibrium, with dimers and tetramers being the most common oligomeric forms. Transcription factors bind DNA in their oligomeric forms, an arrangement that affords cooperativity. In some cases, multiple copies of the oligomeric TFs bind Gs, so as to add a second level of cooperativity. The binding serves to either stimulate or inhibit

transcription according to whether it promotes or inhibits the binding of RNA polymerase to the DNA. This mechanism of associative binding with the polymerase adds a third level of cooperativity. Using the λ Repressor TF as an example, Ptashne has shown that cooperative binding increases the sensitivity of the transcriptional "switch" to changes within a particular repressor concentration region (as set by the equilibrium constant associated with the binding of TF to G), and it decreases this sensitivity outside of that region. Cooperativity resulting from the binding of multiple TF copies also increases the specificity of binding to particular genetic loci.

Cascades. Transcription and translation may be viewed as a two-enzyme cascade, in which allosteric binding of an effector molecule to one enzyme activates it to catalyze the activation of a second enzyme. The advantage of this arrangement is that sensitivity of the rate of the reaction catalyzed by the second enzyme to changes in effector concentration is amplified relative to what it would be if the effector bound directly to the second enzyme. The number of levels and the rates of catalysis of each enzyme determine the level of amplification. Transcription factors correspond to the effectors to be amplified. DNA, RNA polymerase, and any other factors required for transcription together constitute the first enzyme in the cascade (serving to catalyze the synthesis of mRNA from nucleotides). The resulting mRNA transcript, ribosomes, and any other factors required for translation together constitute the second enzyme in the cascade (serving to catalyze the synthesis of proteins from amino acids). Assuming that the concentrations of the polymerase, ribosomes, and the other unspecified factors required for transcription and translation remain constant, this cascade may be described by the reaction in Figure 4.

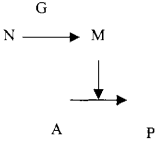


Figure 4. A Simple Cascade Mechanism.

The presence of a two-level reaction cascade in transcription/translation mechanisms should affect the regulatory/homeostatic properties of these systems.

Regulation in Cells. Naively, proteolysis might be expected to play an important role in the homeostasis of proteins, because the effects of perturbing processes that would tend to increase or decrease levels of proteins could be minimized by having rapid rates of both protein degradation and biosynthesis. Although some proteins, especially those involved in cell cycle processes or metabolic control points, do have short lifetimes (approximately ten minutes), the majority of proteins have long lifetimes (approximately 8000 minutes) relative to the cellular reproduction rate (about forty minutes). This suggests that for the majority of proteins, the predominant process leading to declining concentrations is dilution due to the increasing cell volume that accompanies cell growth. This situation must diminish the ability of these stable-protein systems to maintain homeostasis in the presence of positive perturbations (those tending to increase levels of P).

It appears that the dominant mechanisms for regulating protein levels are implemented at the level of transcription. The biosynthesis rate of mRNA is relatively fast (a typical transcript is made in about two minutes), and the lifetime of a typical mRNA in prokaryotes is quite short (also about two minutes) relative to the cell cycle period. Moreover, for a given transcript, the steady-state copy numbers per cell are very low (two to three per cell), allowing small changes in absolute numbers to be a large percentage change. These rapid rates and low copy numbers bode well for effective regulation. In fact, within homeostatic mechanisms, this step appears to be most highly regulated. It is controlled by the negative feedback mechanisms mentioned above, as well as by other reactions that serve to either stabilize or destabilize the transcript for degradation. Given the cascade relationship mentioned above, the regulation of transcription will be able to indirectly control the rate of translation.

THE SIX REGULATORY MECHANISM MODELS

Methods of Analysis. In order to study and compare the properties of regulatory mechanisms, simple systems of chemical reactions were written for six basic models, each regulating a single protein. These models are illustrated in Figure 5. Each system consists of a regulatory "core" and a perturbation. This perturbation is the same for each model, consisting of the synthesis of the protein P at a rate k_3 and the degradation of P at a rate k_4. These reactions represent the sum of all processes involving P other than those which attempt to regulate it. Thus it would include reactions in which P is bound or unbound into structural polymers or consumed or made in any way, including "degradation" in concentration due to

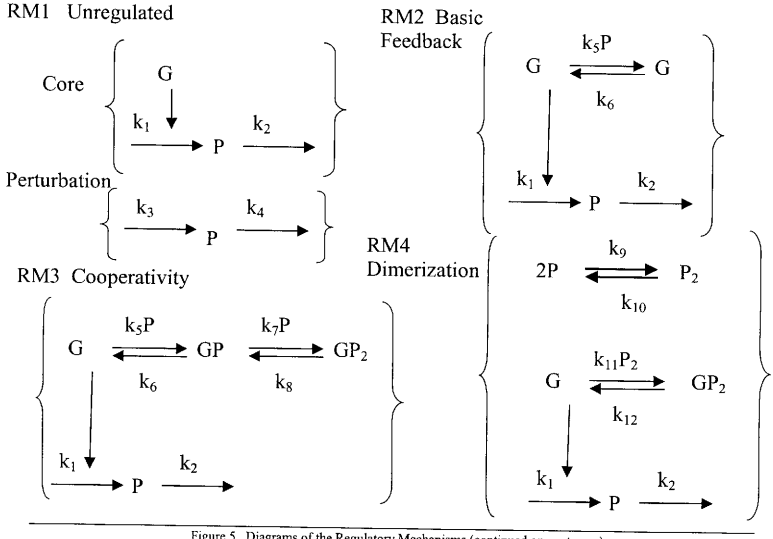
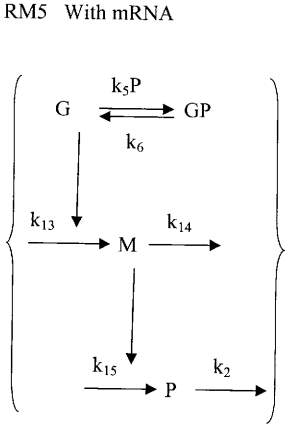


Figure 5. Diagrams of the Regulatory Mechanisms (continued on next page)



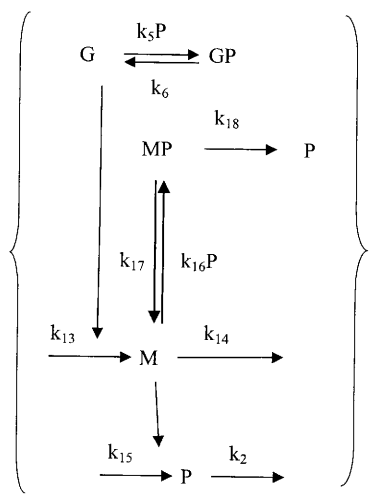


Figure 5 (continued)

volume increases (since concentration is number per unit volume). The goal of the regulatory "core" is to maintain the concentration of P regardless of these external rates k_3 and k_4. Of course, perfect regulation is not possible; if k_3 and k_4 are much greater than the rates involved in the core reactions, P cannot be regulated (unless k_3 and k_4 exactly balance each other out, degrading at exactly the same rate as synthesizing, but this is highly improbable in any real system). The mechanisms can be compared, however, based on how large these external rates can be without perturbing the concentration of P outside of an acceptable range.

Description of the Models. The first of the six models, RM1, is completely unregulated. The gene G simply catalyzes the synthesis of the protein P at some rate k_1, and P degrades at rate k_2. This mechanism serves as a "control group." RM2 uses basic negative feedback to regulate P. The protein can bind the gene which encodes it at a rate dependent on k_5 and the concentration of P, and can unbind it at a rate k_6. Thus, when the concentration of P high, it binds G at a rapid rate, preventing further synthesis of P (since unbound G is a catalyst for the synthesis of P). When the concentration of P decreases, less G is bound, so it can catalyze the synthesis of more P, bringing its concentration back up. RM3 employs cooperativity, as a second protein molecule can bind the singly-bound gene. Intuitively, this should increase the sensitivity of the system to changes in the concentration of P because the binding effect is amplified. RM4 incorporates dimerization, and is similar to RM3, except that the two copies of the protein bind to each other first and then bind together to the gene, rather than each binding the gene sequentially. RM5 is the first to distinguish between the processes of transcription and translation. The gene is a catalyst in the synthesis of an mRNA molecule M which is itself a catalyst for the synthesis of P, rather than G catalyzing the synthesis of P directly. This two-step cascade should also amplify the effect of changes in P. For example, if the gene synthesizes ten mRNAs, each of which can then synthesize ten proteins, then a total of one hundred proteins have been created as a result of the unbinding of one gene. RM6 adds negative feedback control to the degradation of mRNA in addition to the gene itself. P can bind and unbind M, so if the concentration of P increases, P can bind both G and M, quickly shutting down further synthesis of P.

ANALYSIS OF THE SENSITIVITY OF GENE BINDING

G/G_{total} vs. log(P) Plots. The ability of a regulatory system to regulate a protein depends on the sensitivity of changes in the percent of genes that are unbound to changes in the concentration of the protein. This can be examined by plotting the percent of G that is bound (G divided by the sum of all components including G, such as the unbound G and the bound GP) against P, as is shown in Figure 6 with P plotted on a logarithmic scale to highlight the sensitivity of changes in G/G_{total} to large changes in P. A function of G/G_{total} in terms of P can be found from the steady-state and conservation equations of the system.

Using the steady state equations means that we are examining the system in its "final" state (e.g., when all of the concentrations have stopped changing).

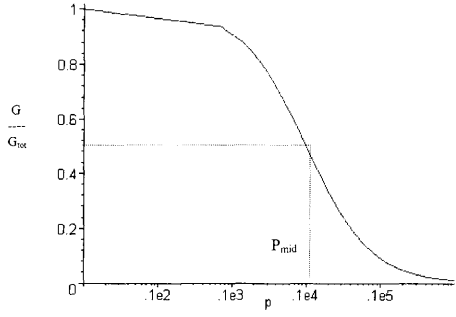


Figure 6. Plot of log([P]) vs. G/G_{total} for RM2 (K = 1/1000).

RM2. The reaction mechanisms neglect raw materials and waste, which are assumed to be present in quantities large enough that they are virtually unaffected by RM2 processes, allowing them to be considered constant and therefore "absorbed" into the rate constants. The core of RM2 contains the reactions

$$G \xrightarrow{k_1} G + P \tag{3-1}$$

$$P \xrightarrow{k_2} \tag{3-2}$$

$$G + P \xrightarrow{k_3} GP \tag{3-3}$$

$$GP \xrightarrow{k_6} G + P \tag{3-4}$$

One of the differential equations of the system is then

$$\frac{d[G]}{dt} = -k_5[P][G] + k_6[GP] \tag{3-5}$$

Since at steady state the concentrations are no longer changing, all derivatives are zero, so

$$0 = -k_5[P][G] + k_6[GP] \tag{3-6}$$

$$\frac{k_5}{k_6} = \frac{[GP]}{[P][G]} = K_{eq} \tag{3-7}$$

Since matter can be neither created nor destroyed, the sum of the concentrations of all components including G must be constant, so at all times

$$[G_{total}] = [GP] + [G] \tag{3-8}$$

For convenience, G_{total} can be assumed to be one, yielding

$$1 = [GP] + [G] \tag{3-9}$$

Therefore,

$$[GP] = 1 - [G] \tag{3-10}$$

may be substituted into (3-7),

$$K_{eq} = \frac{1-[G]}{[P][G]} \tag{3-11}$$

and solved for [G]

$$[G] = \frac{1}{K_{eq}[P] + 1} \tag{3-12}$$

Since G_{total} is taken to be one, $G/G_{total} = G$. The steeper the slope of the G/G_{total} vs. log(P) curve, the more sensitive the concentration of G is to this concentration of P. The steepest slope will always occur when half of the genes are bound and half unbound (G = 1/2), providing the greatest flexibility in both directions. Thus P can be best regulated when G = 1/2. Solving (3-12) for this case,

$$\frac{1}{2} = \frac{1}{K_{eq}[P] + 1} \tag{3-13}$$

$$[P] = \frac{1}{K_{eq}} \tag{3-14}$$

The slope at this point can be determined by taking the derivative of (3-12)

$$\frac{d}{d[P]}\left(\frac{1}{K_{eq}[P] + 1}\right) = \frac{-K_{eq}}{(K_{eq}[P] + 1)^2} \tag{3-15}$$

and substituting in (3-14)

$$\frac{-K_{eq}}{(K_{eq}\dfrac{1}{K_{eq}} + 1)^2} = \frac{-K_{eq}}{4} = \frac{-1}{4[P]} \tag{3-16}$$

RM1. A similar analysis may be performed with the other mechanisms. In RM1, G is never bound or unbound, so G/G_{total} always remains constant. Therefore its graph would be a horizontal line, as it would be completely unresponsive to changes in P.

RM3. Two steady-state equations and one conservation equation,

$$\frac{k_5}{k_6} = \frac{[GP]}{[P][G]} = K_1 \tag{3-17}$$

$$\frac{k_7}{k_8} = \frac{[GP_2]}{[P][GP]} = K_2 \tag{3-18}$$

$$[G_{total}] = 1 = [G] + [GP] + [GP_2] \tag{3-19}$$

are needed in RM3 to obtain the G vs. P curve

$$[G] = \frac{1}{1 + K_1[P] + K_2K_1[P]^2} \tag{3-20}$$

A relationship between K_1 and K_2 can be determined at the point of maximum slope by solving (3-20) for K_1 when G = 1/2 and P = P_{mid}, the value (not yet known) of P when G = 1/2, yielding

$$K_1 = \frac{1}{[P_{mid}](1 + K_2[P_{mid}])} \tag{3-21}$$

which can be substituted back into (3-20). The maximal slope, the derivative of (3-20) when P_{mid} = P, is

$$\frac{\dfrac{1}{[P](1 + K_2[P])} + \dfrac{2K_2}{1 + K_2[P]}}{\left(1 + \dfrac{1}{1 + K_2[P]} + \dfrac{K_2[P]}{1 + K_2[P]}\right)^2} \tag{3-22}$$

This value depends on K_2. The larger K_2, the steeper the slope. The limit as K_2 approaches infinity is

$$\frac{-1}{2[P]} \tag{3-23}$$

exactly twice that for RM2 (3-16). Therefore, the mechanism with cooperativity can be up to twice as sensitive to changes in protein concentrations when regulating in the optimal region. The requirement that

K_3 be large makes sense because a K_3 approaching zero would be the same as RM2, since GP_2 would never exist, leaving the same structure as RM2. The stronger the cooperativity, the better, approaching the limit of twice as good (for one extra level of cooperativity).

As more levels of cooperativity are added, this maximal steepness term also increases. A two-level cooperative system would be the same as RM3, with an extra reaction in which GP_2 reversibly binds another P to form GP_3. Any number of levels can be created by continuing to add such reactions. By performing analyses similar to that presented above for RM3, it was determined that each successive level of cooperativity added a term of -(1/4)(1/p) to the maximum slope. In other words, the maximum slope of the G/G_{total} vs. log(P) curve for a system in which n protein molecules can bind the gene (an n-1 level cooperative system) is

$$\left(\frac{-n}{4}\right)\left(\frac{1}{[P]}\right) \tag{3-24}$$

Therefore, there is a linear relationship between the number of levels of cooperativity and the maximal sensitivity of the system, and this sensitivity approaches infinity as the number of levels of cooperativity approaches infinity.

RM4. A similar analysis may also be performed with RM4. The steady-state and conservation equations

$$\frac{k_9}{k_{10}} = \frac{[P]^2}{[P_2]} = K_3 \tag{3-25}$$

$$\frac{k_{11}}{k_{12}} = \frac{[GP_2]}{[G][P_2]} = K_4 \tag{3-26}$$

$$[G_{total}] = 1 = [G] + [GP_2] \tag{3-27}$$

are used to find the G vs. P curve

$$[G] = \frac{K_3}{K_3 + K_4[P]^2} \tag{3-28}$$

A relationship between K_3 and K_4 can be determined at the point of maximum slope by solving (3-28) for K_3 when G = 1/2 and P = P_{mid}, the value (not yet known) of P when G = 1/2, yielding

$$K_3 = K_4[P_{mid}]^2 \tag{3-29}$$

which can be substituted back into (3-28). The maximal slope, the derivative of (3-28) when P_{mid} = P, is

$$\frac{-1}{2[P]} \tag{3-30}$$

This is the same result as obtained for RM3 (3-23). This makes sense because the behavior of the system should be similar regardless of whether the proteins bind to each other and then to the gene or both bind directly to the gene individually; either way, there is in effect cooperativity and increased sensitivity to the concentration of P. However, there is one notable difference between RM4 and RM3 in this analysis. Equation (3-30) was obtained without taking any limits for values of equilibrium constants; this result is independent of the equilibrium constants for the binding of P. Thus, while RM3 required large (limit to infinity) values of the second equilibrium constant in order to approach its best behavior, RM4 obtains this maximal slope without the need for any large equilibrium constants.

RM5 and RM6. The G vs. log(P) plots for RM5 and RM6 are both identical to that for RM2. Although these mechanisms include a cascade involving messenger RNA, equations (3-7) and (3-8) still hold, so the G-binding analysis remains the same. Differences caused by the mRNA cascade only become apparent in the regulatory region analysis, described in the next section.

ANALYSIS OF THE REGION OF REGULATION

Description of the Procedure. A good regulatory mechanism will allow the widest possible variation in the rates of degradation and synthesis of a protein while maintaining the protein's concentration within some tolerance of its initial value. For the purposes of comparison, for each mechanism this initial value will be at the level at which G is most sensitive to changes in P, which was found to be when half of the gene is bound (G/G_{total} = 1/2), the point of greatest slope for the G vs. log(P) graph (see previous section). The value of P when G = 1/2 is called P_{mid}, and we choose $P_0 = P_{mid}$. The protein may be considered to be effectively regulated as long as its concentration is within some range of its initial value, $(1-\varepsilon)P_0 < P < (1+\varepsilon)P_0$. The concentrations of both P and G at the edges of tolerance can be calculated. As indicated by the negative slope of the graph, the maximum percent of G bound will be at the minimum value of P, and

the minimum percent of G bound will be at the maximum value of P. This is consistent with the fact that G is a catalyst in the production of P, so there will be the most P when there is the most unbound G available.

For mechanisms RM1 through RM4, the equation

$$P = \frac{k_1\left(\frac{G}{G_{total}}\right) + k_3}{k_2 + k_4} \tag{3-31}$$

will always hold (at steady-state), because P is synthesized at rates k_3 and $k_1[G_{unbound}]$, and is degraded at rates k_2 and k_4. Similarly, for mechanisms RM5 and RM6,

$$P = \frac{k_1 M + k_3}{k_2 + k_4} \tag{3-32}$$

will always hold (at steady-state) for the same reasons. By solving the other steady-state and conservation equations specific to the individual mechanisms, and then substituting in the P_{min} / G_{max} and P_{max} / G_{min} values for P and G, P and G or M can be expressed as a function of only rate constants, equilibrium constants, and ε. When substituting this into (3-31) or (3-32), an equation relating k_3 and k_4 in terms of the other rate constants can be obtained and solved for either of these terms. Given values of the core mechanism's rate constants, a graph of k_3 vs. k_4 can be plotted for both P_{min} / G_{max} and P_{max} / G_{min} conditions for given values of the other rate constants and for ε. The region between these two lines can be considered the "regulated region", because for these combinations of k_3 and k_4 perturbment rates, P is maintained within the tolerable range. Moreover, both functions will be linear, since k_3 and k_4 are not multiplied by any non-constant terms in (3-31) or (3-32). Therefore a graph very similar to Figure 7 can be plotted for each regulatory mechanism.

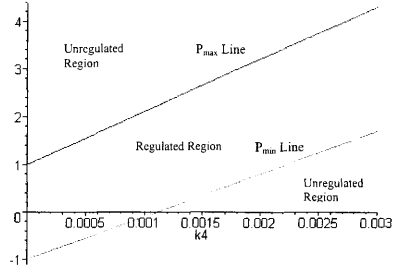


Figure 7. Plot of P_{max} and P_{min} Lines for RM1 (k_1 = 20 min^{-1}, G = ½ nM, ε = 0.1 and $[P]_{mid}$ = 1000 nM (i.e. k_2 = 0.01 $nM^{-1}min^{-1}$). Units of k_3 and k_4 are $nM^{-1}min^{-1}$ and min^{-1}, respectively).

The Method as Applied to RM2. Consider for example RM2. In the G/G_{total} vs. log(P) plot analysis, the value of P when G = 1/2, referred to as P_{mid} , was calculated to be

$$P_{mid} = \frac{1}{K_{eq}} \tag{3-33}$$

by solving the steady-state and conservation equations for G in terms of P and the equilibrium constant,

$$[G] = \frac{1}{K_{eq}[P]+1} \tag{3-34}$$

and then solving for P when G = 1/2 (see Equations 1 through 14).

When attempting to regulate at this value of P, the minimum tolerable value of P is

$$P_{min} = (1-\varepsilon)P_{mid} = \frac{1-\varepsilon}{K_{eq}} \tag{3-35}$$

By substituting P_{min} into (3-34) for P, the maximum tolerable value of G is obtained. The equilibrium constant cancels out, and G_{max} is

$$G_{max} = \frac{-1}{\varepsilon - 2} \tag{3-36}$$

Similarly, the values of P_{max} and G_{min} can be calculated.

$$P_{max} = (1+\varepsilon)P_{mid} = \frac{1+\varepsilon}{K_{eq}} \tag{3-37}$$

$$G_{min} = \frac{1}{2+\varepsilon} \tag{3-38}$$

The P_{min} / G_{max} line is therefore defined by substituting (3-35) and (3-36) into (3-31)

$$(1-\varepsilon)P_{mid} = \frac{\frac{-k_1}{\varepsilon - 2} + k_3}{k_2 + k_4} \tag{3-39}$$

and solving for k_3 (alternatively it could be solved for k_4).

$$P_{min}\text{: } k_3 = \frac{k_1 - P_{mid}\left(2k_2 + 2k_4 - 3k_2\varepsilon - 3k_4\varepsilon + k_2\varepsilon^2 + k_4\varepsilon^2\right)}{\varepsilon - 2} \tag{3-40}$$

The P_{max} / G_{min} line is defined by substituting (3-37) and (3-38) into (3-31)

$$(1+\varepsilon)P_{mid} = \frac{\frac{k_1}{\varepsilon + 2} + k_3}{k_2 + k_4} \tag{3-41}$$

and solving for k_3.

$$P_{max}:\ k_3 = \frac{P_{mid}\left(2k_2 + 2k_4 + 3k_2\varepsilon + 3k_4\varepsilon + k_2\varepsilon^2 + k_4\varepsilon^2\right) - k_1}{\varepsilon + 2} \tag{3-42}$$

Initially, at $P = P_0$, there is no perturbment, so $k_3 = k_4 = 0$. By choice, $P_0 = P_{mid}$, so at $P = P_0$, $G/G_{total} = 1/2$. Therefore (3-31) becomes

$$P_0 = P_{mid} = \frac{k_1}{2k_2} \tag{3-43}$$

This can be substituted into (3-40) and (3-42) to obtain k_3 as a function only of rate constants and ε.

$$P_{min}:\ k_3 = \frac{-\frac{k_1k_4}{k_2} + \frac{3k_1\varepsilon}{2} + \frac{3k_1k_4\varepsilon}{2k_2} - \frac{k_1\varepsilon^2}{2} - \frac{k_1k_4\varepsilon^2}{2k_2}}{\varepsilon - 2} \tag{3-44}$$

$$P_{max}:\ k_3 = \frac{\frac{k_1k_4}{k_2} + \frac{3k_1\varepsilon}{2} + \frac{3k_1k_4\varepsilon}{2k_2} + \frac{k_1\varepsilon^2}{2} + \frac{k_1k_4\varepsilon^2}{2k_2}}{\varepsilon + 2} \tag{3-45}$$

The Uncorrelated Region. These two lines are plotted in Figure 8, using specific values for k_1, k_2, and ε. Although for any combination of k_3 and k_4 between the lines, P is regulated, the region of greatest interest is the rectangle, called the uncorrelated region, bounded by the axes and the y-intercept of the P_{max} linc and the x-intercept of the P_{min} line. Any system can "regulate" large k_3 and k_4 rates if these two values essentially cancel each other; a system in which external synthesis "perturbment" equals external degradation "perturbment", there is no net perturbment, and regulation is unneeded, regardless of how large these rates may be. What is more common and more important is to regulate when either k_3 or k_4 is much greater than the other. The system can regulate along the vertical axis ($k_4 = 0$) up to the k_3 value where P_{max} intersects the axis, and it can regulate along the horizontal axis ($k_3 = 0$) up to the k_4 value where P_{min} intersects the axis. Therefore the area of this "uncorrelated" region in which an external degradation perturbment can be regulated without any "assistance" from an external synthesis perturbment, or vice versa, is an important measure of the effectiveness of a mechanism.

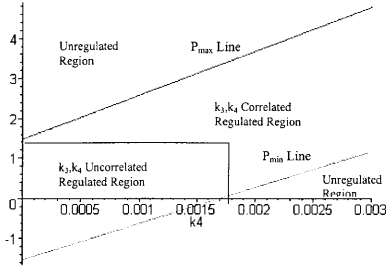


Figure 8. Plot of P_{max} and P_{min} lines for RM2 (values for parameters are as in Figure 7).

This area is easy to calculate because it is a rectangle, and the lengths of its sides are the y-intercept value of the P_{max} line and the x-intercept value of the P_{min} line. To continue the example with RM2, the x (k_4) intercept of (3-44) may be found be setting k_3 to zero and solving for k_4, yielding

$$\frac{k_2(\varepsilon-3)\varepsilon}{(\varepsilon-2)(\varepsilon-1)} \tag{3-46}$$

Setting k_4 to zero gives the y (k_3) intercept of (3-45)

$$\frac{\frac{3k_1\varepsilon}{2}+\frac{k_1\varepsilon^2}{2}}{\varepsilon+2} \tag{3-47}$$

The product of (3-46) and (3-47) is the area of the rectangle.

$$A = \frac{k_2\left(\frac{-3k_1\varepsilon}{2} - \frac{k_1\varepsilon^2}{2}\right)(\varepsilon - 3)\varepsilon}{(\varepsilon - 2)(\varepsilon - 1)(\varepsilon + 2)} \tag{3-48}$$

Results for All Mechanisms. P_{min} and P_{max} lines and the area of the uncorrelated region of regulation may be derived in a similar manner for each of the six regulatory systems from Figure 5. The area equations are listed in Figure 9, and all of the derivations are in the appendix.

Comparisons of the Areas of the Uncorrelated Rectangle. The ε terms make these equations difficult to understand and analyze. The equations may be simplified by selecting a specific value for ε, such as 1/2. In this case, the protein is considered regulated as long as its concentration is between (1-ε) = 1/2 and (1+ε) =3/2 times its initial value, allowing it to increase or decrease by fifty percent. Although this choice is somewhat arbitrary, the relationships thus derived hold for any ε between zero and one, although the exact degree of difference between the mechanisms may depend on ε. It highlights the dependence of the mechanisms on the values of the rate constants. The areas of the uncorrelated region for each mechanism when ε = 1/2 and when ε = 1/10 are listed in Figure 10, and may be obtained by substituting ε = 1/2 or ε = 1/10 into the area formulas from Figure 9.

The areas of RM1 through RM4 are all directly proportional to k_1 and k_2. This is consistent with the fact that core systems are better able to balance large rates of external synthesis or degradation if the core itself is able to synthesize or degrade P rapidly. In addition, all systems regulate best when P_0 is greatest. Thus, the higher the level at which the system is regulating, the more effective all the mechanisms are. This makes sense because a perturbment of a given size is relatively more significant the smaller the value of P_o; the gain or loss of fifty protein molecules is a much greater strain on a system trying to regulate at one hundred molecules than one regulating at one million molecules.

The mechanism that lacks negative feedback (RM1) can still maintain P within useful boundaries if the concentration of P and the rates of its synthesis and degradation within the core system are large relative to perturbments. The mechanism that includes basic negative feedback (RM2) yields a larger region of regulation for uncorrelated k_3 and k_4 than does RM1. The mechanism that includes cooperative-binding feedback (RM3) yields a region even larger than that with basic feedback. This explains why many transcription factors bind DNA in multiple copies. The mechanism that includes a pre-gene-binding dimerization of P (RM4) has the same area as does the two-step cooperative binding mechanism. It does

RM1 : Unregulated

$$-\frac{1}{2}\frac{\varepsilon^2\, k2\, k1}{-1+\varepsilon}$$

RM2 : Simple Negative Feedback

$$-\frac{1}{2}\frac{\varepsilon^2\,(-3+\varepsilon)\, k2\, k1\,(3+\varepsilon)}{(-2+\varepsilon)\,(-1+\varepsilon)\,(2+\varepsilon)}$$

RM3 : Cooperativity

$$-\frac{1}{2}\frac{\varepsilon^2\,(4+\varepsilon^2-3\,\varepsilon)\, k2\, k1\,(4+3\,\varepsilon+\varepsilon^2)}{(-2\,\varepsilon+\varepsilon^2+2)\,(-1+\varepsilon)\,(2+2\,\varepsilon+\varepsilon^2)}$$

RM4 : Dimerization

$$-\frac{1}{2}\frac{\varepsilon^2\,(4-3\,\varepsilon+\varepsilon^2)\, k2\, k1\,(4+3\,\varepsilon+\varepsilon^2)}{(2-2\,\varepsilon+\varepsilon^2)\,(-1+\varepsilon)\,(2+2\,\varepsilon+\varepsilon^2)}$$

RM5 : mRNA Cascade

$$-\frac{1}{2}\frac{\varepsilon^2\,(-3+\varepsilon)\, k2\, k15\, k13\,(3+\varepsilon)}{(-2+\varepsilon)\,(-1+\varepsilon)\, k14\,(2+\varepsilon)}$$

RM6 : mRNA Feedback

$$-\frac{1}{2}\frac{k15\, k13\,(5+\varepsilon^2-4\,\varepsilon)\,\varepsilon^2\, k2^2\,(k18+k17)\,(5+4\,\varepsilon+\varepsilon^2)\sqrt{2}}{(-2+\varepsilon^3+5\,\varepsilon-4\,\varepsilon^2)\,(2+\varepsilon)\sqrt{k2\, k16\, k18\, k15\, k13\,(k18+k17)}\,(1+\varepsilon)}$$

(with k14=0)

Figure 9. Area Equations for the Regulatory Mechanisms

not seem to matter in this analysis whether the two protein molecules bind the gene independently or dimerize and then bind the gene together.

The mechanism that distinguishes between transcription and translation (RM5) can have a larger regulated region than those that do not (RM2), depending on the rate of synthesis and degradation of M. If k_{15} in RM5 and k_1 in RM1 are considered to be the same, since they are both the rate of synthesis of P, RM5 will be more effective than RM1 if $k_{13}/k_{14} > 1$; i.e., if $k_{13} > k_{14}$. The mRNA molecules must not be degraded faster than they are synthesized, or else they will not last long enough to amplify the sensitivity of the system by catalyzing the production of more P. The equations for RM6 are more difficult to directly compare with the other mechanisms. However, when specific self-consistent values are substituted for the rate constants, the area for RM6 may be several times larger than that for RM5. Since RM6 adds the rate constant k_{18} which degrades M (when bound to P) in addition to the M degradation rate k_{14} from RM5, these mechanisms will have the same steady-state concentrations of all components when k_{14} plus k_{18} is a constant, and all other rate constants are the same. For example, for a system in which G=0.5, P=1000, M=2, and MP=2, the rate constants are k_2=0.01, k_{13}=4.0, k_{15}=5.0, k_{16}=0.002, k_{17}=1.0, and $k_{14} + k_{18} = 1.0$. If $k_{14} = 1$ and $k_{18} = 0$, then the situation is exactly the same as in RM5 (since RM5 has no k_{18}), and the area of the rectangle with ε=1/10 is 0.002503. If $k_{18} = 1$ and $k_{14} = 0$, the area is 0.007015, about 2.8 times as large. Therefore, regulation is clearly improved when M can be bound by P and then degraded in this form. Together RM5 and RM6 indicate two important roles of mRNA in regulation. It can amplify sensitivity by forming a cascade (RM5), and can provide a second source of negative feedback control (RM6), with P binding to its mRNA in addition to its gene.

Mechanism	Area with ε = 1/2	Area with ε = 1/10
RM1	$0.25k_1k_2$	$0.00556k_1k_2$
RM2	$0.583k_1k_2$	$0.0504k_1k_2$
RM3	$0.973k_1k_2$	$0.0222k_1k_2$
RM4	$0.973k_1k_2$	$0.0222k_1k_2$
RM5	$0.583\frac{k_{15}k_2k_{13}}{k_{14}}$	$0.0504\frac{k_{15}k_2k_{13}}{k_{14}}$
RM6	$2.09\frac{k_{15}k_{13}k_2^{\,2}(k_{18}+k_{17})\sqrt{2}}{\sqrt{k_2k_{16}k_{18}k_{15}k_{13}(k_{18}+k_{17})}}$	$0.0351\frac{k_{15}k_{13}k_2^{\,2}(k_{18}+k_{17})\sqrt{2}}{\sqrt{k_2k_{16}k_{18}k_{15}k_{13}(k_{18}+k_{17})}}$

Figure 10. The Area of the Uncorrelated Rectangle with ε = 1/2 and 1/10.

When $\varepsilon = 1/2$, the area of the region of regulation with uncorrelated k_3 and k_4 for RM2 is about 2.333 ((7/12)/(1/4)) times larger than that for RM1, and the area for RM3 and RM4 is 3.892 ((253/260)/(1/4)) times larger than that for RM1. When $\varepsilon = 1/10$, these factors are 2.253 and 3.997. These factors may be plotted as a function of ε, for ε between zero and one, as is shown in Figure 11.

As the system becomes more tightly regulated (i.e., the limit as ε approaches zero), the advantage of RM2 over RM1 (i.e., the factor by which the area of RM2 is greater than that of RM1) decreases towards a lower limit of 9/4. The advantage of RM3 and RM4 increases as ε approaches zero towards an upper limit of 4. Of course all areas are decreasing as ε approaches zero; if absolutely no variation in P is allowed, absolutely no perturbations can be tolerated by any system.

As the system becomes less tightly regulated (i.e., the limit as ε approaches one), the advantage of RM2 increases towards 8/3, while the advantage of RM3 and RM4 decreases to 16/5. A value of ε greater than one has meaning for the upper bound (protein concentration is more than double the initial value), but it does not have meaning for the lower bound (yielding a negative concentration).

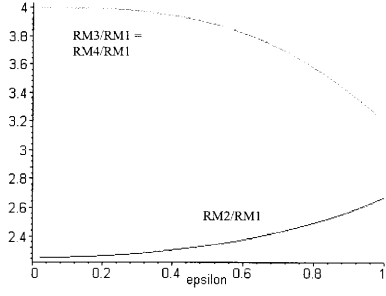


Figure 11. Ratios of the Area of the Region of Regulation with Uncorrelated k_3 and k_4 as a Function of ε.

CHAPTER IV

DATA FITTING

A number of problems in the Biocomplexity Project involve searching for a large number of values (usually rate constants) which give the best fit to real or theoretical data, such as constant concentration functions for regulatory systems or a prescribed reality-based "choreography" for cell cycle reactions. The more equations, the more unknowns, and the larger search space, the longer and harder such searches are. Ideally, searches could be conducted with mathematical analysis to yield quick, exact solutions. In some cases, such as minimizing algebraic equations, this can be done relatively easily with gradients or LaGrange multipliers. However, most of this fitting involves solving the "inverse problem"; finding rate constants that minimize the differences between data and the differential equation of a model. Clearly, this cannot be expressed in a closed algebraic form.

DESCRIPTION OF SIMULATED ANNEALING

Searching Strategy. In many cases, the easiest or only way to find parameter values to minimize a complex function is to use computer searching algorithms. One of the most effective of these algorithms for data fitting problems is simulated annealing. This method is similar to "hill-climbing" or "gradient-descent" search algorithms, which evaluate a cost function with some set of parameter values, re-evaluate it with changes in different directions for each of the parameters, and "move" in the direction of greatest decrease in cost function value. The drawback of such methods is that they tend towards local minima. To avoid this, simulated annealing allows movements to higher cost-function values, jumping around to find better global minima, with a probability that decreases with time, analogous to allowing hot metal to cool slowly so it is malleable (hence its name). The Adaptive Simulated Annealing (ASA) algorithm developed by Lester Ingber has been used for our data fitting. ASA calls a user-defined cost function, passing it an array of parameters. The cost function then returns some value indicative of the "goodness" of these parameter values, the lower the value the better. ASA then updates the parameter values based on this returned cost, and calls the cost function again. This process repeats until no further improvement can be found, or some maximum number of iterations is reached.

The figures and parameter values included in this chapter have been reprinted with permission from "Kinetic Mechanism of Acetyl-CoA Synthase: Steady-State Synthesis at Variable CO/CO2 Pressures", by Ernie Maynard, Christopher Sewell, and Paul A. Lindahl, submitted to the *Journal of the American Chemical Society* for publication in 2001.

Cost Functions. The cost function may evaluate a cost in any way that can be programmed. For regulatory systems, the cost function reads in a series of differential equations and uses the parameters as rate constants to produce a fourth-order Runge-Kutta solution, and evaluates the constancy of the generated concentration function for the protein. For fitting experimental or theoretical choreography data, the cost function compares the concentrations simulated by Runge-Kutta using input ODEs and the parameters as rate constants to the input data at each time point and returns the sum of the squares of the residuals between the two.

Limitations of the Algorithm. Simulated annealing is useful for continuous problems, in which an almost-ideal set of parameters returns an almost-ideal cost. This property holds well for data fitting problems in most cases, since slightly sub-optimal rate constants will produce a simulation slightly off from the data. However, certain "illegal" conditions sometimes present a problem. For example, no valid simulation may allow the concentration of any component to ever be less than zero, even if overall the fit is close to the data. By returning an exceptionally high cost value any time this happens, such simulations may certainly be excluded from consideration. However, returning exactly the same value each time this happens may cause ASA to be unable to choose a direction in which to move, and therefore prematurely terminate, thinking no further improvement is possible. In addition, a high cost value may make ASA jump away, significantly changing its parameter values, even though the previous parameter values may have actually been very close to a good, valid fit if the error was just caused by one low-concentration component briefly dipping slightly below zero. Often it has been best to disregard such illegal conditions and to return the normal cost function, and hope the eventual best fit is legal.

FITTING ACETYL CO-A SYNTHASE EXPERIMENTAL DATA

Simulated annealing has not yet been used extensively to attempt to find rate constants for MC3 (see Chapter One) because additional analysis is first needed, and the division of reactions into modules must be completed in order to start searching for solutions for these modules. However, the program has been used to fit data from two sets of experiments involving the enzyme acetyl-CoA synthase, which is used by certain chemoautotrophic arachae, similar to *Micoplasma genitalium* (the model for MC3), to convert inorganic raw materials into acetyl-CoA, which serves as the cell's source of energy.

$$CO_2 + H_2 \overset{ACS}{\longleftrightarrow} CH_3 - \overset{O}{\overset{||}{C}} - CoA \qquad (4\text{-}1)$$

The [CO] and [CO_2] Dependence of Enzyme Activity. One experiment, performed by Ernie Maynard, studied the dependence of acetyl-CoA synthase's activity on the partial pressures of carbon monoxide and carbon dioxide. When [CO] was varied from 0 to 100 μM in a balance of argon, rates increased sharply from 0.3 to 100 min^{-1}. At [CO] greater than 100 μM, rates declined sharply and eventually stabilized at 10 min^{-1} at 980 μM CO. These results are plotted in Figure 11. However, rates increased as [CO_2] increased for the entire range of [CO_2], coalescing towards a final rate of about 150 min^{-1}, as shown in Figure 12.

Eleven different mechanisms were attempted to fit all experimental data using the simulated annealing program. The simplest model which fit the data well was a mechanism called U4AT (for Uncompetitive, 4 CO bindings, Activation, Two Terms), given by

$$\frac{v}{[E_{total}]} = \frac{\left(\frac{k_{cat}}{K_m}\right)_{CO}[CO] + \left(\frac{k_{cat}}{K_m}\right)_{CO_2}[CO_2]}{T\frac{[CO]}{K_{mCO}} + T\frac{[CO_2]}{K_{mCO_2}} + A} + \frac{k_{res}[CO]}{K_{mres} + [CO]} \qquad (4\text{-}2)$$

where

$$T = 1 + \sum_{j=1}^{n}\left(\frac{[CO]^j}{\prod_{k=1}^{j} K_{ik}}\right) \qquad (4\text{-}3)$$

and

$$A = 1 + \frac{K_{act}\left(k_{a1} + k_{a3}\right)}{k_{a1}[CO] + k_{a3}[CO_2]} \qquad (4\text{-}4)$$

Data sets consisted of $v/[E_{total}]$ rates for varying [CO] and [CO_2], and all other terms in equations (4-2), (4-3), and (4-4) were parameters, and simulated annealing searched for values of these parameters within given reasonable ranges which best fit all the experimental data. These fits are reflected by the closeness of the simulation lines to the data points in Figures 11 and 12.

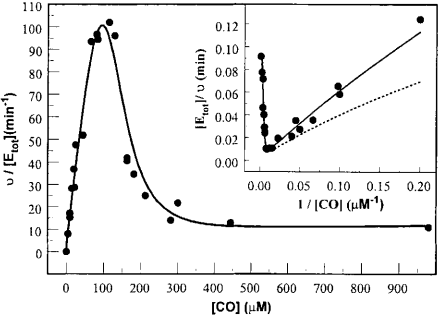


Figure 12. [CO] Dependence of Acetyl CoA Synthase Activity.

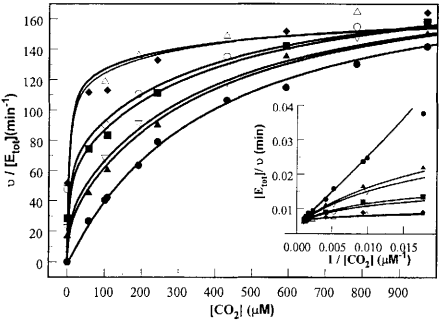


Figure 13. [CO_2] Dependence of Acetyl CoA Synthase Activity.

The uncertainty ranges for these parameters were also determined using simulated annealing. Separately for each parameter, all other parameters were fixed at their best-fit values, and the program searched for the value of the test parameter which would return an error (sum of the squares of the residuals between the model evaluated with those parameters and the data) as close as possible to one-and-a-half times the best-fit error. The difference between this value and the best-fit value was taken as the uncertainty in that parameter.

These best-fit values and their uncertainties for U4AT were $k_{cat,CO} = 900 \pm 300\ min^{-1}$, $K_{m,CO} = 300 \pm 100\ \mu M$, $(k_{cat}/K_m)_{CO} = 3.2 \pm 0.4\ \mu M^{-1}min^{-1}$, $k_{cat,CO2} = 200 \pm 30\ min^{-1}$, $K_{m,CO2} = 380 \pm 40\ \mu M$, $(k_{cat}/K_m)_{CO2} = 0.52 \pm 0.04\ \mu M^{-1}min^{-1}$, $K_{act} = 6 \pm 3\ \mu M$, $k_{a1} = 10 \pm 8\ \mu M^{-1}min^{-1}$, $k_{a3} = 6000 \pm 3000\ \mu M^{-1}min^{-1}$, $k_{res} = 10 \pm 5\ min^{-1}$, and $K_{mres} = 200 \pm 100\ \mu M$. The best-fit inhibition constants were $K_{i1} = 900 \pm 300\ \mu M$, $K_{i2} = 50 \pm 10\ \mu M$, $K_{i3} = 40 \pm 10\ \mu M$, and $K_{i4} = 50 \pm 30\ \mu M$.

In U4AT, the first term of (4-2) represents the major activity and approaches zero as [CO] approaches infinity. The second term of (4-2) represents the residual activity and accounts for the stabilization of the enzyme activity at large [CO] values in Figure 11. Most previous experiments involving acetyl-CoA synthase have been unknowingly performed at partial pressures higher than those at which the major activity is evident. The fact that the last three inhibition constants (K_{i2}, K_{i3}, and K_{i4}) are small relative to K_{i1} indicates a positive-cooperative binding and inhibition of the enzyme by CO. The value of (k_{cat}/K_m) for CO is six times greater than that for CO_2, meaning that CO is a better substrate than CO_2.

Acetyl CoA synthase is known to have several active sites, as shown in Figure 13. At the site called the A-cluster, substrate CO binds the enzyme, activating it for the production of acetyl CoA. At the site called the C-cluster, CO_2 is reduced to CO in a redox reaction, and this CO can then bind to the A-cluster. The CO produced at the C-cluster migrates to the A-cluster through a protein-encapsulated molecular tunnel. CO also acts an inhibitor as well as substrate, binding cooperatively, probably also at the A-cluster. However, these experiments have shown that CO_2 acts only as a substrate, even though it is converted to CO, which can be an inhibitor. It therefore seems that CO which reaches the A-cluster through the molecular tunnel, which would be all of the CO when only CO_2 is present in solution and some of the CO when CO is present, serves as substrate, while CO which reaches the A-cluster directly from solution acts as an inhibitor. Although molecular tunnels have been discovered in other enzymes, this may be the first enzyme known to use such a tunnel to discriminate between two identical molecules with diametrically opposed effects.

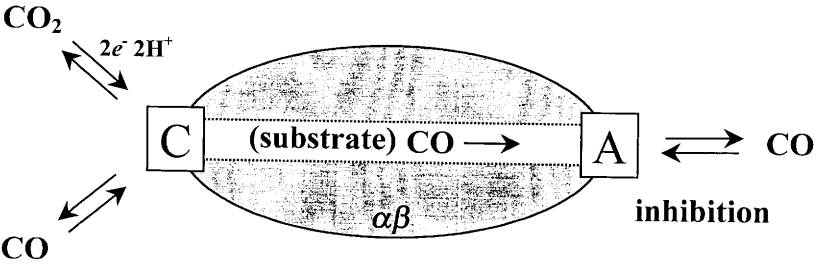


Figure 14. Diagram of Acetyl CoA Synthase Activity.

The [CO] Dependence of Aceytl CoA Synthase Methylation. A second set of experiments, being performed by Dr. Tan, is attempting to determine the strength of the methyl bond to acetyl CoA synthase by studying the reaction

$$ACS + CH_3 - CP \xleftrightarrow{k_1,k_2} Co^{1+} + CH_3 - ACS \tag{4-5}$$

in which the enzyme is methylated by a corn protein. Data sets at different initial concentrations and different temperatures have been collected for both the forward and reverse reactions. By determining the temperature dependence of the equilibrium constant for this reaction, the bond strength may be found by relating the equations

$$-RT\ln(K_{eq}) = \Delta G = \Delta H - T\Delta S \tag{4-6}$$

In order to also determine the [CO] dependence of the reaction, data sets were collected at different [CO] partial pressures. CO can bind the reduced ACS

$$ACS + CO \xleftrightarrow{k_3,k_4} ACS - CO \tag{4-7}$$

Simulated annealing has been used to search for rate constants k_1, k_2, k_3, k_4. The cost function employs a fourth-order Runge-Kutta algorithm to calculate the concentrations at the experimental time points using the ODEs corresponding to (4-5) and (4-7). However, a number of complications have made it very difficult to simultaneously fit all data sets to the same set of rate constants.

One problem is that the data sets are for absorbances (using a stopped flow meter), and the ODE solutions produce concentration values. These concentrations must therefore be converted to equivalent absorbance values in order to compare to the data. This is made more difficult because the absorbances depend upon the reductant used (titanium), which is not included in the basic reactions being studied. Extinction coefficients were experimentally determined for ACS and Co^{1+}, which could then be used to calculate an extinction coefficients for CH_3-CP and CH_3-ACS according to the equation

$$\varepsilon_{CH_3} = \frac{Abs_0 - \varepsilon_{ACS}[ACS_0] - \varepsilon_{Co^+}[Co^{1+}{}_0]}{[CH_3CP]} \tag{4-8}$$

where Abs_0 is the initial absorbance in the data set and the ε terms are the extinction coefficients. Simulated concentrations could then be converted to equivalent absorbances by

$$I_{sim} = \varepsilon_{ACS}[ACS] + \varepsilon_{CH3}[CH_3CP] + \varepsilon_{Co}[Co^{1+}] + \varepsilon_{CH3}[CH_3ACS] \qquad (4\text{-}9)$$

Although data sets for a constant [CO] can all be fit very well, sets at different [CO] cannot be fit to the same parameters. Additional processes, not accounted for in the basic model, are apparently taking place. Several of the possibilities that have been considered include the cooperative binding of two (or more) molecules of CO to ACS, adding the reaction

$$ACS - CO + CO \xleftrightarrow{k_3,k_4} ACS - (CO)_2 \qquad (4\text{-}10)$$

to the model; the equilibriation of ACS with ACS-CO and ACS-$(CO)_2$ in the syringe before being mixed with CH_3-CP; additional processes involving the titanium reductant, adding the reaction

$$ACS_{oxidized} + Ti \xleftrightarrow{k_3,k_4} ACS \qquad (4\text{-}11)$$

to the model; and the further reaction of CH_3-ACS with CO,

$$CH_3\text{-}ACS + CO \longleftrightarrow CH_3 - \overset{\overset{O}{\|}}{C} - ACS \qquad (4\text{-}12)$$

However, none of these modifications have yet been able to satisfactorily fit all data sets simultaneously. Additional experiments designed to eliminate additional complicating processes will be undertaken in the near future, and the new data will be fit.

CHAPTER V

CONCLUSIONS

Work with the Biocomplexity Project has taken a number of new and unexpected directions over the last year, as may be expected for research in a new area.

Substantial progress has been made towards the goal of simulating a mechanical cell. A system of over 17,000 reactions has been written which models the important metabolic and cell-cycle processes in the simplest prokaryotes. A versatile computer program has been created to which cellular mechanisms can be input, analyzed, converted to differential equations, and simulated according to the stochastic model. A 3D computer animation has been produced to visualize the basic cell-cycle reactions. However, a significant amount of analysis must be done in order to find rate constants for the reactions to produce a numerical simulation with realistic results.

One of the most important aspects of this analysis is to determine the best methods of regulation, since the reactions may be divided into many small modules which may be solved independently and then linked together, if each is self-regulating. A study of several common regulatory mechanisms has therefore been completed. Quantitative analytical results indicate that the most effective regulation can take place with high protein concentrations, rapid rates of synthesis and degradation, a cascade which incorporates the separate processes of transcription and translation, negative feedback control of the gene-catalyzed synthesis of mRNA, oligomerizaion of the protein, cooperative binding of multiple copies of the protein to its gene, and negative feedback control of the degradation of mRNA. These results may be useful in a number of contexts because many experiments and theories in biochemistry involve regulated systems.

The development of the mechanical cell also requires solving the "inverse problem", determining rate constants which generate behavior in the time-dependent concentrations of cellular components that is consistent with real cells. The Adaptive Simulated Annealing algorithm has been found to be very effective in searching for values for a number of wide-ranging parameters in a short amount of time. It provides the necessary flexibility by allowing the function to be minimized to be programmed in C, and several such cost functions have been developed including fourth-order Runge-Kutta solvers used to fit data to ODEs. This flexible function-minimization program has also been found to have wide-ranging applications for data fitting. It has been used to fit experimental data describing the [CO] and [CO_2] dependence of acetyl CoA synthase activity, leading to some interesting conclusions about the ability of

this enzyme to use its molecular tunnel to discriminate among [CO] molecules based on their source. It has also been used to help identify problems with the mechanisms being used to model an experimental study of the [CO] dependence of acetyl CoA synthase methylation.

In the future, it is hoped that a fully functional mechanical cell model will be completed and simulated numerically and visually. Such a model would be useful in the study of numerous cellular processes, and to aid in understanding the emergent properties arising from the vast array of genomic, proteomic, and other data that are rapidly being discovered. Meanwhile, it is hoped that a number of the auxiliary studies and developments will be useful in other contexts, as have been the regulatory mechanism analysis and data fitting algorithms.

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APPENDIX B : DERIVATIONS OF REGULATORY EQUATIONS

G vs. Log P Plots

```
> restart;
> with(plots):
```

RM2

```
> rm201:=K1=(-g+1)/(p*g);
```

$$rm201 := K1 = \frac{-g + 1}{p\,g}$$

```
> rm202:=g=solve(rm201,g);
```

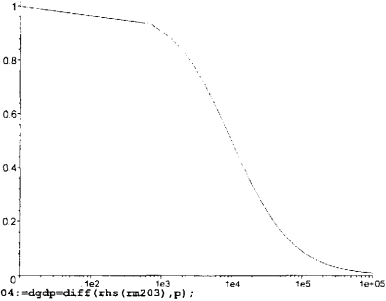
$$rm202 := g = \frac{1}{K1\,p + 1}$$

Numerical Example

```
> rm203:=subs({K1=1/1000},rm202);
```

$$rm203 := g = \frac{1}{\frac{1}{1000}\,p + 1}$$

```
> semilogplot(rhs(rm203),p=1..100000);
```




```
> rm204:=dgdp=diff(rhs(rm203),p);
```

$$rm204 := dgdp = -\frac{1}{1000}\frac{1}{\left(\frac{1}{1000}p + 1\right)^2}$$

```
> rm205:=subs(p=1000,rm204);
```

$$rm205 := dgdp = \frac{-1}{4000}$$

Analytical Solution

```
> rm206:=dgdp=diff(rhs(rm202),p);
```

$$rm206 := dgdp = -\frac{K1}{(K1\,p - 1)^2}$$

```
> rm207:=subs({g=1/2,p=p0},rm201);
```

$$rm207 := K1 = \frac{1}{p0}$$

```
> rm208:=subs(rm207,rm206);
```

$$rm208 := dgdp = -\frac{1}{\left(\frac{p}{p0} + 1\right)^2 p0}$$

```
> rm209:=subs(p0=p,rm208);
```

$$rm209 := dgdp = -\frac{1}{4}\frac{1}{p}$$

RM3

```
> rm301:=g+K1*p*g+K2*K1*p^2*g=1;
```

$$rm301 := g + K1\,p\,g + K2\,K1\,p^2\,g = 1$$

```
> rm302:=g=solve(rm301,g);
```

$$rm302 := g = \frac{1}{1 + K1\,p + K2\,K1\,p^2}$$

Numerical Example

```
> rm303:=subs({g=1/2,p=1000},rm302);
```

$$rm303 := \frac{1}{2} = \frac{1}{1 + 1000\,K1 + 1000000\,K2\,K1}$$

```
> rm304:=K1=solve(rm303,K1);
```

$$rm304 := K1 = \frac{1}{1000}\frac{1}{1 + 1000\,K2}$$

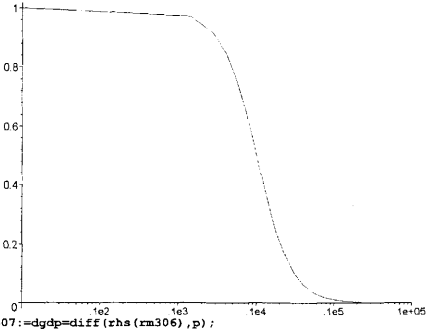
```
> rm305:=subs(rm304,rm302);
```

$$rm305 := g = \frac{1}{1 + \frac{1}{1000}\frac{p}{1 + 1000\,K2} + \frac{1}{1000}\frac{K2\,p^2}{1 + 1000\,K2}}$$

```
> rm306:=subs(K2=1/100,rm305);
```

$$rm306 := g = \frac{1}{1 + \frac{1}{11000}p + \frac{1}{1100000}p^2}$$

```
> semilogplot(rhs(rm306),p=1..100000);
```




```
> rm307:=dgdp=diff(rhs(rm306),p);
```

$$rm307 := dgdp = -\frac{\frac{1}{11000} + \frac{1}{550000}p}{\left(1 + \frac{1}{11000}p + \frac{1}{1100000}p^2\right)^2}$$

```
> rm308:=subs(p=1000.,rm307);
```

$$rm308 := dgdp = -.0004772727273$$

Analytical Solution

```
> rm309:=diff(rhs(rm302),p);
```

$$rm309 := -\frac{K1 + 2\,K2\,K1\,p}{(1 + K1\,p + K2\,K1\,p^2)^2}$$

```
> rm310:=subs({g=1/2,p=p0},rm302);
```

$$rm310 := \frac{1}{2} = \frac{1}{1 + K1\,p0 + K2\,K1\,p0^2}$$

```
> rm311:=K1=solve(rm310,K1);
```

$$rm311 := K1 = \frac{1}{p0\,(1 + K2\,p0)}$$

```
> rm312:=subs(rm311,rm309);
```

$$rm312 := -\frac{\dfrac{1}{p0\,(1 + K2\,p0)} + 2\,\dfrac{K2\,p}{p0\,(1 + K2\,p0)}}{\left(1 + \dfrac{p}{p0\,(1 + K2\,p0)} + \dfrac{K2\,p^2}{p0\,(1 + K2\,p0)}\right)^2}$$

```
> rm313:=subs(p0=p,rm312);
```

$$rm313 := -\frac{\dfrac{1}{p\,(1 + K2\,p)} + 2\,\dfrac{K2}{1 + K2\,p}}{\left(1 + \dfrac{1}{1 + K2\,p} + \dfrac{K2\,p}{1 + K2\,p}\right)^2}$$

```
> rm314:=limit(rm313,K2=infinity);
```

$$rm314 := -\frac{1}{2}\,\frac{1}{p}$$

RM4

```
> rm401:=g+K4*g*p^2/K3 = 1;
```

$$rm401 := g + \frac{K4\,g\,p^2}{K3} = 1$$

```
> rm402:=g=solve(rm401,g);
```

$$rm402 := g = \frac{K3}{K3 + K4\,p^2}$$

Numerical Example

```
> rm403:=subs({g=1/2,p=1000},rm402);
```

$$rm403 := \frac{1}{2} = \frac{K3}{K3 + 1000000\,K4}$$

```
> rm404:=K3=solve(rm403,K3);
```

$$rm404 := K3 = 1000000\,K4$$

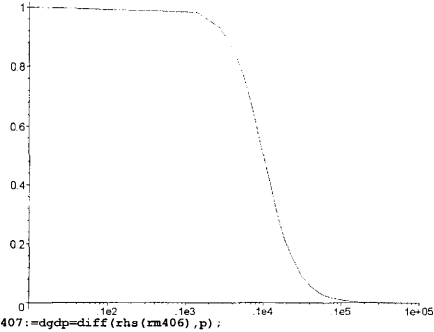
```
> rm405:=subs(rm404,rm402);
```

$$rm405 := g = 1000000\,\frac{K4}{1000000\,K4 + K4\,p^2}$$

```
> rm406:=subs(K4=1/100,rm405);
```

$$rm406 := g = 10000 \frac{1}{10000 + \frac{1}{100} p^2}$$

```
> semilogplot(rhs(rm406),p=1..100000);
```




```
> rm407:=dgdp=diff(rhs(rm406),p);
```

$$rm407 := dgdp = -200 \frac{p}{\left(10000 + \frac{1}{100} p^2\right)^2}$$

```
> rm408:=subs(p=1000.,rm407);
```

$$rm408 := dgdp = -.0005000000000$$

Analytical Solution

```
> rm409:=diff(rhs(rm402),p);
```

$$rm409 := -2 \frac{K3\ K4\ p}{(K3 + K4\ p^2)^2}$$

```
> rm410:=subs({g=1/2,p=p0},rm402);
```

$$rm410 := \frac{1}{2} = \frac{K3}{K3 + K4\ p0^2}$$

```
> rm411:=K3=solve(rm410,K3);
```

$$rm411 := K3 = K4\ p0^2$$

```
> rm412:=subs(rm411,rm409);
```

$$rm412 := -2\frac{K4^2\,p0^2\,p}{\left(K4\,p0^2 + K4\,p^2\right)^2}$$

```
> rm413:=subs(p0=p,rm412);
```

$$rm413 := -\frac{1}{2}\frac{1}{p}$$

Regulatory Mechanism 1

```
> restart;
```

Steady State and Conservation Equations

```
> rm101:=p=(k1*g+k3)/(k2+k4);
```

$$rm101 := p = \frac{k1\,g + k3}{k2 + k4}$$

```
> rm102:=subs({k3=0,k4=0,g=1/2},rm101);
```

$$rm102 := p = \frac{\frac{1}{2}\,k1}{k2}$$

```
> rm103:=pmid=solve(rm102,p);
```

$$rm103 := pmid = \frac{\frac{1}{2}\,k1}{k2}$$

Gmax at Pmin

```
> rm104:=pmin=(1-epsilon)*pmid;
```

$$rm104 := pmin = (1 - \varepsilon)\,pmid$$

```
> rm105:=subs(rm103,rm104);
```

$$rm105 := pmin = \frac{\frac{1}{2}\,(1 - \varepsilon)\,k1}{k2}$$

```
> rm106:=subs(p=rhs(rm105),rm101);
```

$$rm106 := \frac{\frac{1}{2}\,(1 - \varepsilon)\,k1}{k2} = \frac{k1\,g + k3}{k2 + k4}$$

```
> rm107:=subs(g=1/2,rm106);
```

$$rm107 := \frac{\frac{1}{2}\,(1 - \varepsilon)\,k1}{k2} = \frac{\frac{1}{2}\,k1 + k3}{k2 + k4}$$

Gmin at Pmax

```
> rm108:=pmax=(1+epsilon)*pmid;
```

$$rm108 := pmax = (1 + \varepsilon)\,pmid$$

```
> rm109:=subs(rm103,rm108);
```

$$rm109 := pmax = \frac{\frac{1}{2}\,(1 + \varepsilon)\,k1}{k2}$$

```
> rm110:=subs(p=rhs(rm109),rm101);
```

$$rm110 := \frac{\frac{1}{2}\,(1 + \varepsilon)\,k1}{k2} = \frac{k1\,g + k3}{k2 + k4}$$

```
> rm111:=subs(g=1/2,rm110);
```

$$rm111 := \frac{1}{2}\frac{(1+\varepsilon)\,k1}{k2} = \frac{\frac{1}{2}k1 + k3}{k2 + k4}$$

Line Equations

Pmin/Gmax Equation

```
> rm112:=k3=solve(rm107,k3);
```

$$rm112 := k3 = -\frac{1}{2}\frac{k1\,(-k4 + \varepsilon\,k2 + \varepsilon\,k4)}{k2}$$

Pmax/Gmin Equation

```
> rm113:=k3=solve(rm111,k3);
```

$$rm113 := k3 = \frac{1}{2}\frac{k1\,(k4 + \varepsilon\,k2 + \varepsilon\,k4)}{k2}$$

Area of Rectangle

```
> rm114:=subs({g=1/2,k3=0,k4=0},rm101);
```

$$rm114 := p = \frac{1}{2}\frac{k1}{k2}$$

Find k4 intercept when k3=0

```
> rm115:=subs(pmid=rhs(rm114),rm112);
```

$$rm115 := k3 = -\frac{1}{2}\frac{k1\,(-k4 + \varepsilon\,k2 + \varepsilon\,k4)}{k2}$$

```
> rm116:=subs(k3=0,rm115);
```

$$rm116 := 0 = -\frac{1}{2}\frac{k1\,(-k4 + \varepsilon\,k2 + \varepsilon\,k4)}{k2}$$

```
> rm117:=k4=solve(rm116,k4);
```

$$rm117 := k4 = -\frac{\varepsilon\,k2}{-1 + \varepsilon}$$

Find k3 intercept when k4=0

```
> rm118:=subs(pmid=rhs(rm114),rm113);
```

$$rm118 := k3 = \frac{1}{2}\frac{k1\,(k4 + \varepsilon\,k2 + \varepsilon\,k4)}{k2}$$

```
> rm119:=subs(k4=0,rm118);
```

$$rm119 := k3 = \frac{1}{2}k1\,\varepsilon$$

Area equation

```
> rm120:=area=(rhs(rm117))*(rhs(rm119));
```

$$rm120 := area = -\frac{1}{2}\frac{\varepsilon^2\, k2\, k1}{-1+\varepsilon}$$

```
> rm121:=subs(epsilon=1/2,rm120);
```

$$rm121 := area = \frac{1}{4}\, k1\, k2$$

```
> rm122:=subs(epsilon=1/10,rm120);
```

$$rm122 := area = \frac{1}{180}\, k1\, k2$$

Regulatory Mechanism 2

```
> restart;
```

Steady State and Conservation Equations

```
> rm201:=p=(k1*g+k3)/(k2+k4);
```

$$rm201 := p = \frac{k1\,g + k3}{k2 + k4}$$

```
> rm202:=K1=gp/(p*g);
```

$$rm202 := K1 = \frac{gp}{p\,g}$$

```
> rm203:=g+gp=1;
```

$$rm203 := g + gp = 1$$

```
> rm204:=gp=solve(rm203,gp);
```

$$rm204 := gp = -g + 1$$

```
> rm205:=subs(rm204,rm202);
```

$$rm205 := K1 = \frac{-g + 1}{p\,g}$$

```
> rm206:=subs(g=1/2,rm205);
```

$$rm206 := K1 = \frac{1}{p}$$

```
> rm207:=pmid=solve(rm206,p);
```

$$rm207 := pmid = \frac{1}{K1}$$

Gmax at Pmin

```
> rm208:=pmin=(1-epsilon)*pmid;
```

$$rm208 := pmin = (1 - \varepsilon)\,pmid$$

```
> rm209:=subs(rm207,rm208);
```

$$rm209 := pmin = \frac{1 - \varepsilon}{K1}$$

```
> rm210:=subs(p=rhs(rm209),rm205);
```

$$rm210 := K1 = \frac{(-g + 1)\,K1}{(1 - \varepsilon)\,g}$$

```
> rm211:=gmax=solve(rm210,g);
```

$$rm211 := gmax = -\frac{1}{-2 + \varepsilon}$$

Gmin at Pmax

```
> rm212:=pmax=(1+epsilon)*pmid;
```

$$rm212 := pmax = (1 + \varepsilon)\, pmid$$

```
> rm213:=subs(rm207,rm212);
```

$$rm213 := pmax = \frac{1 + \varepsilon}{K1}$$

```
> rm214:=subs(p=rhs(rm213),rm205);
```

$$rm214 := K1 = \frac{(-g + 1)\, K1}{(1 + \varepsilon)\, g}$$

```
> rm215:=gmin=solve(rm214,g);
```

$$rm215 := gmin = \frac{1}{2 - \varepsilon}$$

Line Equations

Pmin/Gmax Equation

```
> rm216:=subs({p=rhs(rm208),g=rhs(rm211)},rm201);
```

$$rm216 := (1 - \varepsilon)\, pmid = \frac{-\frac{k1}{-2 + \varepsilon} + k3}{k2 + k4}$$

```
> rm217:=k3=solve(rm216,k3);
```

$rm217 :=$

$$k3 = -\frac{2\, pmid\, k2 + 2\, pmid\, k4 - 3\, pmid\, \varepsilon\, k2 - 3\, pmid\, \varepsilon\, k4 + pmid\, \varepsilon^2\, k2 + pmid\, \varepsilon^2\, k4 - k1}{-2 + \varepsilon}$$

Pmax/Gmin Equation

```
> rm218:=subs({p=rhs(rm212),g=rhs(rm215)},rm201);
```

$$rm218 := (1 + \varepsilon)\, pmid = \frac{\frac{k1}{2 + \varepsilon} + k3}{k2 + k4}$$

```
> rm219:=k3=solve(rm218,k3);
```

$rm219 :=$

$$k3 = \frac{2\, pmid\, k2 + 2\, pmid\, k4 + 3\, pmid\, \varepsilon\, k2 + 3\, pmid\, \varepsilon\, k4 + pmid\, \varepsilon^2\, k2 + pmid\, \varepsilon^2\, k4 - k1}{2 + \varepsilon}$$

Area of Rectangle

```
> rm220:=subs({g=1/2,k3=0,k4=0},rm201);
```

$$rm220 := p = \frac{1}{2}\frac{k1}{k2}$$

Find k4 intercept when k3=0

```
> rm221:=subs(pmid=rhs(rm220),rm217);
```

$$rm221 := k3 = -\frac{\frac{k1\,k4}{k2} - \frac{3}{2}k1\,\varepsilon - \frac{3}{2}\frac{k1\,\varepsilon\,k4}{k2} - \frac{1}{2}k1\,\varepsilon^2 + \frac{\frac{1}{2}k1\,\varepsilon^2\,k4}{k2}}{-2+\varepsilon}$$

```
> rm222:=subs(k3=0,rm221);
```

$$rm222 := 0 = -\frac{\frac{k1\,k4}{k2} - \frac{3}{2}k1\,\varepsilon - \frac{3}{2}\frac{k1\,\varepsilon\,k4}{k2} + \frac{1}{2}k1\,\varepsilon^2 + \frac{\frac{1}{2}k1\,\varepsilon^2\,k4}{k2}}{-2-\varepsilon}$$

```
> rm223:=k4=solve(rm222,k4);
```

$$rm223 := k4 = -\frac{\varepsilon\,(-3+\varepsilon)\,k2}{(-2+\varepsilon)\,(-1+\varepsilon)}$$

Find k3 intercept when k4=0

```
> rm224:=subs(pmid=rhs(rm220),rm219);
```

$$rm224 := k3 = -\frac{\frac{k1\,k4}{k2} + \frac{3}{2}k1\,\varepsilon + \frac{\frac{3}{2}k1\,\varepsilon\,k4}{k2} + \frac{1}{2}k1\,\varepsilon^2 + \frac{\frac{1}{2}k1\,\varepsilon^2\,k4}{k2}}{2+\varepsilon}$$

```
> rm225:=subs(k4=0,rm224);
```

$$rm225 := k3 = \frac{\frac{3}{2}k1\,\varepsilon + \frac{1}{2}k1\,\varepsilon^2}{2+\varepsilon}$$

Area equation

```
> rm226:=area=simplify((rhs(rm223))*(rhs(rm225)));
```

$$rm226 := area = -\frac{1}{2}\frac{\varepsilon^2\,(-3+\varepsilon)\,k2\,k1\,(3+\varepsilon)}{(-2+\varepsilon)\,(-1+\varepsilon)\,(2+\varepsilon)}$$

```
> rm227:=subs(epsilon=1/2,rm226);
```

$$rm227 := area = \frac{7}{12}k2\,k1$$

```
> rm228:=subs(epsilon=1/10,rm226);
```

$$rm228 := area = \frac{899}{71820}k2\,k1$$

Regulatory Mechanism 3

```
> restart;
```

Steady State and Conservation Equations

```
> rm301:=p=(k1*g+k3)/(k2+k4);
```

$$rm301 := p = \frac{k1\ g + k3}{k2 + k4}$$

```
> rm302:=K1=gp/(p*g);
```

$$rm302 := K1 = \frac{gp}{p\ g}$$

```
> rm303:=K2=gpp/(gp*p);
```

$$rm303 := K2 = \frac{gpp}{gp\ p}$$

```
> rm304:=g+gp+gpp=1;
```

$$rm304 := g + gp + gpp = 1$$

```
> rm305:=gpp=solve(rm303,gpp);
```

$$rm305 := gpp = K2\ gp\ p$$

```
> rm306:=subs(rm305,rm304);
```

$$rm306 := g + gp + K2\ gp\ p = 1$$

```
> rm307:=gp=solve(rm302,gp);
```

$$rm307 := gp = K1\ p\ g$$

```
> rm308:=subs(rm307,rm306);
```

$$rm308 := g + K1\ p\ g + K2\ K1\ p^2\ g = 1$$

```
> rm309:=subs(g=1/2,rm308);
```

$$rm309 := \frac{1}{2} + \frac{1}{2} K1\ p + \frac{1}{2} K2\ K1\ p^2 = 1$$

```
> rm310:=pmid=solve(rm309,p);
```

$$rm310 := pmid = \left(\frac{1}{2} \frac{-K1 + \sqrt{K1^2 + 4\ K2\ K1}}{K2\ K1}, \frac{1}{2} \frac{-K1 - \sqrt{K1^2 + 4\ K2\ K1}}{K2\ K1} \right)$$

```
> rm311:=pmid=rhs(rm310)[1];
```

$$rm311 := pmid = \frac{1}{2} \frac{-K1 + \sqrt{K1^2 + 4\ K2\ K1}}{K2\ K1}$$

Gmax at Pmin

```
> rm312:=K1=solve(rm311,K1);
```

$$rm312 := K1 = \frac{1}{pmid\ (pmid\ K2 + 1)}$$

```
> rm313:=pmin=(1-epsilon)*pmid;
```

$$rm313 := pmin = (1 - \varepsilon)\, pmid$$

```
> rm314:=subs(rm311,rm313);
```

$$rm314 := pmin = \frac{1}{2}\frac{(1-\varepsilon)\left(-K1+\sqrt{K1^2+4\,K2\,K1}\right)}{K2\,K1}$$

```
> rm315:=subs(p=rhs(rm314),rm308);
```

$$rm315 := g + \frac{\frac{1}{2}(1-\varepsilon)\left(-K1+\sqrt{K1^2+4\,K2\,K1}\right) g}{K2} + \frac{\frac{1}{4}(1-\varepsilon)^2\left(-K1+\sqrt{K1^2+4\,K2\,K1}\right)^2 g}{K2\,K1} = 1$$

```
> rm316:=gmax=solve(rm315,g);
```

$$rm316 := gmax = -2\frac{K2}{-4\,K2-\varepsilon\sqrt{K1^2+4\,K2\,K1}+\varepsilon\,K1+4\,\varepsilon\,K2-\varepsilon^2\,K1+\varepsilon^2\sqrt{K1^2+4\,K2\,K1}-2\,\varepsilon^2\,K2}$$

```
> rm317:=subs(rm312,rm316);
```

$$rm317 := gmax = -2\,K2 \Big/ \left(-4\,K2-\varepsilon\sqrt{\frac{1}{pmid^2\,(pmid\,K2+1)^2}+\frac{4\,K2}{pmid\,(pmid\,K2+1)}}\right.$$
$$+\frac{\varepsilon}{pmid\,(pmid\,K2+1)}+4\,\varepsilon\,K2-\frac{\varepsilon^2}{pmid\,(pmid\,K2+1)}$$
$$\left.+\varepsilon^2\sqrt{\frac{1}{pmid^2\,(pmid\,K2+1)^2}+\frac{4\,K2}{pmid\,(pmid\,K2+1)}}-2\,\varepsilon^2\,K2\right)$$

```
> rm318:=limit(rhs(rm317),K2=infinity);
```

$$rm318 := \frac{1}{-2\,\varepsilon+\varepsilon^2+2}$$

Gmin at Pmax

```
> rm319:=pmax=(1+epsilon)*pmid;
```

$$rm319 := pmax = (1+\varepsilon)\, pmid$$

```
> rm320:=subs(rm311,rm319);
```

$$rm320 := pmax = \frac{1}{2}\frac{(1+\varepsilon)\left(-K1+\sqrt{K1^2+4\,K2\,K1}\right)}{K2\,K1}$$

```
> rm321:=subs(p=rhs(rm320),rm308);
```

$$rm321 := g + \frac{\frac{1}{2}(1+\varepsilon)\left(-K1+\sqrt{K1^2+4\,K2\,K1}\right) g}{K2} + \frac{\frac{1}{4}(1+\varepsilon)^2\left(-K1+\sqrt{K1^2+4\,K2\,K1}\right)^2 g}{K2\,K1} = 1$$

```
> rm322:=gmin=solve(rm321,g);
```

$$rm322 := gmin = 2\,\frac{K2}{4\,K2 - \varepsilon\sqrt{K1^2 + 4\,K2\,K1 + \varepsilon\,K1 + 4\,\varepsilon\,K2 + \varepsilon^2\,K1 - \varepsilon^2\sqrt{K1^2 + 4\,K2\,K1 + 2\,\varepsilon^2\,K2}}}$$

```
> rm323:=subs(rm312,rm322);
```

$$rm323 := gmin = 2\,K2 \Big/ \left(4\,K2 - \varepsilon\sqrt{\frac{1}{pmid^2\,(pmid\,K2+1)^2} + \frac{4\,K2}{pmid\,(pmid\,K2+1)}} + \frac{\varepsilon}{pmid\,(pmid\,K2+1)} + 4\,\varepsilon\,K2 + \frac{\varepsilon^2}{pmid\,(pmid\,K2+1)} - \varepsilon^2\sqrt{\frac{1}{pmid^2\,(pmid\,K2+1)^2} + \frac{4\,K2}{pmid\,(pmid\,K2+1)}} + 2\,\varepsilon^2\,K2\right)$$

```
> rm324:=limit(rhs(rm323),K2=infinity);
```

$$rm324 := \frac{1}{2 + 2\,\varepsilon + \varepsilon^2}$$

Line Equations

Pmin/Gmax Equation

```
> rm325:=subs({p=rhs(rm313),g=rm318},rm301);
```

$$rm325 := (1 - \varepsilon)\,pmid = \frac{\dfrac{k1}{-2\,\varepsilon + \varepsilon^2 + 2} + k3}{k2 + k4}$$

```
> rm326:=k3=solve(rm325,k3);
```

$$rm326 := k3 = -(4\,pmid\,\varepsilon\,k2 + 4\,pmid\,\varepsilon\,k4 - 3\,pmid\,\varepsilon^2\,k2 - 3\,pmid\,\varepsilon^2\,k4 - 2\,pmid\,k2 - 2\,pmid\,k4 + pmid\,\varepsilon^3\,k2 + pmid\,\varepsilon^3\,k4 + k1) \Big/ (-2\,\varepsilon + \varepsilon^2 + 2)$$

Pmax/Gmin Equation

```
> rm327:=subs({p=rhs(rm319),g=rm324},rm301);
```

$$rm327 := (1 + \varepsilon)\,pmid = \frac{\dfrac{k1}{2 + 2\,\varepsilon + \varepsilon^2} + k3}{k2 + k4}$$

```
> rm328:=k3=solve(rm327,k3);
```

$$rm328 := k3 = (2\,pmid\,k2 + 2\,pmid\,k4 + 4\,pmid\,\varepsilon\,k2 + 4\,pmid\,\varepsilon\,k4 + 3\,pmid\,\varepsilon^2\,k2 + 3\,pmid\,\varepsilon^2\,k4 + pmid\,\varepsilon^3\,k2 + pmid\,\varepsilon^3\,k4 - k1) \Big/ (2 + 2\,\varepsilon + \varepsilon^2)$$

Area of Rectangle

```
> rm329:=subs({g=1/2,k3=0,k4=0},rm301);
```

$$rm329 := p = \frac{1}{2}\frac{kl}{k2}$$

Find k4 intercept when k3=0

```
> rm330:=subs(pmid=rhs(rm329),rm326);
```

$$rm330 := k3 = -\frac{2\,kl\,\varepsilon + \frac{2\,kl\,\varepsilon\,k4}{k2} - \frac{3}{2}kl\,\varepsilon^2 - \frac{3}{2}\frac{kl\,\varepsilon^2\,k4}{k2} - \frac{kl\,k4}{k2} + \frac{1}{2}kl\,\varepsilon^3 + \frac{\frac{1}{2}kl\,\varepsilon^3\,k4}{k2}}{-2\,\varepsilon + \varepsilon^2 + 2}$$

```
> rm331:=subs(k3=0,rm330);
```

$$rm331 := 0 = -\frac{2\,kl\,\varepsilon + \frac{2\,kl\,\varepsilon\,k4}{k2} - \frac{3}{2}kl\,\varepsilon^2 - \frac{3}{2}\frac{kl\,\varepsilon^2\,k4}{k2} - \frac{kl\,k4}{k2} + \frac{1}{2}kl\,\varepsilon^3 + \frac{\frac{1}{2}kl\,\varepsilon^3\,k4}{k2}}{-2\,\varepsilon + \varepsilon^2 + 2}$$

```
> rm332:=k4=solve(rm331,k4);
```

$$rm332 := k4 = -\frac{\varepsilon\,(4 + \varepsilon^2 - 3\,\varepsilon)\,k2}{(-2\,\varepsilon + \varepsilon^2 + 2)\,(-1 + \varepsilon)}$$

Find k3 intercept when k4=0

```
> rm333:=subs(pmid=rhs(rm329),rm328);
```

$$rm333 := k3 = \frac{\frac{kl\,k4}{k2} + 2\,kl\,\varepsilon - \frac{2\,kl\,\varepsilon\,k4}{k2} + \frac{3}{2}kl\,\varepsilon^2 + \frac{\frac{3}{2}kl\,\varepsilon^2\,k4}{k2} + \frac{1}{2}kl\,\varepsilon^3 + \frac{\frac{1}{2}kl\,\varepsilon^3\,k4}{k2}}{2 + 2\,\varepsilon + \varepsilon^2}$$

```
> rm334:=subs(k4=0,rm333);
```

$$rm334 := k3 = \frac{2\,kl\,\varepsilon + \frac{3}{2}kl\,\varepsilon^2 + \frac{1}{2}kl\,\varepsilon^3}{2 + 2\,\varepsilon + \varepsilon^2}$$

Area equation

```
> rm335:=area=simplify((rhs(rm332))*(rhs(rm334)));
```

$$rm335 := area = -\frac{1}{2}\frac{\varepsilon^2\,(4 + \varepsilon^2 - 3\,\varepsilon)\,k2\,kl\,(4 + 3\,\varepsilon + \varepsilon^2)}{(-2\,\varepsilon + \varepsilon^2 + 2)\,(-1 + \varepsilon)\,(2 + 2\,\varepsilon + \varepsilon^2)}$$

```
> rm336:=subs(epsilon=1/2,rm335);
```

$$rm336 := area = \frac{253}{260}k2\,kl$$

```
> rm337:=subs(epsilon=1/10,rm335);
```

$$rm337 := area = \frac{159901}{7200180}k2\,kl$$

Regulatory Mechanism 4

```
> restart;
```

Steady State and Conservation Equations

```
> rm401:=p=(k1*g+k3)/(k2+k4);
```

$$rm401 := p = \frac{k1\ g + k3}{k2 + k4}$$

```
> rm402:=K3=pp/p^2;
```

$$rm402 := K3 = \frac{pp}{p^2}$$

```
> rm403:=K4=gpp/(g*pp);
```

$$rm403 := K4 = \frac{gpp}{g\ pp}$$

```
> rm404:=g+gpp=1;
```

$$rm404 := g + gpp = 1$$

```
> rm405:=pp=solve(rm402,pp);
```

$$rm405 := pp = K3\ p^2$$

```
> rm406:=gpp=solve(rm403,gpp);
```

$$rm406 := gpp = K4\ g\ pp$$

```
> rm407:=subs(rm405,rm406);
```

$$rm407 := gpp = K4\ g\ K3\ p^2$$

```
> rm408:=subs({rm407,rm405},rm404);
```

$$rm408 := g + K4\ g\ K3\ p^2 = 1$$

```
> rm409:=g=solve(rm408,g);
```

$$rm409 := g = \frac{1}{1 + K4\ K3\ p^2}$$

```
> rm410:=subs(g=1/2,rm409);
```

$$rm410 := \frac{1}{2} = \frac{1}{1 + K4\ K3\ p^2}$$

```
> rm411:=pmid=solve(rm410,p);
```

$$rm411 := pmid = \left(\frac{\sqrt{K4\ K3}}{K4\ K3}, -\frac{\sqrt{K4\ K3}}{K4\ K3} \right)$$

```
> rm412:=pmid=rhs(rm411)[1];
```

$$rm412 := pmid = \frac{\sqrt{K4\ K3}}{K4\ K3}$$

Gmax at Pmin

```
> rm413:=pmin=(1-epsilon)*pmid;
```

$$rm413 := pmin = (1 - \varepsilon)\, pmid$$

```
> rm414:=subs(rm412,rm413);
```

$$rm414 := pmin = \frac{(1-\varepsilon)\sqrt{K4\ K3}}{K4\ K3}$$

```
> rm415:=subs(p=rhs(rm414),rm408);
```

$$rm415 := g + g\,(1-\varepsilon)^2 = 1$$

```
> rm416:=gmax=solve(rm415,g);
```

$$rm416 := gmax = \frac{1}{2 - 2\,\varepsilon + \varepsilon^2}$$

Gmin at Pmax

```
> rm417:=pmax=(1+epsilon)*pmid;
```

$$rm417 := pmax = (1 + \varepsilon)\, pmid$$

```
> rm418:=subs(rm412,rm417);
```

$$rm418 := pmax = \frac{(1+\varepsilon)\sqrt{K4\ K3}}{K4\ K3}$$

```
> rm419:=subs(p=rhs(rm418),rm408);
```

$$rm419 := g + g\,(1+\varepsilon)^2 = 1$$

```
> rm420:=gmin=solve(rm419,g);
```

$$rm420 := gmin = \frac{1}{2 + 2\,\varepsilon + \varepsilon^2}$$

Line Equations

Pmin/Gmax Equation

```
> rm421:=subs({p=rhs(rm413),g=rhs(rm416)},rm401);
```

$$rm421 := (1-\varepsilon)\, pmid = \frac{\frac{k1}{2 - 2\,\varepsilon + \varepsilon^2} + k3}{k2 + k4}$$

```
> rm422:=k3=solve(rm421,k3);
```

$$rm422 := k3 = -(-2\, pmid\, k2 - 2\, pmid\, k4 + 4\, pmid\, \varepsilon\, k2 + 4\, pmid\, \varepsilon\, k4 - 3\, pmid\, \varepsilon^2\, k2 - 3\, pmid\, \varepsilon^2\, k4 + pmid\, \varepsilon^3\, k2 + pmid\, \varepsilon^3\, k4 + k1) \Big/ (2 - 2\,\varepsilon + \varepsilon^2)$$

Pmax/Gmin Equation

```
> rm423:=subs({p=rhs(rm417),g=rhs(rm420)},rm401);
```

$$rm423 := (1+\varepsilon)\, pmid = \frac{\frac{k1}{2 + 2\,\varepsilon + \varepsilon^2} + k3}{k2 + k4}$$

```
> rm424:=k3=solve(rm423,k3);
```

$$rm424 := k3 = -(-2\,pmid\,k2 - 2\,pmid\,k4 - 4\,pmid\,\varepsilon\,k2 - 4\,pmid\,\varepsilon\,k4 - 3\,pmid\,\varepsilon^2\,k2 - 3\,pmid\,\varepsilon^2\,k4 - pmid\,\varepsilon^3\,k2 - pmid\,\varepsilon^3\,k4 + kl) \Big/ (2 + 2\,\varepsilon + \varepsilon^2)$$

Area of Rectangle

```
> rm425:=subs({g=1/2,k3=0,k4=0},rm401);
```

$$rm425 := p = \frac{1}{2}\frac{kl}{k2}$$

Find k4 intercept when k3=0

```
> rm426:=subs(pmid=rhs(rm425),rm422);
```

$$rm426 := k3 = -\frac{-\frac{kl\,k4}{k2} + 2\,kl\,\varepsilon + \frac{2\,kl\,\varepsilon\,k4}{k2} - \frac{3}{2}kl\,\varepsilon^2 - \frac{3}{2}\frac{kl\,\varepsilon^2\,k4}{k2} + \frac{1}{2}kl\,\varepsilon^3 + \frac{\frac{1}{2}kl\,\varepsilon^3\,k4}{k2}}{2 - 2\,\varepsilon + \varepsilon^2}$$

```
> rm427:=subs(k3=0,rm426);
```

$$rm427 := 0 = -\frac{-\frac{kl\,k4}{k2} + 2\,kl\,\varepsilon + \frac{2\,kl\,\varepsilon\,k4}{k2} - \frac{3}{2}kl\,\varepsilon^2 - \frac{3}{2}\frac{kl\,\varepsilon^2\,k4}{k2} + \frac{1}{2}kl\,\varepsilon^3 + \frac{\frac{1}{2}kl\,\varepsilon^3\,k4}{k2}}{2 - 2\,\varepsilon + \varepsilon^2}$$

```
> rm428:=k4=solve(rm427,k4);
```

$$rm428 := k4 = -\frac{\varepsilon\,(4 - 3\,\varepsilon + \varepsilon^2)\,k2}{(2 - 2\,\varepsilon + \varepsilon^2)(-1 + \varepsilon)}$$

Find k3 intercept when k4=0

```
> rm429:=subs(pmid=rhs(rm425),rm424);
```

$$rm429 := k3 = -\frac{-\frac{kl\,k4}{k2} - 2\,kl\,\varepsilon - \frac{2\,kl\,\varepsilon\,k4}{k2} - \frac{3}{2}kl\,\varepsilon^2 - \frac{3}{2}\frac{kl\,\varepsilon^2\,k4}{k2} - \frac{1}{2}kl\,\varepsilon^3 - \frac{1}{2}\frac{kl\,\varepsilon^3\,k4}{k2}}{2 + 2\,\varepsilon + \varepsilon^2}$$

```
> rm430:=subs(k4=0,rm429);
```

$$rm430 := k3 = -\frac{-2\,kl\,\varepsilon - \frac{3}{2}kl\,\varepsilon^2 - \frac{1}{2}kl\,\varepsilon^3}{2 + 2\,\varepsilon + \varepsilon^2}$$

Area equation

```
> rm431:=area=simplify((rhs(rm428))*(rhs(rm430)));
```

$$rm431 := area = -\frac{1}{2}\frac{\varepsilon^2\,(4 - 3\,\varepsilon + \varepsilon^2)\,k2\,kl\,(4 + 3\,\varepsilon + \varepsilon^2)}{(2 - 2\,\varepsilon + \varepsilon^2)(-1 + \varepsilon)(2 + 2\,\varepsilon + \varepsilon^2)}$$

```
> rm432:=subs(epsilon=1/2,rm431);
```

$$rm432 := area = \frac{253}{260} k2\ k1$$

```
> rm433:=subs(epsilon=1/10,rm431);
```

$$rm433 := area = \frac{159901}{7200180} k2\ k1$$

Regulatory Mechanism 5

```
> restart;
```

Steady State and Conservation Equations

```
> rm501:=p=(k15*m+k3)/(k2+k4);
```

$$rm501 := p = \frac{k15\,m + k3}{k2 + k4}$$

```
> rm502:=K1=gp/(g*p);
```

$$rm502 := K1 = \frac{gp}{g\,p}$$

```
> rm503:=k13*g=k14*m;
```

$$rm503 := k13\,g = k14\,m$$

```
> rm504:=g+gp=1;
```

$$rm504 := g + gp = 1$$

```
> rm505:=gp=solve(rm502,gp);
```

$$rm505 := gp = K1\,g\,p$$

```
> rm506:=subs(rm505,rm504);
```

$$rm506 := g + K1\,g\,p = 1$$

```
> rm507:=g=solve(rm506,g);
```

$$rm507 := g = \frac{1}{1 + K1\,p}$$

```
> rm508:=subs(g=1/2,rm507);
```

$$rm508 := \frac{1}{2} = \frac{1}{1 + K1\,p}$$

```
> rm509:=pmid=solve(rm508,p);
```

$$rm509 := pmid = \frac{1}{K1}$$

Gmax at Pmin

```
> rm510:=pmin=(1-epsilon)*pmid;
```

$$rm510 := pmin = (1 - \varepsilon)\,pmid$$

```
> rm511:=subs(rm509,rm510);
```

$$rm511 := pmin = \frac{1 - \varepsilon}{K1}$$

```
> rm512:=subs(p=rhs(rm511),rm506);
```

$$rm512 := g + g\,(1 - \varepsilon) = 1$$

```
> rm513:=gmax=solve(rm512,g);
```

$$rm513 := gmax = -\frac{1}{-2+\varepsilon}$$

Gmin at Pmax

```
> rm514:=pmax=(1+epsilon)*pmid;
```

$$rm514 := pmax = (1+\varepsilon)\,pmid$$

```
> rm515:=subs(rm509,rm514);
```

$$rm515 := pmax = \frac{1+\varepsilon}{k1}$$

```
> rm516:=subs(p=rhs(rm515),rm506);
```

$$rm516 := g + g(1+\varepsilon) = 1$$

```
> rm517:=gmin=solve(rm516,g);
```

$$rm517 := gmin = \frac{1}{2+\varepsilon}$$

Line Equations

Solve rm501 for p in terms of g

```
> rm518:=m=solve(rm503,m);
```

$$rm518 := m = \frac{k13\,g}{k14}$$

```
> rm519:=subs(rm518,rm501);
```

$$rm519 := p = \frac{\frac{k15\,k13\,g}{k14} + k3}{k2 + k4}$$

Pmin/Gmax Equation

```
> rm520:=subs({p=rhs(rm510),g=rhs(rm513)},rm519);
```

$$rm520 := (1-\varepsilon)\,pmid = \frac{-\frac{k15\,k13}{(-2+\varepsilon)\,k14} + k3}{k2 + k4}$$

```
> rm521:=k3=expand(solve(rm520,k3));
```

$$rm521 := k3 = -2\frac{pmid\,k2}{-2+\varepsilon} - \frac{2\,pmid\,k4}{-2+\varepsilon} + \frac{3\,pmid\,\varepsilon\,k2}{-2+\varepsilon} + \frac{3\,pmid\,\varepsilon\,k4}{-2+\varepsilon} - \frac{pmid\,\varepsilon^2\,k2}{-2+\varepsilon}$$
$$- \frac{pmid\,\varepsilon^2\,k4}{-2+\varepsilon} + \frac{k15\,k13}{(-2+\varepsilon)\,k14}$$

Pmax/Gmin Equation

```
> rm522:=subs({p=rhs(rm514),g=rhs(rm517)},rm519);
```

$$rm522 := (1+\varepsilon)\,pmid = \frac{\frac{k15\ k13}{(2+\varepsilon)\ k14} + k3}{k2 + k4}$$

```
> rm523:=k3=expand(solve(rm522,k3));
```

$$rm523 := k3 = 2\frac{pmid\ k2}{2+\varepsilon} + \frac{2\,pmid\ k4}{2+\varepsilon} + \frac{3\,pmid\ \varepsilon\ k2}{2+\varepsilon} + \frac{3\,pmid\ \varepsilon\ k4}{2+\varepsilon} + \frac{pmid\ \varepsilon^2\ k2}{2+\varepsilon} + \frac{pmid\ \varepsilon^2\ k4}{2+\varepsilon} - \frac{k15\ k13}{(2+\varepsilon)\ k14}$$

Area of Rectangle

```
> rm524:=subs({g=1/2,k3=0,k4=0},rm519);
```

$$rm524 := p = \frac{1}{2}\frac{k15\ k13}{k14\ k2}$$

Find k4 intercept when k3=0.

```
> rm525:=subs(pmid=rhs(rm524),rm521);
```

$$rm525 := k3 = -\frac{k15\ k13\ k4}{(-2+\varepsilon)\ k14\ k2} + \frac{\frac{3}{2}k15\ k13\ \varepsilon}{(-2+\varepsilon)\ k14} + \frac{\frac{3}{2}k15\ k13\ \varepsilon\ k4}{(-2+\varepsilon)\ k14\ k2} - \frac{1}{2}\frac{k15\ k13\ \varepsilon^2}{(-2+\varepsilon)\ k14} - \frac{1}{2}\frac{k15\ k13\ \varepsilon^2\ k4}{(-2+\varepsilon)\ k14\ k2}$$

```
> rm526:=subs(k3=0,rm525);
```

$$rm526 := 0 = -\frac{k15\ k13\ k4}{(-2+\varepsilon)\ k14\ k2} + \frac{\frac{3}{2}k15\ k13\ \varepsilon}{(-2+\varepsilon)\ k14} + \frac{\frac{3}{2}k15\ k13\ \varepsilon\ k4}{(-2+\varepsilon)\ k14\ k2} - \frac{1}{2}\frac{k15\ k13\ \varepsilon^2}{(-2+\varepsilon)\ k14} - \frac{1}{2}\frac{k15\ k13\ \varepsilon^2\ k4}{(-2+\varepsilon)\ k14\ k2}$$

```
> rm527:=k4=solve(rm526,k4);
```

$$rm527 := k4 = -\frac{\varepsilon\,(-3+\varepsilon)\,k2}{(-2+\varepsilon)\,(-1+\varepsilon)}$$

Find k3 intercept when k4=0.

```
> rm528:=subs(pmid=rhs(rm524),rm523);
```

$$rm528 := k3 = \frac{k15\ k13\ k4}{(2+\varepsilon)\ k14\ k2} + \frac{\frac{3}{2}k15\ k13\ \varepsilon}{(2+\varepsilon)\ k14} + \frac{\frac{3}{2}k15\ k13\ \varepsilon\ k4}{(2+\varepsilon)\ k14\ k2} + \frac{\frac{1}{2}k15\ k13\ \varepsilon^2}{(2+\varepsilon)\ k14} + \frac{\frac{1}{2}k15\ k13\ \varepsilon^2\ k4}{(2+\varepsilon)\ k14\ k2}$$

```
> rm529:=subs(k4=0,rm528);
```

$$rm529 := k3 = \frac{3}{2}\frac{k15\ k13\ \varepsilon}{(2+\varepsilon)\ k14} + \frac{\frac{1}{2}k15\ k13\ \varepsilon^2}{(2+\varepsilon)\ k14}$$

Area equation

```
> rm530:=area=simplify((rhs(rm527))*(rhs(rm529)));
```

$$rm530 := area = -\frac{1}{2}\frac{\varepsilon^2(-3+\varepsilon)\,k2\,k15\,k13\,(3+\varepsilon)}{(-2+\varepsilon)(-1+\varepsilon)\,k14\,(2+\varepsilon)}$$

```
> rm531:=subs(epsilon=1/2,rm530);
```

$$rm531 := area = -\frac{7}{12}\frac{k2\,k15\,k13}{k14}$$

```
> rm532:=subs(epsilon=1/10,rm530);
```

$$rm532 := area = -\frac{899}{71820}\frac{k2\,k15\,k13}{k14}$$

Regulatory Mechanism 6

```
> restart;
```

Steady State and Conservation Equations

```
> rm601:=p=(k15*m+k3)/(k2+k4);
```

$$rm601 := p = \frac{k15\,m + k3}{k2 + k4}$$

```
> rm602:=K1=gp/(g*p);
```

$$rm602 := K1 = \frac{gp}{g\,p}$$

```
> rm603:=K2=mp/(m*p);
```

$$rm603 := K2 = \frac{mp}{m\,p}$$

```
> rm604:=k13*g+k17*mp=k14*m+k16*p*m;
```

$$rm604 := k13\,g + k17\,mp = k14\,m + k16\,p\,m$$

```
> rm605:=g+gp=1;
```

$$rm605 := g + gp = 1$$

```
> rm606:=k16*p*m=(k18+k17)*mp;
```

$$rm606 := k16\,p\,m = (k18 + k17)\,mp$$

```
> rm607:=gp=solve(rm602,gp);
```

$$rm607 := gp = K1\,g\,p$$

```
> rm608:=subs(rm607,rm605);
```

$$rm608 := g + K1\,g\,p = 1$$

```
> rm609:=g=solve(rm608,g);
```

$$rm609 := g = \frac{1}{1 + K1\,p}$$

```
> rm610:=subs(g=1/2,rm609);
```

$$rm610 := \frac{1}{2} = \frac{1}{1 + K1\,p}$$

```
> rm611:=pmid=solve(rm610,p);
```

$$rm611 := pmid = \frac{1}{K1}$$

Gmax at Pmin

```
> rm612:=pmin=(1-epsilon)*pmid;
```

$$rm612 := pmin = (1 - \varepsilon)\,pmid$$

```
> rm613:=subs(rm611,rm612);
```

$$rm613 := pmin = \frac{1-\varepsilon}{K1}$$

```
> rm614:=subs(p=rhs(rm613),rm608);
```

$$rm614 := g + g(1-\varepsilon) = 1$$

```
> rm615:=gmax=solve(rm614,g);
```

$$rm615 := gmax = -\frac{1}{-2+\varepsilon}$$

Gmin at Pmax

```
> rm616:=pmax=(1+epsilon)*pmid;
```

$$rm616 := pmax = (1+\varepsilon)\,pmid$$

```
> rm617:=subs(rm611,rm616);
```

$$rm617 := pmax = \frac{1+\varepsilon}{K1}$$

```
> rm618:=subs(p=rhs(rm617),rm608);
```

$$rm618 := g + g(1+\varepsilon) = 1$$

```
> rm619:=gmin=solve(rm618,g);
```

$$rm619 := gmin = \frac{1}{2+\varepsilon}$$

Final Equations

Solve rm601 for p in terms of g

```
> rm620:=mp=solve(rm606,mp);
```

$$rm620 := mp = \frac{k16\,p\,m}{k18+k17}$$

```
> rm621:=subs(rm620,rm604);
```

$$rm621 := k13\,g + \frac{k17\,k16\,p\,m}{k18+k17} = k14\,m + k16\,p\,m$$

```
> rm622:=m=solve(rm621,m);
```

$$rm622 := m = \frac{k13\,g\,(k18+k17)}{k14\,k18+k14\,k17+k16\,p\,k18}$$

```
> rm623:=subs(rm622,rm601);
```

$$rm623 := p = \frac{\dfrac{k15\,k13\,g\,(k18+k17)}{k14\,k18+k14\,k17+k16\,p\,k18}+k3}{k2+k4}$$

Pmin/Gmax Equation

```
> rm624:=subs({p=rhs(rm612),g=rhs(rm615)},rm623);
```

$$rm624 := (1-\varepsilon)\,pmid = \frac{-\dfrac{k15\,k13\,(k18+k17)}{(-2+\varepsilon)\,(k14\,k18+k14\,k17+k16\,(1-\varepsilon)\,pmid\,k18)} + k3}{k2+k4}$$

```
> rm625:=k3=solve(rm624,k3);
```

$$\begin{aligned} rm625 := k3 = &-(-2\,pmid\,k14\,k18\,k2 - 2\,pmid\,k14\,k18\,k4 - 2\,pmid\,k14\,k17\,k2 \\ &- 2\,pmid\,k14\,k17\,k4 - 2\,k16\,pmid^2\,k18\,k2 - 2\,k16\,pmid^2\,k18\,k4 + 5\,k16\,pmid^2\,k18\,\varepsilon\,k2 \\ &+ 5\,k16\,pmid^2\,k18\,\varepsilon\,k4 + 3\,pmid\,\varepsilon\,k14\,k18\,k2 + 3\,pmid\,\varepsilon\,k14\,k18\,k4 + 3\,pmid\,\varepsilon\,k14\,k17\,k2 \\ &+ 3\,pmid\,\varepsilon\,k14\,k17\,k4 - 4\,k16\,pmid^2\,k18\,\varepsilon^2\,k2 - 4\,k16\,pmid^2\,k18\,\varepsilon^2\,k4 - pmid\,\varepsilon^2\,k14\,k18\,k2 \\ &- pmid\,\varepsilon^2\,k14\,k18\,k4 - pmid\,\varepsilon^2\,k14\,k17\,k2 - pmid\,\varepsilon^2\,k14\,k17\,k4 + \varepsilon^3\,k16\,pmid^2\,k18\,k2 \\ &+ \varepsilon^3\,k16\,pmid^2\,k18\,k4 + k15\,k13\,k18 + k15\,k13\,k17)\,/\,(\\ &(-2+\varepsilon)\,(-k14\,k18 - k14\,k17 - k16\,pmid\,k18 - k16\,pmid\,k18\,\varepsilon)) \end{aligned}$$

PmaxiGmin Equation

```
> rm626:=subs({p=rhs(rm616),g=rhs(rm619)},rm623);
```

$$rm626 := (1+\varepsilon)\,pmid = \frac{-\dfrac{k15\,k13\,(k18+k17)}{(2+\varepsilon)\,(k14\,k18+k14\,k17-k16\,(1+\varepsilon)\,pmid\,k18)} + k3}{k2+k4}$$

```
> rm627:=k3=solve(rm626,k3);
```

$$\begin{aligned} rm627 := k3 = &-(-2\,pmid\,k14\,k18\,k2 - 2\,pmid\,k14\,k18\,k4 - 2\,pmid\,k14\,k17\,k2 \\ &- 2\,pmid\,k14\,k17\,k4 - 2\,k16\,pmid^2\,k18\,k2 - 2\,k16\,pmid^2\,k18\,k4 - 5\,k16\,pmid^2\,k18\,\varepsilon\,k2 \\ &- 5\,k16\,pmid^2\,k18\,\varepsilon\,k4 - 3\,pmid\,\varepsilon\,k14\,k18\,k2 - 3\,pmid\,\varepsilon\,k14\,k18\,k4 - 3\,pmid\,\varepsilon\,k14\,k17\,k2 \\ &- 3\,pmid\,\varepsilon\,k14\,k17\,k4 - 4\,k16\,pmid^2\,k18\,\varepsilon^2\,k2 - 4\,k16\,pmid^2\,k18\,\varepsilon^2\,k4 - pmid\,\varepsilon^2\,k14\,k18\,k2 \\ &- pmid\,\varepsilon^2\,k14\,k18\,k4 - pmid\,\varepsilon^2\,k14\,k17\,k2 - pmid\,\varepsilon^2\,k14\,k17\,k4 - \varepsilon^3\,k16\,pmid^2\,k18\,k2 \\ &- \varepsilon^3\,k16\,pmid^2\,k18\,k4 + k15\,k13\,k18 + k15\,k13\,k17)\,/\,(\\ &(2+\varepsilon)\,(k14\,k18+k14\,k17+k16\,pmid\,k18+k16\,pmid\,k18\,\varepsilon)) \end{aligned}$$

Area of Rectangle

```
> rm628:=subs({g=1/2,k3=0,k4=0},rm623);
```

$$rm628 := p = \frac{1}{2}\,\frac{k15\,k13\,(k18+k17)}{(k14\,k18+k14\,k17+k16\,p\,k18)\,k2}$$

```
> rm629:=solve(rm628,p);
```

$$\begin{aligned} rm629 := &\frac{1}{4}\,(-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 \\ &+ k14^2\,k18^2\,k2^2 + 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17))\,/\,(k16\,k18\,k2),\frac{1}{4}\,(\\ &-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 - 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2 \\ &+ 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17))\,/\,(k16\,k18\,k2) \end{aligned}$$

Find k4 intercept when k3=0.

```
> rm630:=subs(pmid=rm629[1],rm625);
```

$$rm630 := k3 = -\left(-\frac{1}{2}\left((-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2 + 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17)\right)k14\right)/k16 - \frac{1}{2}\left((-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2 + 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17)\right)k14\,k4\right)/(k16\,k2) - \frac{1}{2}\left((-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2 + 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17)\right)k14\,k17\right)/(k16\,k18) - \frac{1}{2}\left(\left(-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2 + 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17)\right)k14\,k17\,k4\right)/(k16\,k18\,k2) - \frac{1}{8}\left(-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2 + 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17)\right)^2/(k16\,k18\,k2) - \frac{1}{8}(-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2 + 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17))^3\,k4\,/\,(k16\,k18\,k2^2) + \frac{5}{16}\left(-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2 + 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17)\right)^3\varepsilon/(k16\,k18\,k2) + \frac{5}{16}(-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2 + 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17))^3\,\varepsilon\,k4\,/\,(k16\,k18\,k2^2) + \frac{3}{4}\left(-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2 + 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17)\right)\varepsilon\,k14/k16 + \frac{3}{4}(-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2 + 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17))\,\varepsilon\,k14\,k4\,/\,(k16\,k2) + \frac{3}{4}(-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2$$

$$+ 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17))\,\varepsilon;\,k14\,k17\,/\,(k16\,k18)) + \frac{3}{4}($$

$$-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2$$

$$+ 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17))\,\varepsilon;\,k14\,k17\,k4\,/\,(k16\,k18\,k2) - \frac{1}{4}($$

$$-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2$$

$$+ 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17))\,\varepsilon^2\,/\,(k16\,k18\,k2) - \frac{1}{4}(-2\,k14\,k17\,k2$$

$$- 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2$$

$$+ 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17))^2\,\varepsilon^2\,k4\,/\,(k16\,k18\,k2^2) - \frac{1}{4}((-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2$$

$$+ 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17))\,\varepsilon^2\,k14)\,/\,k16 - \frac{1}{4}((-2\,k14\,k17\,k2$$

$$- 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2$$

$$+ 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17))\,\varepsilon^2\,k14\,k4)\,/\,(k16\,k2) - \frac{1}{4}((-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2$$

$$+ 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17))\,\varepsilon^2\,k14\,k17)\,/\,(k16\,k18) - \frac{1}{4}((-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2$$

$$+ 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17))\,\varepsilon^2\,k14\,k17\,k4)\,/\,(k16\,k18\,k2) + \frac{1}{16}\varepsilon^3\,($$

$$-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2$$

$$+ 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17))^2\,/\,(k16\,k18\,k2) + \frac{1}{16}\varepsilon^3\,(-2\,k14\,k17\,k2$$

$$- 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2$$

$$+ 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17))^2\,k4\,/\,(k16\,k18\,k2^2) + k15\,k13\,k18$$

$$+ k15\,k13\,k17\Big) \Big/ \Big(\Big(-2+\varepsilon\Big)\Big(-k14\,k18 - k14\,k17 - \frac{1}{4}(-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 + 2$$

$$\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2 + 2\,k16\,k18^2\,k2\,k15\,k13$$

$$+ 2\,k16\,k18\,k2\,k15\,k13\,k17))\,/\,k2 + \frac{1}{4}(-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2$$

$$+ 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2 + 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17))\,\varepsilon$$

```
   / k2))
> rm631:=subs(k3=0,rm630);
rm631 := 0 = -(-1/2 ((-2 k14 k17 k2 - 2 k14 k18 k2 + 2 sqrt(k14^2 k17^2 k2^2
   + 2 k14^2 k17 k2^2 k18 + k14^2 k18^2 k2^2 + 2 k16 k18^3 k2 k15 k13 + 2 k16 k18 k2 k15 k13 k17))
   k14) / k16 - 1/2 ((-2 k14 k17 k2 - 2 k14 k18 k2 + 2 sqrt(k14^2 k17^2 k2^2 + 2 k14^2 k17 k2^2 k18
   + k14^2 k18^2 k2^2 + 2 k16 k18^2 k2 k15 k13 + 2 k16 k18 k2 k15 k13 k17)) k14 k4) / (k16 k2) - 1/2
   ((-2 k14 k17 k2 - 2 k14 k18 k2 + 2 sqrt(k14^2 k17^2 k2^2 + 2 k14^2 k17 k2^2 k18 + k14^2 k18^2 k2^2
   + 2 k16 k18^2 k2 k15 k13 + 2 k16 k18 k2 k15 k13 k17)) k14 k17) / (k16 k18) - 1/2 ((
   -2 k14 k17 k2 - 2 k14 k18 k2 + 2 sqrt(k14^2 k17^2 k2^2 + 2 k14^2 k17 k2^2 k18 + k14^2 k18^2 k2^2
   + 2 k16 k18^2 k2 k15 k13 + 2 k16 k18 k2 k15 k13 k17)) k14 k17 k4) / (k16 k18 k2) - 1/8 (
   -2 k14 k17 k2 - 2 k14 k18 k2 + 2 sqrt(k14^2 k17^2 k2^2 + 2 k14^2 k17 k2^2 k18 + k14^2 k18^2 k2^2
   + 2 k16 k18^2 k2 k15 k13 + 2 k16 k18 k2 k15 k13 k17))^2 / (k16 k18 k2) - 1/8 (-2 k14 k17 k2
   - 2 k14 k18 k2 + 2 sqrt(k14^2 k17^2 k2^2 + 2 k14^2 k17 k2^2 k18 + k14^2 k18^2 k2^2
   + 2 k16 k18^2 k2 k15 k13 + 2 k16 k18 k2 k15 k13 k17))^2 k4 / (k16 k18 k2^2) + 5/16 (
   -2 k14 k17 k2 - 2 k14 k18 k2 + 2 sqrt(k14^2 k17^2 k2^2 + 2 k14^2 k17 k2^2 k18 + k14^2 k18^2 k2^2
   + 2 k16 k18^2 k2 k15 k13 + 2 k16 k18 k2 k15 k13 k17))^2 ε / (k16 k18 k2) + 5/16 (-2 k14 k17 k2
   - 2 k14 k18 k2 + 2 sqrt(k14^2 k17^2 k2^2 + 2 k14^2 k17 k2^2 k18 + k14^2 k18^2 k2^2
   + 2 k16 k18^2 k2 k15 k13 + 2 k16 k18 k2 k15 k13 k17))^2 ε k4 / (k16 k18 k2^2) + 3/4 (
   -2 k14 k17 k2 - 2 k14 k18 k2 + 2 sqrt(k14^2 k17^2 k2^2 + 2 k14^2 k17 k2^2 k18 + k14^2 k18^2 k2^2
   + 2 k16 k18^2 k2 k15 k13 + 2 k16 k18 k2 k15 k13 k17)) ε k14 / k16 - 3/4 (-2 k14 k17 k2
   - 2 k14 k18 k2 + 2 sqrt(k14^2 k17^2 k2^2 + 2 k14^2 k17 k2^2 k18 + k14^2 k18^2 k2^2
   + 2 k16 k18^2 k2 k15 k13 + 2 k16 k18 k2 k15 k13 k17)) ε k14 k4 / (k16 k2) + 3/4 (-2 k14 k17 k2
   - 2 k14 k18 k2 + 2 sqrt(k14^2 k17^2 k2^2 + 2 k14^2 k17 k2^2 k18 + k14^2 k18^2 k2^2
```

$$+ 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17)\big)\,\varepsilon\,k14\,k17 \,/\, (k16\,k18) + \frac{2}{4}($$

$$-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 - 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2$$

$$+ 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17))\,\varepsilon\,k14\,k17\,k4 \,/\, (k16\,k18\,k2) - \frac{1}{4}($$

$$-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2$$

$$+ 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17))^2\,\varepsilon^2 \,/\, (k16\,k18\,k2) - \frac{1}{4}(-2\,k14\,k17\,k2$$

$$- 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2$$

$$+ 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17))^2\,\varepsilon^2\,k4 \,/\, (k16\,k18\,k2^2) - \frac{1}{4}(($$

$$-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2$$

$$+ 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17))\,\varepsilon^2\,k14) \,/\, k16 - \frac{1}{4}(($$

$$-2\,k14\,k17\,k2$$

$$- 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2$$

$$+ 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17))\,\varepsilon^2\,k14\,k4) \,/\, (k16\,k2) - \frac{1}{4}(($$

$$-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2$$

$$+ 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17))\,\varepsilon^2\,k14\,k17) \,/\, (k16\,k18) - \frac{1}{4}(($$

$$-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2$$

$$+ 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17))\,\varepsilon^3\,k14\,k17\,k4) \,/\, (k16\,k18\,k2) + \frac{1}{16}\varepsilon^3\,($$

$$-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2$$

$$+ 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17))^2 \,/\, (k16\,k18\,k2) + \frac{1}{16}\varepsilon^3\,(-2\,k14\,k17\,k2$$

$$- 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2$$

$$+ 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17))^2\,k4 \,/\, (k16\,k18\,k2^2) + k15\,k13\,k18$$

$$+ k15\,k13\,k17\Big) \,/\, \Big((-2+\varepsilon)\Big(-k14\,k18 - k14\,k17 - \frac{1}{4}(-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 + 2$$

$$\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2 + 2\,k16\,k18^2\,k2\,k15\,k13$$

$$+ 2\,k16\,k18\,k2\,k15\,k13\,k17)) \,/\, k2 + \frac{1}{4}(-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2$$

$$+ 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2 + 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17))\,\varepsilon$$

```
    / k2 ) ) ;
> rm632:=k4=solve(rm631,k4) ;
rm632 := k4 = -((-k17 ε^2 k14^2 k2 - 2 k14^2 k2 k17 + 3 k17 ε k14^2 k2 + 3 ε k14^2 k2 k18
  - 2 k14^2 k2 k18 - 5 k15 k13 k16 k18 - ε^2 k16 k18 k15 k13 - ε^2 k14^2 k2 k18 + 2 k14 sqrt(
  k14^2 k17^2 k2^2 + 2 k14^2 k17 k2^2 k18 + k14^2 k18^2 k2^2 + 2 k16 k18^2 k2 k15 k13
  + 2 k16 k18 k2 k15 k13 k17) - 3 ε k14 sqrt(k14^2 k17^2 k2^2 + 2 k14^2 k17 k2^2 k18
  + k14^2 k18^2 k2^2 + 2 k16 k18^2 k2 k15 k13 + 2 k16 k18 k2 k15 k13 k17) + 4 ε k16 k18 k15 k13
  + ε^2 k14 sqrt(k14^2 k17^2 k2^2 + 2 k14^2 k17 k2^2 k18 + k14^2 k18^2 k2^2 + 2 k16 k18^2 k2 k15 k13
  + 2 k16 k18 k2 k15 k13 k17)) ε k2) / (-k17 ε^2 k14^2 k2 - 2 k17 ε k14^2 k2 + 3 k17 ε^2 k14^2 k2
  - 3 ε^2 k14 sqrt(k14^2 k17^2 k2^2 + 2 k14^2 k17 k2^2 k18 + k14^2 k18^2 k2^2 + 2 k16 k18^2 k2 k15 k13
  + 2 k16 k18 k2 k15 k13 k17) + ε^3 k14 sqrt(k14^2 k17^2 k2^2 + 2 k14^2 k17 k2^2 k18 + k14^2 k18^2 k2^2
  + 2 k16 k18^2 k2 k15 k13 + 2 k16 k18 k2 k15 k13 k17) + 2 k15 k13 k16 k18
  - ε^3 k16 k18 k15 k13 + 2 ε k14 sqrt(k14^2 k17^2 k2^2 + 2 k14^2 k17 k2^2 k18 + k14^2 k18^2 k2^2
  + 2 k16 k18^2 k2 k15 k13 + 2 k16 k18 k2 k15 k13 k17) - ε^2 k14^2 k2 k18 + 3 ε^2 k14^2 k2 k18
  - 5 ε k16 k18 k15 k13 + 4 ε^2 k16 k18 k15 k13 - 2 ε k14^2 k2 k18)
Find k3 intercept when k4=0.
> rm633:=subs(pmid=rm629[1],rm627) ;
rm633 := k3 = -(-1/2 ((-2 k14 k17 k2 - 2 k14 k18 k2 + 2 sqrt(k14^2 k17^2 k2^2
  + 2 k14^2 k17 k2^2 k18 + k14^2 k18^2 k2^2 + 2 k16 k18^2 k2 k15 k13 + 2 k16 k18 k2 k15 k13 k17))
  k14) / k16 - 1/2 ((-2 k14 k17 k2 - 2 k14 k18 k2 + 2 sqrt(k14^2 k17^2 k2^2 + 2 k14^2 k17 k2^2 k18
  + k14^2 k18^2 k2^2 + 2 k16 k18^2 k2 k15 k13 + 2 k16 k18 k2 k15 k13 k17)) k14 k4) / (k16 k2) - 1/2
  ((-2 k14 k17 k2 - 2 k14 k18 k2 + 2 sqrt(k14^2 k17^2 k2^2 + 2 k14^2 k17 k2^2 k18 + k14^2 k18^2 k2^2
  + 2 k16 k18^2 k2 k15 k13 + 2 k16 k18 k2 k15 k13 k17)) k14 k17) / (k16 k18) - 1/2 ((
  -2 k14 k17 k2 - 2 k14 k18 k2 + 2 sqrt(k14^2 k17^2 k2^2 + 2 k14^2 k17 k2^2 k18 + k14^2 k18^2 k2^2
  + 2 k16 k18^2 k2 k15 k13 + 2 k16 k18 k2 k15 k13 k17)) k14 k17 k4) / (k16 k18 k2) - 1/8 (
  -2 k14 k17 k2 - 2 k14 k18 k2 + 2 sqrt(k14^2 k17^2 k2^2 + 2 k14^2 k17 k2^2 k18 + k14^2 k18^2 k2^2
  + 2 k16 k18^2 k2 k15 k13 + 2 k16 k18 k2 k15 k13 k17))^2 / (k16 k18 k2) - 1/8 (-2 k14 k17 k2
```

$$-2\,k14\,k18\,k2+2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2+2\,k14^2\,k17\,k2^2\,k18+k14^2\,k18^2\,k2^2$$

$$+2\,k16\,k18^2\,k2\,k15\,k13+2\,k16\,k18\,k2\,k15\,k13\,k17))^2\,k4\,/\,(k16\,k18\,k2^2)-\frac{5}{16}($$

$$-2\,k14\,k17\,k2-2\,k14\,k18\,k2+2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2+2\,k14^2\,k17\,k2^2\,k18+k14^2\,k18^2\,k2^2$$

$$+2\,k16\,k18^2\,k2\,k15\,k13+2\,k16\,k18\,k2\,k15\,k13\,k17))^2\,\varepsilon/(k16\,k18\,k2)-\frac{5}{16}(-2\,k14\,k17\,k2$$

$$-2\,k14\,k18\,k2+2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2+2\,k14^2\,k17\,k2^2\,k18+k14^2\,k18^2\,k2^2$$

$$+2\,k16\,k18^2\,k2\,k15\,k13+2\,k16\,k18\,k2\,k15\,k13\,k17))^2\,\varepsilon\,k4\,/\,(k16\,k18\,k2^2)-\frac{3}{4}(($$

$$-2\,k14\,k17\,k2-2\,k14\,k18\,k2+2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2+2\,k14^2\,k17\,k2^2\,k18+k14^2\,k18^2\,k2^2$$

$$+2\,k16\,k18^2\,k2\,k15\,k13+2\,k16\,k18\,k2\,k15\,k13\,k17))\,\varepsilon\,k14)/k16-\frac{3}{4}(($$

$$-2\,k14\,k17\,k2$$

$$-2\,k14\,k18\,k2+2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2+2\,k14^2\,k17\,k2^2\,k18+k14^2\,k18^2\,k2^2$$

$$+2\,k16\,k18^2\,k2\,k15\,k13+2\,k16\,k18\,k2\,k15\,k13\,k17))\,\varepsilon\,k14\,k4)/(k16\,k2)-\frac{3}{4}(($$

$$-2\,k14\,k17\,k2-2\,k14\,k18\,k2+2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2+2\,k14^2\,k17\,k2^2\,k18+k14^2\,k18^2\,k2^2$$

$$+2\,k16\,k18^2\,k2\,k15\,k13+2\,k16\,k18\,k2\,k15\,k13\,k17))\,\varepsilon\,k14\,k17)/(k16\,k18)-\frac{3}{4}(($$

$$-2\,k14\,k17\,k2-2\,k14\,k18\,k2+2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2+2\,k14^2\,k17\,k2^2\,k18+k14^2\,k18^2\,k2^2$$

$$+2\,k16\,k18^2\,k2\,k15\,k13+2\,k16\,k18\,k2\,k15\,k13\,k17))\,\varepsilon\,k14\,k17\,k4)/(k16\,k18\,k2)-\frac{1}{4}($$

$$-2\,k14\,k17\,k2-2\,k14\,k18\,k2+2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2+2\,k14^2\,k17\,k2^2\,k18+k14^2\,k18^2\,k2^2$$

$$+2\,k16\,k18^2\,k2\,k15\,k13+2\,k16\,k18\,k2\,k15\,k13\,k17))^2\,\varepsilon^2/(k16\,k18\,k2)-\frac{1}{4}(-2\,k14\,k17\,k2$$

$$-2\,k14\,k18\,k2+2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2+2\,k14^2\,k17\,k2^2\,k18+k14^2\,k18^2\,k2^2$$

$$+2\,k16\,k18^2\,k2\,k15\,k13+2\,k16\,k18\,k2\,k15\,k13\,k17))^2\,\varepsilon^2\,k4\,/\,(k16\,k18\,k2^2)-\frac{1}{4}(($$

$$-2\,k14\,k17\,k2-2\,k14\,k18\,k2+2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2+2\,k14^2\,k17\,k2^2\,k18+k14^2\,k18^2\,k2^2$$

$$+2\,k16\,k18^2\,k2\,k15\,k13+2\,k16\,k18\,k2\,k15\,k13\,k17))\,\varepsilon^2\,k14)/k16-\frac{1}{4}(($$

$$-2\,k14\,k17\,k2$$

$$-2\,k14\,k18\,k2+2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2+2\,k14^2\,k17\,k2^2\,k18+k14^2\,k18^2\,k2^2$$

$$+2\,k16\,k18^2\,k2\,k15\,k13+2\,k16\,k18\,k2\,k15\,k13\,k17))\,\varepsilon^2\,k14\,k4)/(k16\,k2)-\frac{1}{4}(($$

$$-2\,k14\,k17\,k2-2\,k14\,k18\,k2+2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2+2\,k14^2\,k17\,k2^2\,k18+k14^2\,k18^2\,k2^2$$

```
    + 2 k16 k18^2 k2 k15 k13 + 2 k16 k18 k2 k15 k13 k17)) ε^2 k14 k17) / (k16 k18) - 1/4 ((
    -2 k14 k17 k2 - 2 k14 k18 k2 + 2 sqrt(k14^2 k17^2 k2^2 + 2 k14^2 k17 k2^2 k18 + k14^2 k18^2 k2^2
    + 2 k16 k18^2 k2 k15 k13 + 2 k16 k18 k2 k15 k13 k17)) ε^2 k14 k17 k4) / (k16 k18 k2) - 1/16 ε^3 (
    -2 k14 k17 k2 - 2 k14 k18 k2 + 2 sqrt(k14^2 k17^2 k2^2 + 2 k14^2 k17 k2^2 k18 + k14^2 k18^2 k2^2
    + 2 k16 k18^2 k2 k15 k13 + 2 k16 k18 k2 k15 k13 k17))^2 / (k16 k18 k2) - 1/16 ε^3 (-2 k14 k17 k2
    - 2 k14 k18 k2 + 2 sqrt(k14^2 k17^2 k2^2 + 2 k14^2 k17 k2^2 k18 + k14^2 k18^2 k2^2
    + 2 k16 k18^2 k2 k15 k13 + 2 k16 k18 k2 k15 k13 k17))^2 k4 / (k16 k18 k2^2) + k15 k13 k18
    + k15 k13 k17) / ((2 + ε) (k14 k18 + k14 k17 + 1/4 (-2 k14 k17 k2 - 2 k14 k18 k2 + 2
    sqrt(k14^2 k17^2 k2^2 + 2 k14^2 k17 k2^2 k18 + k14^2 k18^2 k2^2 + 2 k16 k18^2 k2 k15 k13
    + 2 k16 k18 k2 k15 k13 k17)) / k2 + 1/4 (-2 k14 k17 k2 - 2 k14 k18 k2 + 2 sqrt(k14^2 k17^2 k2^2
    + 2 k14^2 k17 k2^2 k18 + k14^2 k18^2 k2^2 + 2 k16 k18^2 k2 k15 k13 + 2 k16 k18 k2 k15 k13 k17)) ε
    / k2)))
> rm634:=subs(k4=0,rm633);
rm634 := k3 = -(-1/2 ((-2 k14 k17 k2 - 2 k14 k18 k2 + 2 sqrt(k14^2 k17^2 k2^2
    + 2 k14^2 k17 k2^2 k18 + k14^2 k18^2 k2^2 + 2 k16 k18^2 k2 k15 k13 + 2 k16 k18 k2 k15 k13 k17))
    k14) / k16 - 1/2 ((-2 k14 k17 k2 - 2 k14 k18 k2 + 2 sqrt(k14^2 k17^2 k2^2 + 2 k14^2 k17 k2^2 k18
    + k14^2 k18^2 k2^2 + 2 k16 k18^2 k2 k15 k13 + 2 k16 k18 k2 k15 k13 k17)) k14 k17) / (k16 k18)
    - 1/8 (-2 k14 k17 k2 - 2 k14 k18 k2 + 2 sqrt(k14^2 k17^2 k2^2 + 2 k14^2 k17 k2^2 k18
    + k14^2 k18^2 k2^2 + 2 k16 k18^2 k2 k15 k13 + 2 k16 k18 k2 k15 k13 k17))^2 / (k16 k18 k2) - 5/16 (
    -2 k14 k17 k2 - 2 k14 k18 k2 + 2 sqrt(k14^2 k17^2 k2^2 + 2 k14^2 k17 k2^2 k18 + k14^2 k18^2 k2^2
    + 2 k16 k18^2 k2 k15 k13 + 2 k16 k18 k2 k15 k13 k17))^2 ε / (k16 k18 k2) - 3/4 ((-2 k14 k17 k2
    - 2 k14 k18 k2 + 2 sqrt(k14^2 k17^2 k2^2 + 2 k14^2 k17 k2^2 k18 + k14^2 k18^2 k2^2
    + 2 k16 k18^2 k2 k15 k13 + 2 k16 k18 k2 k15 k13 k17)) ε k14) / k16 - 3/4 ((-2 k14 k17 k2
```

$$-2\,k14\,k18\,k2+2\,\sqrt{(k14^2\,k17^2\,k2^2+2\,k14^2\,k17\,k2^2\,k18+k14^2\,k18^2\,k2^2}$$

$$+2\,k16\,k18^2\,k2\,k15\,k13+2\,k16\,k18\,k2\,k15\,k13\,k17))\,\varepsilon\,k14\,k17)/(k16\,k18)-\frac{1}{4}($$

$$-2\,k14\,k17\,k2-2\,k14\,k18\,k2+2\,\sqrt{(k14^2\,k17^2\,k2^2-2\,k14^2\,k17\,k2^2\,k18+k14^2\,k18^2\,k2^2}$$

$$+2\,k16\,k18^2\,k2\,k15\,k13+2\,k16\,k18\,k2\,k15\,k13\,k17))^2\,\varepsilon^2/(k16\,k18\,k2)-\frac{1}{4}((-2\,k14\,k17\,k2$$

$$-2\,k14\,k18\,k2+2\,\sqrt{(k14^2\,k17^2\,k2^2+2\,k14^2\,k17\,k2^2\,k18+k14^2\,k18^2\,k2^2}$$

$$+2\,k16\,k18^2\,k2\,k15\,k13+2\,k16\,k18\,k2\,k15\,k13\,k17))\,\varepsilon^2\,k14)/k16-\frac{1}{4}((-2\,k14\,k17\,k2$$

$$-2\,k14\,k18\,k2+2\,\sqrt{(k14^2\,k17^2\,k2^2+2\,k14^2\,k17\,k2^2\,k18-k14^2\,k18^2\,k2^2}$$

$$+2\,k16\,k18^2\,k2\,k15\,k13-2\,k16\,k18\,k2\,k15\,k13\,k17))\,\varepsilon^2\,k14\,k17)/(k16\,k18)-\frac{1}{16}\varepsilon^3\,($$

$$-2\,k14\,k17\,k2-2\,k14\,k18\,k2+2\,\sqrt{(k14^2\,k17^2\,k2^2+2\,k14^2\,k17\,k2^2\,k18+k14^2\,k18^2\,k2^2}$$

$$+2\,k16\,k18^2\,k2\,k15\,k13+2\,k16\,k18\,k2\,k15\,k13\,k17))^2/(k16\,k18\,k2)+k15\,k13\,k18$$

$$+k15\,k13\,k17\Bigg)\Bigg/\Bigg(\Bigg((2+\varepsilon)\Bigg(k14\,k18+k14\,k17+\frac{1}{4}(-2\,k14\,k17\,k2-2\,k14\,k18\,k2+2$$

$$\sqrt{(k14^2\,k17^2\,k2^2+2\,k14^2\,k17\,k2^2\,k18-k14^2\,k18^2\,k2^2+2\,k16\,k18^2\,k2\,k15\,k13}$$

$$+2\,k16\,k18\,k2\,k15\,k13\,k17))/k2+\frac{1}{4}(-2\,k14\,k17\,k2-2\,k14\,k18\,k2+2\,\sqrt{(k14^2\,k17^2\,k2^2}$$

$$+2\,k14^2\,k17\,k2^2\,k18+k14^2\,k18^2\,k2^2+2\,k16\,k18^2\,k2\,k15\,k13+2\,k16\,k18\,k2\,k15\,k13\,k17))\,\varepsilon$$

$$/k2\Bigg)\Bigg)$$

Area equation

```
> rm635:=area=rhs(rm632)*rhs(rm634);
```

$$rm635:=area=\Bigg((-k17\,\varepsilon^2\,k14^2\,k2-2\,k14^3\,k2\,k17+3\,k17\,\varepsilon\,k14^2\,k2+3\,\varepsilon\,k14^2\,k2\,k18$$

$$-2\,k14^2\,k2\,k18-5\,k15\,k13\,k16\,k18-\varepsilon^2\,k16\,k18\,k15\,k13-\varepsilon^2\,k14^2\,k2\,k18+2\,k14\,\sqrt{(}$$

$$k14^2\,k17^2\,k2^2+2\,k14^2\,k17\,k2^2\,k18+k14^2\,k18^2\,k2^2+2\,k16\,k18^2\,k2\,k15\,k13$$

$$+2\,k16\,k18\,k2\,k15\,k13\,k17)-3\,\varepsilon\,k14\,\sqrt{(k14^2\,k17^2\,k2^2+2\,k14^2\,k17\,k2^2\,k18}$$

$$+k14^2\,k18^2\,k2^2+2\,k16\,k18^2\,k2\,k15\,k13+2\,k16\,k18\,k2\,k15\,k13\,k17)+4\,\varepsilon\,k16\,k18\,k15\,k13$$

$$+\varepsilon^2\,k14\,\sqrt{(k14^2\,k17^2\,k2^2+2\,k14^2\,k17\,k2^2\,k18+k14^2\,k18^2\,k2^2+2\,k16\,k18^2\,k2\,k15\,k13}$$

$$+2\,k16\,k18\,k2\,k15\,k13\,k17))\,\varepsilon\,k2\Bigg(-\frac{1}{2}((-2\,k14\,k17\,k2-2\,k14\,k18\,k2+2\,\sqrt{(}$$

$$k14^2\,k17^2\,k2^2+2\,k14^2\,k17\,k2^2\,k18+k14^2\,k18^2\,k2^2+2\,k16\,k18^2\,k2\,k15\,k13$$

$$+ 2\,k16\,k18\,k2\,k15\,k13\,k17))\,k14) : k16 - \frac{1}{2}((-2\,k14\,k17^2\,k2 - 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}($$

$$k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17^2\,k2^2\,k18 + k14^2\,k18^2\,k2^2 + 2\,k16\,k18^2\,k2\,k15\,k13$$

$$+ 2\,k16\,k18\,k2\,k15\,k13\,k17))\,k14\,k17) / (k16\,k18) - \frac{1}{8}(-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 + 2$$

$$\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17^2\,k2^2\,k18 + k14^2\,k18^2\,k2^2 + 2\,k16\,k18^2\,k2\,k15\,k13$$

$$+ 2\,k16\,k18\,k2\,k15\,k13\,k17))^2 / (k16\,k18\,k2) - \frac{5}{16}(-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}($$

$$k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2 + 2\,k16\,k18^2\,k2\,k15\,k13$$

$$+ 2\,k16\,k18\,k2\,k15\,k13\,k17))^2\,\varepsilon / (k16\,k18\,k2) - \frac{3}{4}((-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}($$

$$k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17^2\,k2^2\,k18 + k14^2\,k18^2\,k2^2 + 2\,k16\,k18^2\,k2\,k15\,k13$$

$$+ 2\,k16\,k18\,k2\,k15\,k13\,k17))\,\varepsilon\,k14) / k16 - \frac{3}{4}((-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}($$

$$k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2 + 2\,k16\,k18^2\,k2\,k15\,k13$$

$$+ 2\,k16\,k18\,k2\,k15\,k13\,k17))\,\varepsilon\,k14\,k17) / (k16\,k18) - \frac{1}{4}(-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 + 2$$

$$\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2 + 2\,k16\,k18^2\,k2\,k15\,k13$$

$$+ 2\,k16\,k18\,k2\,k15\,k13\,k17))^2\,\varepsilon^2 / (k16\,k18\,k2) - \frac{1}{4}((-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}($$

$$k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2 + 2\,k16\,k18^2\,k2\,k15\,k13$$

$$+ 2\,k16\,k18\,k2\,k15\,k13\,k17))\,\varepsilon^2\,k14) / k16 - \frac{1}{4}((-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}($$

$$k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2 + 2\,k16\,k18^2\,k2\,k15\,k13$$

$$+ 2\,k16\,k18\,k2\,k15\,k13\,k17))\,\varepsilon^2\,k14\,k17) / (k16\,k18) - \frac{1}{16}\varepsilon^3(-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2$$

$$+ 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17^2\,k2^2\,k18 + k14^2\,k18^2\,k2^2 + 2\,k16\,k18^2\,k2\,k15\,k13$$

$$+ 2\,k16\,k18\,k2\,k15\,k13\,k17))^2 / (k16\,k18\,k2) + k15\,k13\,k18 + k15\,k13\,k17))\Big) \Big/ \Big((\,$$

$$-k17\,\varepsilon^3\,k14^2\,k2 - 2\,k17\,\varepsilon\,k14^2\,k2 + 3\,k17\,\varepsilon^2\,k14^2\,k2 - 3\,\varepsilon^2\,k14\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2$$

$$+ 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2 + 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17) +$$

$$\varepsilon^3\,k14\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2 + 2\,k16\,k18^2\,k2\,k15\,k13$$

$$+ 2\,k16\,k18\,k2\,k15\,k13\,k17) + 2\,k15\,k13\,k16\,k18 - \varepsilon^3\,k16\,k18\,k15\,k13 + 2\,\varepsilon\,k14\,\mathrm{sqrt}($$

$$k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2 + 2\,k16\,k18^2\,k2\,k15\,k13$$

$$+ 2\,k16\,k18\,k2\,k15\,k13\,k17) - \varepsilon^3\,k14^2\,k2\,k18 + 3\,\varepsilon^2\,k14^2\,k2\,k18 - 5\,\varepsilon\,k16\,k18\,k15\,k13$$
$$+ 4\,\varepsilon^2\,k16\,k18\,k15\,k13 - 2\,\varepsilon\,k14^2\,k2\,k18)\,(2+\varepsilon)\left(k14\,k18 - k14\,k17 + \frac{1}{4}(-2\,k14\,k17\,k2\right.$$
$$- 2\,k14\,k18\,k2 + 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 - k14^2\,k18^2\,k2^2$$
$$+ 2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17)) / k2 + \frac{1}{4}(-2\,k14\,k17\,k2 - 2\,k14\,k18\,k2$$
$$+ 2\,\mathrm{sqrt}(k14^2\,k17^2\,k2^2 + 2\,k14^2\,k17\,k2^2\,k18 + k14^2\,k18^2\,k2^2 + 2\,k16\,k18^2\,k2\,k15\,k13$$
$$\left.+ 2\,k16\,k18\,k2\,k15\,k13\,k17))\,\varepsilon / k2\right)\Big)$$

```
> rm636:=subs(k14=0,rm635);
```

$$rm636 := area = \left(\left(-5\,k15\,k13\,k16\,k18 - \varepsilon^2\,k16\,k18\,k15\,k13 + 4\,\varepsilon\,k16\,k18\,k15\,k13\right)\varepsilon\,k2\left(\right.\right.$$
$$-\frac{1}{2}\frac{2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17}{k16\,k18\,k2}$$
$$-\frac{5}{4}\frac{(2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17)\,\varepsilon}{k16\,k18\,k2}$$
$$-\frac{(2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17)\,\varepsilon^2}{k16\,k18\,k2}$$
$$\left.-\frac{1}{4}\frac{\varepsilon^3\,(2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17)}{k16\,k18\,k2} + k15\,k13\,k18 + k15\,k13\,k17\right)\Big)$$
$$\Big/\left(\right.$$
$$(2\,k13\,k13\,k16\,k18 - \varepsilon^3\,k16\,k18\,k15\,k13 - 5\,\varepsilon\,k16\,k18\,k15\,k13 + 4\,\varepsilon^2\,k16\,k18\,k15\,k13)$$
$$(2+\varepsilon)\left(\frac{1}{2}\frac{\sqrt{2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17}}{k2}\right.$$
$$\left.\left.+\frac{\frac{1}{2}\sqrt{2\,k16\,k18^2\,k2\,k15\,k13 + 2\,k16\,k18\,k2\,k15\,k13\,k17}\,\varepsilon}{k2}\right)\right)$$

```
> rm637:=simplify(rm636);
```

$$rm637 := area = -\frac{1}{2}\frac{k15\,k13\,(5+\varepsilon^2-4\,\varepsilon)\,\varepsilon^2\,k2^2\,(k18+k17)\,(5+4\,\varepsilon+\varepsilon^2)\sqrt{2}}{(-2+\varepsilon^3+5\,\varepsilon-4\,\varepsilon^2)\,(2+\varepsilon)\sqrt{k16\,k18\,k2\,k15\,k13\,(k18+k17)}\,(1+\varepsilon)}$$

```
> rm638:=subs(epsilon=1/2,rm637);
```

$$rm638 := area = \frac{377}{180}\,\frac{k15\,k13\,k2^2\,(k18+k17)\sqrt{2}}{\sqrt{k16\,k18\,k2\,k15\,k13\,(k18+k17)}}$$

```
> rm639:=subs(epsilon=1/10,rm637);
```

$$rm639 := area = \frac{249401}{7110180}\,\frac{k15\,k13\,k2^2\,(k18+k17)\sqrt{2}}{\sqrt{k16\,k18\,k2\,k15\,k13\,(k18+k17)}}$$

```
> rm640:=evalf(eval(rm635,{k14=0.0,k2=0.01,k13=4.0,k18=1.0,k15=5.
  0,k16=0.002,k17=1.0,epsilon=0.1}));
```

$$rm640 := area = .007015321695$$

```
> rm641:=limit(evalf(eval(rm635,{k14=1.0,k2=0.01,k13=4.0,k15=5.0,
  k16=0.002,k17=1.0,epsilon=0.1})),k18=0);
```

$$rm641 := area = .002503480925$$

Multi-Level Cooperativity

```
> restart;
```

Two-Level Cooperativity

```
> rm101:=K1=gp/(g*p);
```

$$rm101 := K1 = \frac{gp}{g\,p}$$

```
> rm102:=K2=gpp/(gp*p);
```

$$rm102 := K2 = \frac{gpp}{gp\,p}$$

```
> rm103:=K3=gppp/(gpp*p);
```

$$rm103 := K3 = \frac{gppp}{gpp\,p}$$

```
> rm104:=g+gp+gpp+gppp=1;
```

$$rm104 := g + gp + gpp + gppp = 1$$

```
> rm105:=gppp=solve(rm103,gppp);
```

$$rm105 := gppp = K3\,gpp\,p$$

```
> rm106:=gpp=solve(rm102,gpp);
```

$$rm106 := gpp = K2\,gp\,p$$

```
> rm107:=gp=solve(rm101,gp);
```

$$rm107 := gp = K1\,g\,p$$

```
> rm108:=subs(rm107,rm106);
```

$$rm108 := gpp = K2\,K1\,g\,p^2$$

```
> rm109:=subs(rm108,rm105);
```

$$rm109 := gppp = K3\,K2\,K1\,g\,p^3$$

```
> rm110:=subs({rm107,rm108,rm109},rm104);
```

$$rm110 := g + K1\,g\,p + K2\,K1\,g\,p^2 + K3\,K2\,K1\,g\,p^3 = 1$$

```
> rm111:=g=solve(rm110,g);
```

$$rm111 := g = \frac{1}{K1\,p + K2\,K1\,p^2 + K3\,K2\,K1\,p^3 + 1}$$

```
> rm112:=subs({g=1/2,p=q},rm111);
```

$$rm112 := \frac{1}{2} = \frac{1}{K1\,q + K2\,K1\,q^2 + K3\,K2\,K1\,q^3 + 1}$$

```
> rm113:=K1=solve(rm112,K1);
```

$$rm113 := K1 = \frac{1}{q\,(1 + K2\,q + K3\,K2\,q^2)}$$

```
> rm114:=subs(rm113,rm111);
```

$$rm114 := g = \frac{1}{\frac{p}{q\,(1 + K2\,q + K3\,K2\,q^2)} + \frac{K2\,p^2}{q\,(1 + K2\,q + K3\,K2\,q^2)} - \frac{K3\,K2\,p^3}{q\,(1 + K2\,q + K3\,K2\,q^2)} + 1}$$

```
> rm115:=diff(rhs(rm114),p);
```

$$rm115 := -\frac{\frac{1}{q\,(1 + K2\,q + K3\,K2\,q^2)} + 2\frac{K2\,p}{q\,(1 + K2\,q + K3\,K2\,q^2)} + 3\frac{K3\,K2\,p^2}{q\,(1 + K2\,q + K3\,K2\,q^2)}}{\left(\frac{p}{q\,(1 + K2\,q + K3\,K2\,q^2)} + \frac{K2\,p^2}{q\,(1 + K2\,q + K3\,K2\,q^2)} + \frac{K3\,K2\,p^3}{q\,(1 + K2\,q + K3\,K2\,q^2)} + 1\right)^2}$$

```
> rm116:=subs(q=p,rm115);
```

$$rm116 := -\frac{\frac{1}{p\,(1 + K2\,p + K3\,K2\,p^2)} + 2\frac{K2}{1 + K2\,p + K3\,K2\,p^2} + 3\frac{K3\,K2\,p}{1 + K2\,p + K3\,K2\,p^2}}{\left(\frac{1}{1 + K2\,p + K3\,K2\,p^2} + \frac{K2\,p}{1 + K2\,p + K3\,K2\,p^2} + \frac{K3\,K2\,p^2}{1 + K2\,p + K3\,K2\,p^2} + 1\right)^2}$$

```
> rm117:=limit(rm116,{K2=infinity,K3=infinity});
```

$$rm117 := -\frac{3}{4}\frac{1}{p}$$

Three-Level Cooperativity

```
> rm101:=K1=gp/(g*p);
```

$$rm101 := K1 = \frac{gp}{g\,p}$$

```
> rm102:=K2=gpp/(gp*p);
```

$$rm102 := K2 = \frac{gpp}{gp\,p}$$

```
> rm103:=K3=gppp/(gpp*p);
```

$$rm103 := K3 = \frac{gppp}{gpp\,p}$$

```
> rm104:=K4=gpppp/(gppp*p);
```

$$rm104 := K4 = \frac{gpppp}{gppp\,p}$$

```
> rm105:=g+gp+gpp+gppp+gpppp=1;
```

$$rm105 := g + gp + gpp + gppp + gpppp = 1$$

```
> rm106:=gpppp=solve(rm104,gpppp);
```

$$rm106 := gpppp = K4\ gppp\ p$$

```
> rm107:=gppp=solve(rm103,gppp);
```

$$rm107 := gppp = K3\ gpp\ p$$

```
> rm108:=gpp=solve(rm102,gpp);
```

$$rm108 := gpp = K2\ gp\ p$$

```
> rm109:=gp=solve(rm101,gp);
```

$$rm109 := gp = K1\ g\ p$$

```
> rm110:=subs(rm109,rm108);
```

$$rm110 := gpp = K2\ K1\ g\ p^2$$

```
> rm111:=subs(rm110,rm107);
```

$$rm111 := gppp = K3\ K2\ K1\ g\ p^3$$

```
> rm112:=subs(rm111,rm106);
```

$$rm112 := gpppp = K4\ K3\ K2\ K1\ g\ p^4$$

```
> rm113:=subs({rm109,rm110,rm111,rm112},rm105);
```

$$rm113 := g + K1\ g\ p + K2\ K1\ g\ p^2 + K3\ K2\ K1\ g\ p^3 + K4\ K3\ K2\ K1\ g\ p^4 = 1$$

```
> rm114:=g=solve(rm113,g);
```

$$rm114 := g = \frac{1}{1 + K2\ K1\ p^2 + K3\ K2\ K1\ p^3 + K4\ K3\ K2\ K1\ p^4 + K1\ p}$$

```
> rm115:=subs({g=1/2,p=q},rm114);
```

$$rm115 := \frac{1}{2} = \frac{1}{1 + K2\ K1\ q^2 + K3\ K2\ K1\ q^3 + K4\ K3\ K2\ K1\ q^4 + K1\ q}$$

```
> rm116:=K1=solve(rm115,K1);
```

$$rm116 := K1 = \frac{1}{q\ (K2\ q + K3\ K2\ q^2 + K4\ K3\ K2\ q^3 + 1)}$$

```
> rm117:=subs(rm116,rm114);
```

$$rm117 := g = 1 / \left(1 - \frac{K2\ p^2}{q\ (K2\ q + K3\ K2\ q^2 + K4\ K3\ K2\ q^3 + 1)} + \frac{K3\ K2\ p^3}{q\ (K2\ q + K3\ K2\ q^2 + K4\ K3\ K2\ q^3 + 1)} + \frac{K4\ K3\ K2\ p^4}{q\ (K2\ q + K3\ K2\ q^2 + K4\ K3\ K2\ q^3 + 1)} + \frac{p}{q\ (K2\ q + K3\ K2\ q^2 + K4\ K3\ K2\ q^3 + 1)} \right)$$

```
> rm118:=diff(rhs(rm117),p);
```

$$rm118 := -\left(2\ \frac{K2\ p}{q\ (K2\ q + K3\ K2\ q^2 + K4\ K3\ K2\ q^3 + 1)} + 3\ \frac{K3\ K2\ p^2}{q\ (K2\ q + K3\ K2\ q^2 + K4\ K3\ K2\ q^3 + 1)} + 4\ \frac{K4\ K3\ K2\ p^3}{q\ (K2\ q + K3\ K2\ q^2 + K4\ K3\ K2\ q^3 + 1)} \right.$$

$$+\frac{1}{q\,(K2\,q+K3\,K2\,q^2+K4\,K3\,K2\,q^3+1)}\Bigg)\Bigg/\Bigg(1$$

$$+\frac{K2\,p^2}{q\,(K2\,q+K3\,K2\,q^2+K4\,K3\,K2\,q^3+1)}+\frac{K3\,K2\,p^3}{q\,(K2\,q+K3\,K2\,q^2+K4\,K3\,K2\,q^3+1)}$$

$$+\frac{K4\,K3\,K2\,p^4}{q\,(K2\,q+K3\,K2\,q^2+K4\,K3\,K2\,q^3+1)}+\frac{p}{q\,(K2\,q+K3\,K2\,q^2+K4\,K3\,K2\,q^3+1)}\Bigg)^2$$

```
> rm119:=subs(q=p,rm118);
```

$$rm119 := -\left(2\frac{K2}{K2\,p+K3\,K2\,p^2+K4\,K3\,K2\,p^3+1}+3\frac{K3\,K2\,p}{K2\,p+K3\,K2\,p^2+K4\,K3\,K2\,p^3+1}\right.$$

$$\left.+4\frac{K4\,K3\,K2\,p^2}{K2\,p+K3\,K2\,p^2+K4\,K3\,K2\,p^3+1}+\frac{1}{p\,(K2\,p+K3\,K2\,p^2+K4\,K3\,K2\,p^3+1)}\right)\Bigg/\Bigg($$

$$1+\frac{K2\,p}{K2\,p+K3\,K2\,p^2+K4\,K3\,K2\,p^3+1}+\frac{K3\,K2\,p^2}{K2\,p+K3\,K2\,p^2+K4\,K3\,K2\,p^3+1}$$

$$+\frac{K4\,K3\,K2\,p^3}{K2\,p+K3\,K2\,p^2+K4\,K3\,K2\,p^3+1}+\frac{1}{K2\,p+K3\,K2\,p^2+K4\,K3\,K2\,p^3+1}\Bigg)^2$$

```
> rm120:=limit(rm119,{K2=infinity,K3=infinity,K4=infinity});
```

$$rm120 := -\frac{1}{p}$$

Four-Level Cooperativity

```
> rm101:=K1=gp/(g*p);
```

$$rm101 := K1 = \frac{gp}{g\,p}$$

```
> rm102:=K2=gpp/(gp*p);
```

$$rm102 := K2 = \frac{gpp}{gp\,p}$$

```
> rm103:=K3=gppp/(gpp*p);
```

$$rm103 := K3 = \frac{gppp}{gpp\,p}$$

```
> rm104:=K4=gpppp/(gppp*p);
```

$$rm104 := K4 = \frac{gpppp}{gppp\,p}$$

```
> rm105:=K5=gppppp/(gpppp*p);
```

$$rm105 := K5 = \frac{gppppp}{gpppp\,p}$$

```
> rm106:=g+gp+gpp+gppp+gpppp+gppppp=1;
```

$$rm106 := g + gp + gpp + gppp + gpppp + gppppp = 1$$

```
> rm107:=gppppp=solve(rm105,gppppp);
```

$$rm107 := gppppp = K5\ gpppp\ p$$

```
> rm108:=gpppp=solve(rm104,gpppp);
```

$$rm108 := gpppp = K4\ gppp\ p$$

```
> rm109:=gppp=solve(rm103,gppp);
```

$$rm109 := gppp = K3\ gpp\ p$$

```
> rm110:=gpp=solve(rm102,gpp);
```

$$rm110 := gpp = K2\ gp\ p$$

```
> rm111:=gp=solve(rm101,gp);
```

$$rm111 := gp = K1\ g\ p$$

```
> rm112:=subs(rm111,rm110);
```

$$rm112 := gpp = K2\ K1\ g\ p^2$$

```
> rm113:=subs(rm112,rm109);
```

$$rm113 := gppp = K3\ K2\ K1\ g\ p^3$$

```
> rm114:=subs(rm113,rm108);
```

$$rm114 := gpppp = K4\ K3\ K2\ K1\ g\ p^4$$

```
> rm115:=subs(rm114,rm107);
```

$$rm115 := gppppp = K5\ K4\ K3\ K2\ K1\ g\ p^5$$

```
> rm115:=subs({rm115,rm111,rm112,rm113,rm114},rm106);
```

$$rm115 :=$$

$$g + K1\ g\ p + K2\ K1\ g\ p^2 + K3\ K2\ K1\ g\ p^3 + K4\ K3\ K2\ K1\ g\ p^4 + K5\ K4\ K3\ K2\ K1\ g\ p^5 = 1$$

```
> rm116:=g=solve(rm115,g);
```

$$rm116 := g = \frac{1}{1 + K1\ p + K3\ K2\ K1\ p^3 + K4\ K3\ K2\ K1\ p^4 + K5\ K4\ K3\ K2\ K1\ p^5 + K2\ K1\ p^2}$$

```
> rm117:=subs({g=1/2,p=q},rm116);
```

$$rm117 := \frac{1}{2} = \frac{1}{1 + K1\ q + K3\ K2\ K1\ q^3 + K4\ K3\ K2\ K1\ q^4 + K5\ K4\ K3\ K2\ K1\ q^5 + K2\ K1\ q^2}$$

```
> rm118:=K1=solve(rm117,K1);
```

$$rm118 := K1 = \frac{1}{q\,(1 + K3\ K2\ q^2 + K4\ K3\ K2\ q^3 + K5\ K4\ K3\ K2\ q^4 + K2\ q)}$$

```
> rm119:=subs(rm118,rm116);
```

$$rm119 := g = 1 \Big/ \left(1 + \frac{p}{q\,(1 + K3\ K2\ q^2 + K4\ K3\ K2\ q^3 + K5\ K4\ K3\ K2\ q^4 + K2\ q)} + \frac{K3\ K2\ p^3}{q\,(1 + K3\ K2\ q^2 + K4\ K3\ K2\ q^3 + K5\ K4\ K3\ K2\ q^4 + K2\ q)} \right.$$

$$+\frac{K4\,K3\,K2\,p^4}{q\,(1+K3\,K2\,q^2+K4\,K3\,K2\,q^3+K5\,K4\,K3\,K2\,q^4+K2\,q)}$$
$$+\frac{K5\,K4\,K3\,K2\,p^5}{q\,(1+K3\,K2\,q^2+K4\,K3\,K2\,q^3+K5\,K4\,K3\,K2\,q^4+K2\,q)}$$
$$+\frac{K2\,p^2}{q\,(1+K3\,K2\,q^2+K4\,K3\,K2\,q^3+K5\,K4\,K3\,K2\,q^4+K2\,q)}\Bigg)$$

```
> rm120:=diff(rhs(rm119),p);
```

$$rm120 := -\left(\frac{1}{q\,(1+K3\,K2\,q^2+K4\,K3\,K2\,q^3+K5\,K4\,K3\,K2\,q^4+K2\,q)}\right.$$
$$+3\,\frac{K3\,K2\,p^2}{q\,(1+K3\,K2\,q^2+K4\,K3\,K2\,q^3+K5\,K4\,K3\,K2\,q^4+K2\,q)}$$
$$+4\,\frac{K4\,K3\,K2\,p^3}{q\,(1+K3\,K2\,q^2+K4\,K3\,K2\,q^3+K5\,K4\,K3\,K2\,q^4+K2\,q)}$$
$$+5\,\frac{K5\,K4\,K3\,K2\,p^4}{q\,(1+K3\,K2\,q^2+K4\,K3\,K2\,q^3+K5\,K4\,K3\,K2\,q^4+K2\,q)}$$
$$\left.+2\,\frac{K2\,p}{q\,(1+K3\,K2\,q^2+K4\,K3\,K2\,q^3+K5\,K4\,K3\,K2\,q^4+K2\,q)}\right)\Bigg/\left(1\right.$$
$$+\frac{p}{q\,(1+K3\,K2\,q^2+K4\,K3\,K2\,q^3+K5\,K4\,K3\,K2\,q^4+K2\,q)}$$
$$+\frac{K3\,K2\,p^2}{q\,(1+K3\,K2\,q^2+K4\,K3\,K2\,q^3+K5\,K4\,K3\,K2\,q^4+K2\,q)}$$
$$+\frac{K4\,K3\,K2\,p^3}{q\,(1+K3\,K2\,q^2+K4\,K3\,K2\,q^3+K5\,K4\,K3\,K2\,q^4+K2\,q)}$$
$$+\frac{K5\,K4\,K3\,K2\,p^4}{q\,(1+K3\,K2\,q^2+K4\,K3\,K2\,q^3+K5\,K4\,K3\,K2\,q^4+K2\,q)}$$
$$\left.+\frac{K2\,p^2}{q\,(1+K3\,K2\,q^2+K4\,K3\,K2\,q^3+K5\,K4\,K3\,K2\,q^4+K2\,q)}\right)^2$$

```
> rm121:=subs(q=p,rm120);
```

$$rm121 := -\left(\frac{1}{p\,(1+K3\,K2\,p^2+K4\,K3\,K2\,p^3+K5\,K4\,K3\,K2\,p^4+K2\,p)}\right.$$
$$+3\,\frac{K3\,K2\,p}{1+K3\,K2\,p^2+K4\,K3\,K2\,p^3+K5\,K4\,K3\,K2\,p^4+K2\,p}$$

$$+4\frac{K4\,K3\,K2\,p^2}{1+K3\,K2\,p^2+K4\,K3\,K2\,p^3+K5\,K4\,K3\,K2\,p^4+K2\,p}$$

$$+5\frac{K5\,K4\,K3\,K2\,p^3}{1+K3\,K2\,p^2+K4\,K3\,K2\,p^3+K5\,K4\,K3\,K2\,p^4+K2\,p}$$

$$+2\frac{K2}{1+K3\,K2\,p^2+K4\,K3\,K2\,p^3+K5\,K4\,K3\,K2\,p^4+K2\,p}\Bigg)\Bigg/\Bigg(1$$

$$+\frac{1}{1+K3\,K2\,p^2+K4\,K3\,K2\,p^3+K5\,K4\,K3\,K2\,p^4+K2\,p}$$

$$+\frac{K3\,K2\,p^2}{1+K3\,K2\,p^2+K4\,K3\,K2\,p^3+K5\,K4\,K3\,K2\,p^4+K2\,p}$$

$$+\frac{K4\,K3\,K2\,p^3}{1+K3\,K2\,p^2+K4\,K3\,K2\,p^3+K5\,K4\,K3\,K2\,p^4+K2\,p}$$

$$+\frac{K5\,K4\,K3\,K2\,p^4}{1+K3\,K2\,p^2+K4\,K3\,K2\,p^3+K5\,K4\,K3\,K2\,p^4+K2\,p}$$

$$+\frac{K2\,p}{1+K3\,K2\,p^2+K4\,K3\,K2\,p^3+K5\,K4\,K3\,K2\,p^4+K2\,p}\Bigg)^2$$

```
> rm122:=limit(rm121,{K2=infinity,K3=infinity,K4=infinity,K5=infi
  nity});
```

$$rm122 := -\frac{5}{4}\frac{1}{p}$$

Uncorrelated Rectangles

```
> restart;
```

Comparison of Areas

```
> rm1 := -1/2*epsilon^2*k2*k1/(-1+epsilon);
```

$$rm1 := -\frac{1}{2}\frac{\varepsilon^2\, k2\, k1}{-1+\varepsilon}$$

```
> rm2 :=
-epsilon*(-3+epsilon)*k2*(3/2*k1*epsilon+1/2*k1*epsilon^2)/((-2
+epsilon)*(-1+epsilon)*(2+epsilon));
```

$$rm2 := -\frac{\varepsilon\,(-3+\varepsilon)\,k2\left(\frac{3}{2}k1\,\varepsilon+\frac{1}{2}k1\,\varepsilon^2\right)}{(-2+\varepsilon)(-1+\varepsilon)(2+\varepsilon)}$$

```
> rm3 :=
-8*epsilon^2*k2*k1/((2-2*epsilon+epsilon^2)*(-1+epsilon)*(2+2*e
psilon+epsilon^2))+1/2*epsilon^4*k2*k1/((2-2*epsilon+epsilon^2)
*(-1+epsilon)*(2+2*epsilon+epsilon^2))-1/2*epsilon^6*k2*k1/((2-
2*epsilon+epsilon^2)*(-1+epsilon)*(2+2*epsilon+epsilon^2));
```

$$rm3 := -8\frac{\varepsilon^2\, k2\, k1}{(2-2\varepsilon+\varepsilon^2)(-1+\varepsilon)(2+2\varepsilon+\varepsilon^2)}+\frac{\frac{1}{2}\varepsilon^4\, k2\, k1}{(2-2\varepsilon+\varepsilon^2)(-1+\varepsilon)(2+2\varepsilon+\varepsilon^2)}$$
$$-\frac{1}{2}\frac{\varepsilon^6\, k2\, k1}{(2-2\varepsilon+\varepsilon^2)(-1+\varepsilon)(2+2\varepsilon+\varepsilon^2)}$$

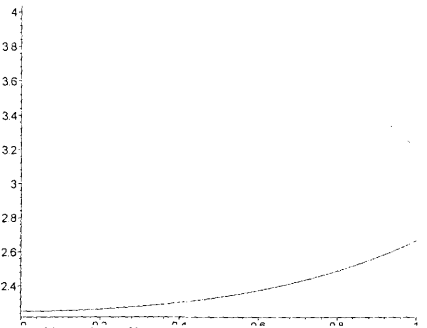
```
> rmc01:=rm2/rm1;
```

$$rmc01 := 2\frac{(-3+\varepsilon)\left(\frac{3}{2}k1\,\varepsilon+\frac{1}{2}k1\,\varepsilon^2\right)}{\varepsilon(-2+\varepsilon)(2+\varepsilon)\,k1}$$

```
> rmc02:=rm3/rm1;
```

$$rmc02 := -2\left(\left(-8\frac{\varepsilon^2\, k2\, k1}{(2-2\varepsilon+\varepsilon^2)(-1+\varepsilon)(2+2\varepsilon+\varepsilon^2)}\right.\right.$$
$$\left.\left.+\frac{\frac{1}{2}\varepsilon^4\, k2\, k1}{(2-2\varepsilon+\varepsilon^2)(-1+\varepsilon)(2+2\varepsilon+\varepsilon^2)}-\frac{1}{2}\frac{\varepsilon^6\, k2\, k1}{(2-2\varepsilon+\varepsilon^2)(-1+\varepsilon)(2+2\varepsilon+\varepsilon^2)}\right)(-1+\varepsilon)\right)$$
$$\Big/(\varepsilon^2\, k2\, k1)$$

```
> plot({rmc01,rmc02},epsilon=0..1);
```




```
> limit(rmc01,epsilon=0);
```

$$\frac{9}{4}$$

```
> limit(rmc02,epsilon=0);
```

$$4$$

```
> limit(rmc01,epsilon=1);
```

$$\frac{8}{3}$$

```
> limit(rmc02,epsilon=1);
```

$$\frac{16}{5}$$

VITA

Christopher Sewell was born in Houston in 1980 and graduated from Franklin High School in El Paso in 1998. He anticipates graduation from Texas A&M University in December 2001 or May 2002. His permanent mailing address is

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