THE UNIVERSITY OF WARWICK

Modelling the microscopic evolution of molecular aggregates

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Contents

Summary

Endocytosis is an essential function of a living cell. The successful transport of molecules across membranes and through the cytosol depends on the successful formation and disintegration of the transport vesicles which, in turn, depends on the actions of the coat proteins enveloping the vesicles. Our interest lies in clathrin-coated vesicles and their temporal uncoating in the presence of ATP and the proteins, auxilin and Hsc70.

Previous research involved conducting light-scattering experiments on the disintegration of clathrin cages, formed in vitro. The purpose of this project was to analyse the experimental data and obtain a mathematical model which describes the process. The analytical package Origin® was used to obtain timescales for the uncoating and two mathematical models have been presented. The graphs resulting from the mathematical models were compared with the experimental graphs to assess their accuracy.

The results from Origin® and the Parameter model imply that there are two underlying exponential decay processes in the system, one of a consistent timescale averaging 40 seconds and the other more chaotic and of a much larger value. Another conclusion reached was that the mathematical model based on Michaelis-Menten kinetics was the more satisfactory match to the experimental data, giving average kinetic rate constants of $k_1 = 0.00165$ $(\text{moldm}^{-3})^{-1}\text{s}^{-1}$ and $k_3 = 0.002$ s^{-1} for the forward reactions in the two-step process.

More information regarding the correct stoichiometry process would allow for a more accurate and representative model of the system. This would require more experimental data. Repeated experiments would also provide satisfactory statistics to confirm the conclusions reached.

Acknowledgements

I would like to thank

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Abbreviations

MM	–	Michaelis-Menten
C_n	–	Concentration of clathrin cages containing *n* triskelions
C_1	–	Concentration of clathrin triskelions

Chapter 1 – Introduction

This chapter will provide an introduction to the research project with some basic background information on the biological system studied, the motivation for the research and an outline of the research conducted.

1.1 – Biological background

The fundamental need of a living organism to communicate with its environment and access external nutrition sources is accomplished in cells through endocytosis. The successful transport of molecules from membranes to the relevant target depends on the transport vesicles which are formed from specialized, coated regions of the membrane. The coat proteins play an important role in that they concentrate the specific membrane proteins to the site on the membrane and then mould them into vesicles around the required molecule, which bud off from the membrane and pass through the cytosol. These coat proteins must then be discarded from the vesicle at the target, allowing the two cytosolic surfaces to interact and fuse, resulting in the successful transfer of the molecule. Our research concentrated on the temporal aspect of this secondary feature of the coat protein.

At present, there are three different types of coat protein, with the most thoroughly studied being that of clathrin. As such, our research was based on clathrin-coated vesicles. Each subunit of clathrin consists of three large and three small polypeptide chains which combine to form a three-legged structure, a triskelion, as seen in Figure 1.1. These triskelions assemble to form pentagon- and hexagon-faced cages around the vesicles.

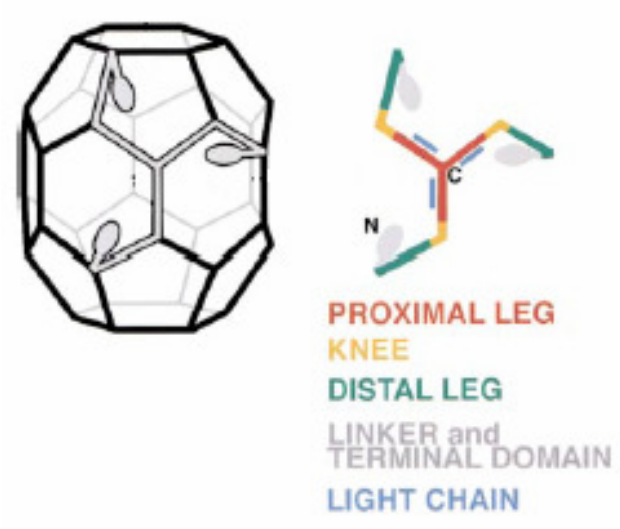

Figure 1.1: An individual triskelion and its positioning in a clathrin cage
(Image from Musacchio et.al., *Molecular Cell* **3** [1999])

This diagram shows the different sections of a clathrin triskelion, including the positioning of the C– and N–termini of the peptide. The names are given in the corresponding colours on the image. It also shows the position of an individual triskelion in the clathrin cage.

The uncoating process is activated by the Hsc70, a chaperone protein of the hsp70 heat shock protein family. Hsc70 binds at critical junctions in the clathrin lattice, thus breaking the connection between the subunits and causing the disintegration of the cage into the separate triskelions. This process requires energy which is obtained from the hydrolysis of ATP. It is believed that three Hsc70–ATP molecules are involved in the disengaging of one triskelion from the cage [Heymann, J. B., Iwasaki, K., et. al. (2005)]

The protein, auxilin is also present in the complete clathrin cage. It is a DNA J homolog and is believed to act as a co-chaperone to Hsc70 during the uncoating process. Each auxilin molecule binds within the lattice at a point between an inward-projecting C–terminal helical tripod and the crossing of two 'knee' segments (see Figures 1.1 and 1.2), and contacts the terminal domain of another clathrin 'leg'. Fotin et. al. suggest that the auxilin molecule recruits Hsc70 to this critical point where it will interact with the triskelions, releasing them from the lattice [Fotin, A., Cheng, Y., et. al. (2004)].

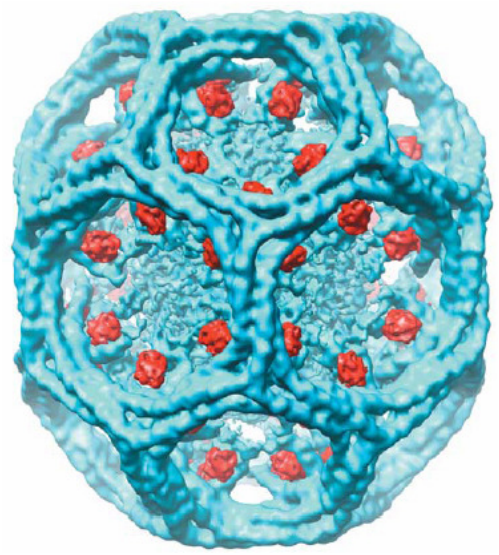

Figure 1.2: Location of auxilin within the clathrin cage
(Image from Fotin et.al., *Nature* **432** [2004])

A clathrin D6 barrel with bound auxilin. This is a 3D reconstruction at 12 Å resolution. The auxilin molecules are shown in red, whereas the rest of the structure is in blue. The location of the auxilin molecules was evaluated by comparing the electron micrograph of an auxilin-bound cage with that of a cage that was known to be free of auxilin.

Since our interest lay in the time-dependent uncoating of vesicles, experiments were conducted on clathrin cages, formed in vitro using clathrin obtained from pig brain. The timescale on which the disintegration of the cages occurred in the presence of auxilin, Hsc70 and ATP was measured.

1.2 – Project Outline

As previously stated, endocytosis is a central process in a living cell and, as such, of great importance to molecular biologists. Although much research has been done on the subject, key questions, such as 'why does uncoating occur at the target but not before in the cytosol when the same activating agents exist?' remain unanswered. In order to address issues such as this, it is integral that we understand more about the uncoating process, in particular the time-scale over which it occurs as this would allow us to examine the relation between transportation and uncoating times. Our research aims to discover the kinetics behind the uncoating process.

The experimental portion of this research was conducted previously, providing a range of data with which the results provided by our models could be compared. The experimental data is a collection of graphs, obtained from a fluorimeter, which indicate the quantity of auxilin-bound clathrin cages, as opposed to triskelions, over a time period during which Hsc70 and ATP are added. The typical graph shows a decay, as will be explained in detail in Chapter 3, which was analysed using the analysis package Origin® (Chapter 4) and then used to design our mathematical models.

There are two mathematical models. The first was derived from a basic reaction scheme and was dependent on parameters related to the conditions of the experiment and the stoichiometry of the reactions. This is discussed further in Chapter 5. The second was formulated by applying Michaelis-Menten kinetics to the system and was dependent on the rates of the reactions, as will be described in Chapter 6.

Ultimately, the two models were compared, with significant observation and suggestions for improvement in Chapter 7. Since the majority of graphs and comparisons were very similar, for ease of reading only a representative graph is presented in each chapter. However, all the graphs worked on can be found in Appendix A.

Chapter 2 – Materials and Methods

This chapter gives a brief overview of the experimental methods and materials used in the obtaining of the experimental data, as well as information on the computational packages used in the analysis of said data and the implementation of the models in order to provide theoretical graphs to be compared with the experimental graphs.

2.1 – Obtaining Experimental Data

Clathrin was purified from pig brain cells via a series of centrifugations and column filtrations. This supply of triskelions was spun down to form cages, which they do preferably at a pH of 6. Auxilin and Hsc70 were expressed in *E.Coli*, courtesy of Sarah Batson and Dr. Corinne Smith. The experiments were conducted in Barouch buffer of pH 7, with auxilin added to the clathrin in the cuvette first. The sample was then placed in a LS-50 Perkin-Elmer fluorimeter and a timedrive of the intensity, with excitation and emission wavelengths of 390 nm, an emission slit width of 20 nm, an excitation slit width of 15nm and a response time of 2 s. Once a constant signal was obtained, first Hsc70 and then ATP were added to the mixture. Although extreme care was taken to avoid the possibility of air bubbles in the mixture when the pipette was inserted into the cuvette, at times these could not be avoided. Therefore, such 'contaminated' results were omitted from the analysis and comparisons.

2.2 – Mathematical and Analytical Packages

The data points resulting from the fluorimeter timedrives were plotted in Miscrosoft Excel.

These data points were directly imported into Origin® 6.1 (copyright © 1991–2000 OriginLab Corporation) where they were analysed using the variety of graph fitting techniques.

The mathematical models were implemented using MATLAB Version 6.0.0.88 Release 12 (copyright © 1984–2000 The MathWorks, Inc.).

Comparisons between the graphs were made by directly overlaying the theoretical graph on the experimental one and looking for the best fit.

Chapter 3 – Experimental Data

This chapter explains the selection of the specific wavelengths for the light scattering experiments and also the implications of the resulting graph.

3.1 – Light Scattering

Light is made up of particles known as photons. To shine a beam of light on the sample is to effectively direct a stream of photons through the mixture. As they pass through the sample, they are scattered by the molecules in the sample. The wavelength of the light determines the manner in which the molecules affect the beam. If the wavelength is much smaller than the size of the molecules, the light beam will pass right through without being affected, giving us a saturated signal. However, should the wavelength be of the same order as the size of the molecule, the light will be scattered and give a reasonable signal.

To ascertain the most suitable wavelength, a series of experiments were conducted on a sample of clathrin cages and resulted in the value of 390 nm being decided upon as it gave the largest intensity without saturation. Although wavelengths slightly to either side of 390 nm also gave a significant signal, using this wavelength meant that less clathrin would give a higher signal.

Since this value for the wavelength was used throughout the timedrive, as the cages disintegrated into triskelions, which are of smaller dimensions, we would expect the signal to reduce in intensity. This is due to the smaller molecules absorbing the energy from the light.

The theory behind the experiments was that of measuring the intensity of the light scattered off the clathrin cages. To do this successfully and accurately it is necessary to use the appropriate excitation wavelength.

3.2 – Typical Result and Explanation thereof

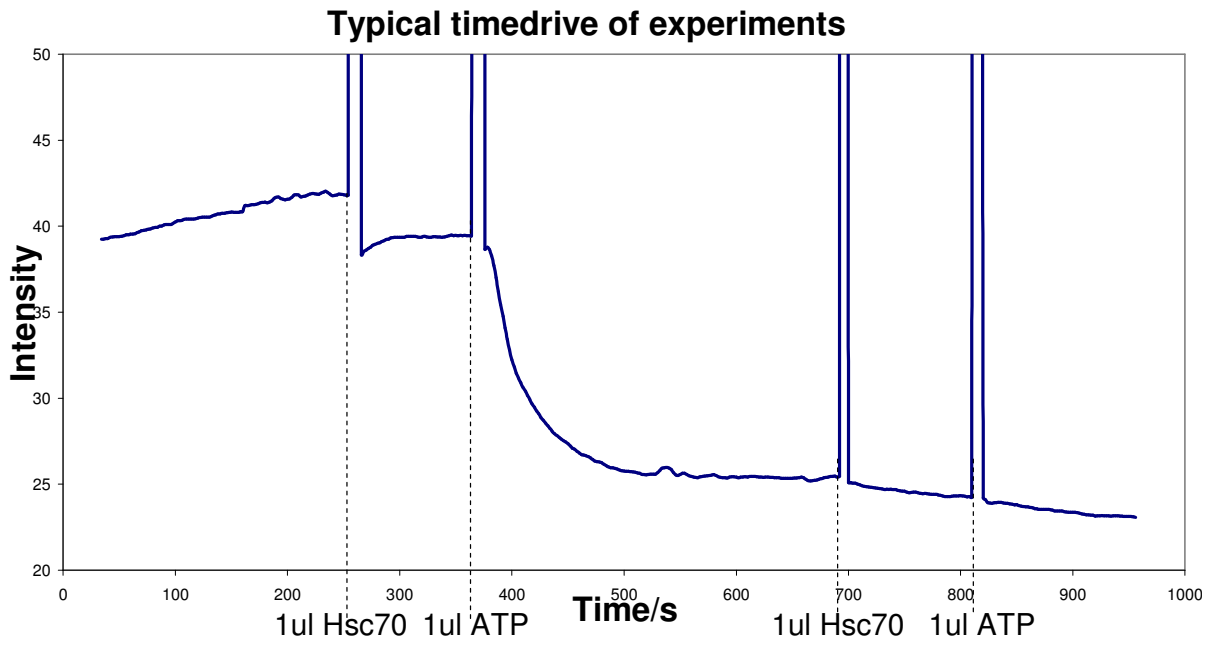

Figure 3.1: Typical timedrive graph of the experiments carried out

The intensity of the scattered light is plotted against time in a typical experiment, where the saturation peaks correspond to the insertion of the pipette with the Hsc70 or ATP.

As can be seen in Figure 3.1, the intensity of the light scattered off the sample is detected over time. The initial sample contains auxilin-bound clathrin cages. The signal is allowed to settle to a constant level before the addition of the other compounds. The slight variations are due to the Brownian motion of the molecules in the solution. Then, Hsc70 is introduced to the system using a pipette, which causes the intensity to reach the saturation level. Again the signal is allowed to settle before the addition of the ATP.

It is clear that the signal does not decay without the addition of ATP, which confirms the belief that energy is required for the uncoating process and is obtained from ATP hydrolysis. The instant drop in intensity seen at the first addition is not consistent throughout the experiments. In some instances the intensity decreased, at others it increased and sometimes remained unchanged. Reductions in intensity can be attributed to proteins being picked out (unintentionally and unavoidably) by the pipette tip and increments to the addition of proteins to the solution.

Once the ATP is added, there is a definite decay of the signal. This is clearly due to the disintegration of the cages to triskelions. The triskelions which are smaller in size than the cages do not scatter light of wavelength 390 nm effectively and, as a result, do not give a signal. Hence, the gradual decay of the signal as the cages disintegrate into their separate subunits.

This decay curve is integral to the kinetics of the process of uncoating and provides information as to the reaction rates and timescales of decay. And, these values are that which we hope to obtain from our analysis and mathematical modelling. A key assumption throughout our research and analysis was that the intensity of the signal was directly proportional to the quantity if clathrin cages in the system.

Chapter 4 – Analysis using Origin®

Origin® is an analytical package which can be used to find an equation to fit a given curve. Since it provides a R^2-value for each fit, selecting the most appropriate fit is straight-forward and not completely random. Of course, the fact that the equation fits the curve does not confirm that this is the absolute equation because the program cannot provide all possible equations for each curve. An advantage of using Origin® was the ability to omit any peaks that arise due to bubbles from the points evaluated for the match. However, even though this was interesting, in order to maintain a certain level of accuracy and to improve the statistics these results have been omitted from the mathematical part of the research.

In our analysis, we have attempted to fit an exponential decay to the curves, simply because decays in most biological systems tend to be exponential. Both a first order decay and a second order decay were tried.

A first order exponential decay curve would satisfy the equation

$$I = I_0 + Ae^{-\left(t/T_1\right)}$$

and a second order exponential decay curve would satisfy the equation

$$I = I_0 + A_1 e^{-\left(t/T_1\right)} + A_2 e^{-\left(t/T_2\right)}$$

where I is intensity, t is time, I_0 is the initial intensity, T_1 and T_2 are the time constants and A_1 and A_2 are constants.

The results obtained were as shown in Table 4.1. The experiments were categorized according to the ratio of clathrin cages to auxilin molecules in the initial sample. Hsc70 and ATP were always added in excess, in terms of molarity, thus ensuring that the cages disintegrated completely.

Table 4.1: Time constants for 1st and 2nd order exponential decays

This table contains the time constants predicted by Origin® to fit the graphs obtained from experiment. Each experiment is described in terms of the ratio of clathrin cages to auxilin molecules in the initial sample before the addition of Hsc70 and ATP.

	1st Order	2nd Order	
Clathrin:Auxilin Molar Ratio	T_1 /seconds	T_1 /seconds	T_2 /seconds
10:15	–	33	720
10:9	–	40	350
10:9	–	72	133
10:8	–	30	426
10:6	–	37	246
10:4	–	40	323
10:2	39†	37	43
10:1	–	36	22912
10:1	–	43	199
20:1	–	25	276
30:1	–	57	1492
30:1	–	65	693
40:1	169†	167	281282
50:1	184†	131	530

† *Origin® analysis of these graphs gave good fits for 1st order exponentials as well as 2nd order exponentials.*

These results imply that the experimental curves are more likely to be the result of a 2nd order exponential decay. The values for T_1 are consistently of a similar value and average 40 s, with the judicious omission of a few values, specifically those of the experiments of ratio above 30:1. As can be seen, T_1 increases rapidly for these experiments. This affirms the idea that auxilin recruits Hsc70 to the critical junctions of the cage and, therefore, accelerates the uncoating process since it clearly slows down when the auxilin content is low. Unfortunately, the values for T_2 do not follow an obvious pattern, seeming rather chaotic and impractically large for a few graphs.

This preference for 2nd order decays implies the possibility of two significant processes occurring in the system, one on a faster timescale than the other. This is a useful idea to be used when designing the mathematical models.

Chapter 5 – Mathematical Model 1: Parameter Model

This chapter details the first mathematical model including the reasoning behind the design, the comparison to the experimental data and a discussion of the results.

5.1 – Mathematical Model and MATLAB Code

This model is based on the idea that each clathrin cage comes apart one triskelion at a time. We have only considered the first step in the reaction, since the value of n is unknown. However, since the cages were formed artificially, it is reasonable to assume that n is the same for all the cages. Hence the elemental equation behind the model is

$$C_n \leftrightarrow C_{n-1} + C_1$$

where C_n represents the concentration of clathrin cages consisting of n triskelions,

C_{n-1} is the concentration of cages missing one triskelion and

C_1 is the concentration of clathrin triskelions.

The only possibilities considered when constructing the equations to describe the system were the formation and destruction of the respective entities. Since the experimentation does not provide adequate information on the stoichiometry of the reactions in the system, as both Hsc70 and ATP were always added in excess, or the necessary conditions such as pH and temperature, this method was considered suitable since all these could be included in the parameters of the system. The resulting differential equations describing the rate of change of the components of the system are

$$\frac{dC_n}{dt} = -aC_n + \varepsilon C_1 C_{n-1} \qquad (5.1)$$

$$\frac{dC_{n-1}}{dt} = bC_n - \xi C_1 C_{n-1} \qquad (5.2)$$

$$\frac{dC_1}{dt} = dC_n - \eta C_1 C_{n-1} \qquad (5.3)$$

$$C = C_n + C_{n-1} + C_1 \qquad (5.4)$$

C represents the total concentration of the system and is a constant since this is a closed system. The parameters of the system are a, b, c, ε, ξ and η. The C_1C_{n-1} terms represent the triskelions recombining with the partially disintegrated cage (C_{n-1}) to reform the complete

clathrin cage. However, this is an extremely unlikely occurrence and allows the assumption that ε, ξ and η have very small values, compared to a, b and c. All the parameters are considered to be positive because the term it represents is given a positive or negative sign depending on whether it increases or decreases the concentration of the corresponding component. In equation (5.1), the presence of complete cages implies the forward reaction which decreases the concentration of complete cages and is, therefore a negative contribution, whereas the backward reaction provides a positive contribution to the concentration of complete cages. Conversely, in equations (5.2) and (5.3) the presence of complete cages encourages the forward reaction, producing more partial cages and triskelions which is a positive contribution.

To implement this system of differential equations in MATLAB, it was necessary to discretise the equations, as the Euler method of integration was being used by the programme. This resulted in the following system of equations.

$$t(n+1) = nh \tag{5.5}$$

$$\dot{x} = \frac{x(n+1) - x(n)}{h} = -ax(n) + \varepsilon y(n)[C - x(n) - y(n)] \tag{5.6}$$

$$\dot{y} = \frac{y(n+1) - y(n)}{h} = dx(n) - \eta y(n)[C - x(n) - y(n)] \tag{5.7}$$

where, x and y are equivalent to C_n and C_1. Equation (5.4) was used to eliminate the C_{n-1} term. The integration is done stepwise using steps of size h = 0.1 for N steps, where N = T/h and T is the total integration time. ε and η were fixed at very small values of 0.0000000001 and 0.00000000001. The complete code can be found in Appendix B.

5.2 – Graph Fitting

The values of a and d were varied until graphs were obtained which were as similar as possible to the experimental graphs. This process was repeated for every experiment, all of which are in Appendix A. Figure 5.1 is a representative of the typical graph obtained, with the theoretical graph overlaid on the experimental one. Figure 5.2 shows an almost perfect, but rare fit. The complete set of parameter values obtained is presented in Table 5.1 and all the graphs are in Appendix A.

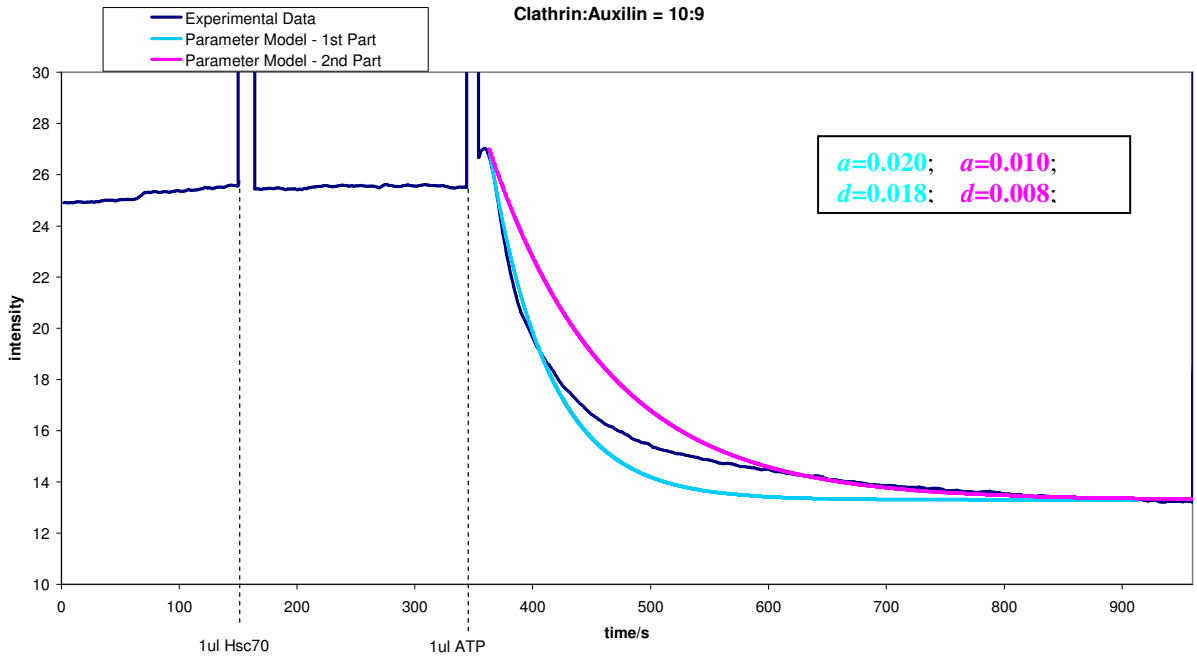

Figure 5.1: A typical example of a graph fitting

These are the graphs obtained from the Parameter Model overlaid on the experimental data of the clathrin to auxilin ratio 10:9 experiment. The experimental data is in dark blue. The 2 theoretical graphs are in light blue and pink, with the former fitting better at the initial stage and the latter fitting the end stage. The corresponding parameters are as shown.

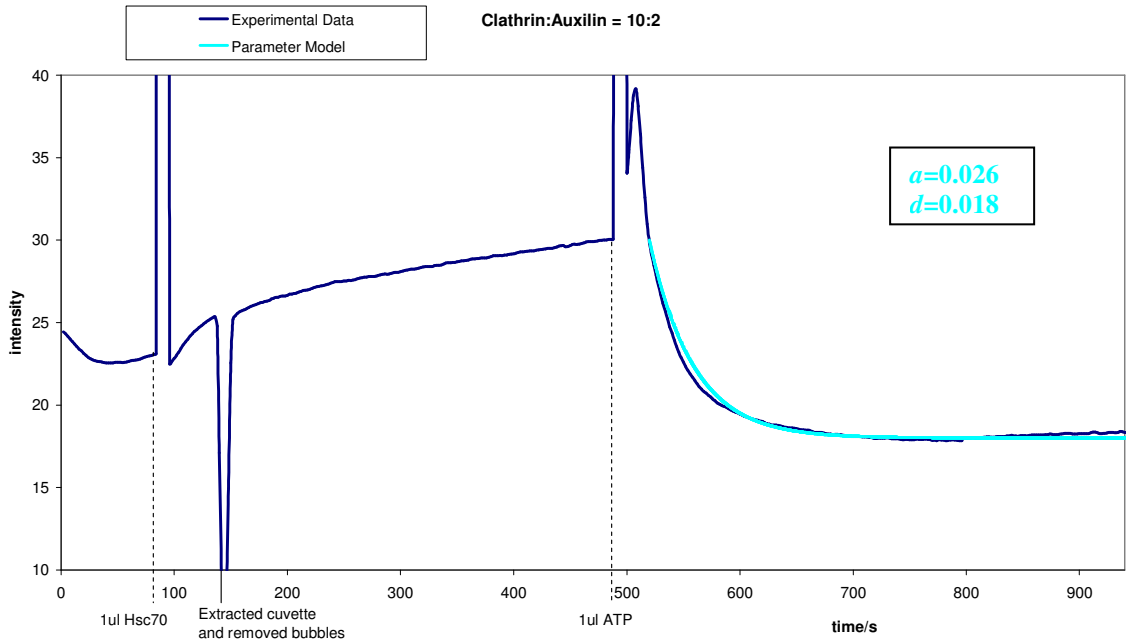

Figure 5.2: The best fit for the Parameter Model

This is the graph obtained from the Parameter Model overlaid on the experimental data of the clathrin to auxilin ratio 10:2. The experimental and theoretical data are in dark and light blue, respectively. This was the best fit for this model with the given parameters.

5.3 – Results and Analysis

Table 5.1: The complete set of parameters

This is a list of the most appropriate parameters obtained for all the experiments from the Parameter Model. Some showed better fits than others as can be seen in Appendix A.

	Fit to initial rapid uncoating		Fit to final slow uncoating	
Clathrin:Auxilin Molar Ratio	***a***	***d***	***a***	***d***
10:15	0.028	0.018	0.018	0.008
10:9	0.020	0.018	0.010	0.008
10:9	0.019	0.018	0.017	0.008
10:8	0.024	0.018	0.010	0.008
10:6	0.022	0.018	0.017	0.008
10:4	0.020	0.018	0.010	0.008
10:2	0.026*	0.018*	–	–
10:1	0.026	0.018	0.010	0.008
10:1	0.019	0.018	–	–
20:1	0.023	0.018	0.010	0.009
30:1	0.015	0.010	0.010	0.008
30:1	0.013	0.010	0.009	0.007
40:1	0.006	0.001	0.010	0.005
50:1	0.006*	0.001*	–	–

* *These values for the parameters gave a fit for the entire graph.*

As can be seen from Figure 5.1, it was not possible to obtain parameters that gave a good fit for the entire graph, except for just two experiments (see Figure 5.2). Therefore, attempts were made to find parameters for graphs that fit, firstly, the initial stages of the decay and, then, the latter stages of the decay, as it was observed that any value for the parameter would result in a graph that fit only one part of the experimental data. As such, the parameter values seen in Table 5.1 were obtained.

These show quite a clear consistency in the values for *d* in both theoretical graphs. The value for *a* is less consistent, yet is quite similar except for a few instances. There is a gradual decrease in the value for *a* and *d* as the clathrin to auxilin ratio of the experiments increases above 30:1. This agrees with the Origin® result, which shows an increase in the time constant at about the same ratio.

5.4 – Problems and Improvements

The primary observation arising from this model is that it does not provide graphs that fit very well with the experimental data and this is obviously not satisfactory. We can conclude that the system of equations does not fully describe the biological system. This is expected, as we have grouped all possible effects from the other components in the system into the parameters. Unfortunately, this was an unavoidable necessity since there was insufficient information about these possible effects available from previous research and experimentation.

However, this model is not totally ineffectual as it does corroborate the hypothesis, arising from the Origin® analysis, that there appears to be two separate sub-processes occurring in the uncoating process. To further explore this interesting observation, we would require more experimentation and possibly a more in depth look at the system. Including this extra information in the form of more equations or constraints to the above equations would definitely give a more accurate portrayal of the process. In our next model, we have attempted to do just this by including the effect of Hsc70 on the process.

Chapter 6 – Mathematical Model 2: Michaelis-Menten Model

This second model is based on Michaelis-Menten (MM) kinetics and attempts to portray the uncoating process in more detail than the previous model by including Hsc70 in the reaction.

6.1 – Mathematical Model and MATLAB Code

This model uses the Michaelis-Menten method of formulating a system of equations based on a multiple-step reaction equation, linking the different components of the equation through rate constants. Our reaction equation suggests the reversible formation of an active substrate, a combination of Hsc70 and the clathrin cage, before the disintegration of the cage begins.

$$C_n + H \underset{k_2}{\overset{k_1}{\longleftrightarrow}} C_n \text{^} H \xrightarrow{k_3} C_{n-1} + H + C_1$$

H represents the concentration of Hsc70 in the system and C_n^H is the compound formed in the intermediate step, with the other symbols having the same meaning as in the previous model. k_1, k_2 and k_3 are the reaction rate constants for the indicated reactions.

This reaction equation gives rise to the following system of equation.

$$\dot{C}_n = -k_1 C_n H + k_2 C_n \text{^} H \qquad (6.1)$$

$$\dot{C}_{n-1} = k_3 C_n \text{^} H \qquad (6.2)$$

$$\dot{C}_n \text{^} H = k_1 C_n H - k_2 C_n \text{^} H - k_3 C_n \text{^} H \qquad (6.3)$$

$$\dot{C}_1 = k_3 C_n \text{^} H \qquad (6.4)$$

$$\dot{H} = -k_1 C_n H + k_2 C_n \text{^} H + k_3 C_n \text{^} H \qquad (6.5)$$

These equations are obtained by considering whether each compound exerts a positive or negative influence on the compound being considered. For example, the reverse reaction of C_n^H causes the formation of C_n, at a rate proportional to the rate constant k_2, which is a gain for C_n resulting in a positive $k_2 C_n$^H term. Conversely, C_n and H combine to reduce the number of C_n in the system and is, therefore assigned a negative sign.

As in the previous model, these equations are discretised to implement in the MATLAB code, which can be found in full in Appendix B. With C_n, C_1, C_n^H, C_{n-1} and H represented by *x, y, z, v* and *w,* the discretised equations are

$$\frac{x(n+1)-x(n)}{h} = k_2 z(n) - k_1 x(n) w(n) \qquad (6.6)$$

$$\frac{v(n+1)-v(n)}{h} = k_3 z(n) \qquad (6.7)$$

$$\frac{z(n+1)-z(n)}{h} = k_1 x(n) w(n) - (k_2 + k_3) z(n) \qquad (6.8)$$

$$\frac{y(n+1)-y(n)}{h} = k_3 z(n) \qquad (6.9)$$

$$\frac{w(n+1)-w(n)}{h} = (k_2 + k_3) z(n) - k_1 x(n) w(n) \qquad (6.8)$$

Again, since the reverse reaction is rather unlikely, the value of k_2 was kept constant at 0.00000005, a much lower value than those of k_1 and k_3.

6.2 – Graph Fitting

The graph fitting procedure followed was the same as in the previous model, with the values of k_1 and k_3 being changed until the resulting graph agreed with the experimental graph.

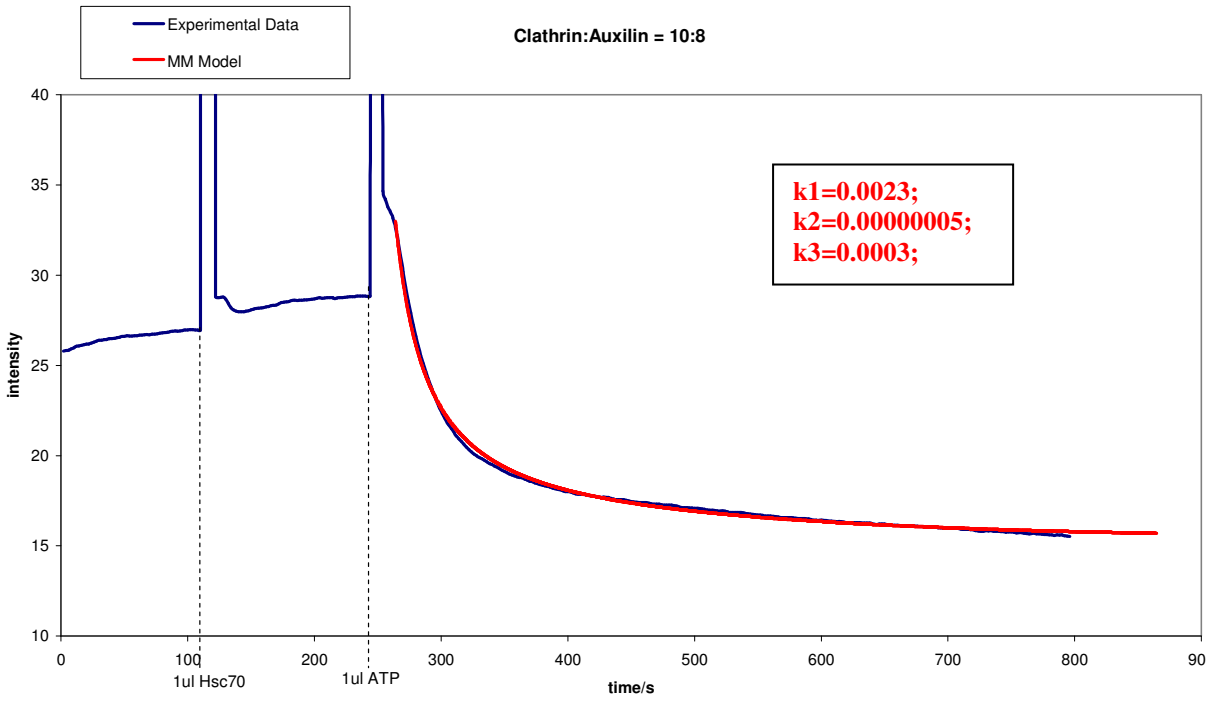

Figure 6.1: A typical example of a graph fitting

This is the graph obtained from the MM Model overlaid on the experimental data of the clathrin to auxilin ratio 10:8 experiment. The experimental data is in dark blue. The theoretical graph is in red. The corresponding parameters are as shown.

6.3 – Results and Analysis

Figure 6.1 is an example of the typical overlaying of the theoretical graph obtained from the Michaelis-Menten (MM) Model on the experimental data. In general, the graphs fit extremely well and the complete set of values obtained for the reaction rate constants are presented in Table 6.1.

Table 6.1: The complete set of reaction rate constants

This is a list of the most appropriate reaction rate constants obtained for all the experiments from the MM Model. The complete set of graphs is in Appendix A.

Clathrin:Auxilin Molar Ratio	k_1	k_2	k_3
10:15	0.00300	0.00000005	0.0015
10:9	0.00250	0.00000005	0.0010
10:9	0.00300	0.00000005	0.0010
10:8	0.00230	0.00000005	0.0003
10:6	0.00220	0.00000005	0.0050
10:4	0.00170	0.00000005	0.0020
10:2	0.00450	0.00000005	0.0040
10:1	0.00022	0.00000005	0.0010
10:1	0.00012	0.00000005	0.0030
20:1	0.00010	0.00000005	0.0004
30:1	0.00013	0.00000005	0.0020
30:1	0.00007	0.00000005	0.0025
40:1	0.00004	0.00000005	0.0080
50:1	0.00004	0.00000005	0.0040

Having kept the value of k_2 constant at 0.00000005, it is observed that k_1 shows a general decrease in value with the decrease in quantity of auxilin in the system (with an average of 0.00165 $(moldm^{-3})^{-1}s^{-1}$) and the values of k_3, averaging 0.002 s^{-1}, do not appear to have any obvious pattern. This first observation agrees with the idea that auxilin aids in recruiting Hsc70 to the critical points on the clathrin lattice, since the decrease in auxilin results in a slower reaction rate for the first reaction which is that of the formation of the clathrin-Hsc70 intermediate. The second observation is disappointing, in that this is the disintegration step and we would have hoped for some correlation between the values. Of course, this could

imply that the quantity of auxilin in the system has no effect on the actual disintegration step in the process, but merely recruits the Hsc70 to the specific points on the cage, thus speeding up the overall process. As such, it would definitely be beneficial to investigate the relationship between Hsc70 and auxilin.

6.4 – Problems and Improvements

The Michaelis-Menten model provides much better fits than the Parameter Model and also more useful information in the form of reaction rate constants. However, it is still not complete and would undoubtedly benefit from the addition of more information, such as the stoichiometry of the Hsc70 in the reaction equation. This would provide a more representative model of the system and result in more accurate values for the reaction rate constants. It is likely that this would result in a correlation between the values of k_3 and the amount of Hsc70 in the system. Inclusion of the effects of ATP would further improve the model since the energy providing step would influence the kinetics of the entire process significantly. All these improvements would require further experimentation of the same kind but with variations of Hsc70 and ATP concentration rather than auxilin.

Chapter 7 – Discussion and Conclusions

Our aim at the start of this research project was to ascertain the kinetics of the process of uncoating of a clathrin-coated vesicle. This was done through analysis of the experiments conducted prior to the beginning of this research and by modelling the system mathematically, in a manner that agreed with the experimental data. Experimental data analysis was conducted using Origin® and two mathematical models, the Parameter Model and the Michaelis-Menten Model, were implemented to analyse different aspects of the system. The results are tabulated in full in Table 7.1.

Three key conclusions have been reached at the end of this research.

1. Auxilin increases the speed of the overall uncoating process by recruiting the activating agent, Hsc70 to the critical sites on the clathrin lattice. This was supported by both the analysis via Origin® and the rate constants of the MM model. Since the effect of auxilin is clear from the research, it is expected that the quantity of Hsc70, the primary activator of uncoating, and ATP, the essential energy-providing compound, present in the system would also provide useful insights into the relation between all the compounds.

2. There are two separate sub-processes occurring within the total uncoating process, with one taking place on a considerably faster timescale than the other. This is evident from the Origin® analysis and the fact that the Parameter model shows two separate fits for the initial and end stages of the data. However, these two sub-processes are probably not the two-steps described in the MM model, as the time constants, parameter values and rate constants do not show any obvious relationship. The explanation to the second sub-process, the first being the actual decay of the cage, could lie in the interaction between auxilin and Hsc70 or the energy-producing reaction between ATP and Hsc70.

3. It is evident that considerably more experimentation needs to be conducted in order to discover more about the system which, in turn, would allow for the significant improvement of the mathematical models, resulting in more accurate and representative parameters and rate constants. Obtaining the stoichiometry of the uncoating process is essential to the progress of the research.

Table 7.1: The complete set of constants obtained from the analysis and mathematical models

	Parameter Model				Michaelis-Menten Model			Origin®		
	Fit to initial rapid uncoating		Fit to final slow uncoating					1st Order	2nd Order	
Clathrin:Auxilin Molar Ratio	***a***	***d***	***a***	***d***	k_1	k_2	k_3	T_1 /seconds	T_1 /seconds	T_2 /seconds
10:15	0.028	0.018	0.018	0.008	0.00300	0.00000005	0.0015	–	33	720
10:9	0.020	0.018	0.010	0.008	0.00250	0.00000005	0.0010	–	40	350
10:9	0.019	0.018	0.017	0.008	0.00300	0.00000005	0.0010	–	72	133
10:8	0.024	0.018	0.010	0.008	0.00230	0.00000005	0.0003	–	30	426
10:6	0.022	0.018	0.017	0.008	0.00220	0.00000005	0.0050	–	37	246
10:4	0.020	0.018	0.010	0.008	0.00170	0.00000005	0.0020	–	40	323
10:2	0.026*	0.018*	–	–	0.00450	0.00000005	0.0040	39†	37	43
10:1	0.026	0.018	0.010	0.008	0.00022	0.00000005	0.0010	–	36	22912
10:1	0.019	0.018	–	–	0.00012	0.00000005	0.0030	–	43	199
20:1	0.023	0.018	0.010	0.009	0.00010	0.00000005	0.0004	–	25	276
30:1	0.015	0.010	0.010	0.008	0.00013	0.00000005	0.0020	–	57	1492
30:1	0.013	0.010	0.009	0.007	0.00007	0.00000005	0.0025	–	65	693
40:1	0.006	0.001	0.010	0.005	0.00004	0.00000005	0.0080	169†	167	281282
50:1	0.006*	0.001*	–	–	0.00004	0.00000005	0.0040	184†	131	530

* These values for the parameters gave a fit for the entire graph.
† Origin® analysis of these graphs gave very good fits for first order exponentials as well as 2nd order exponentials.

To conclude, I believe this project has been very instructive in the possible methods of modelling the uncoating process of clathrin-coated vesicles. Naturally, they are not complete or perfect, but that is an aspect to be studied in the future. Further improvements to the research can be made by looking into the absolute relationship between the intensity of the fluorimeter reading and the concentration of the clathrin cages. A tentative step in this direction during the research resulted in the discovery that information such as the viscosity of the sample would be required and as this was not readily available, further research was temporarily abandoned. The most essential step in any future work, as stated before, would be to discover the stoichiometry of the process. I believe that the key to the successful modelling of this system lies in the reaction between auxilin and Hsc70 and this is one aspect that should be explored in depth. Similarly, the energy production via ATP should also be examined. Should these steps be taken to obtain more detailed information of the system, the modelling of the system would provide conclusive results which would help us understand the process of uncoating and, therefore, bring us closer to understanding the more substantial subject of endocytosis.

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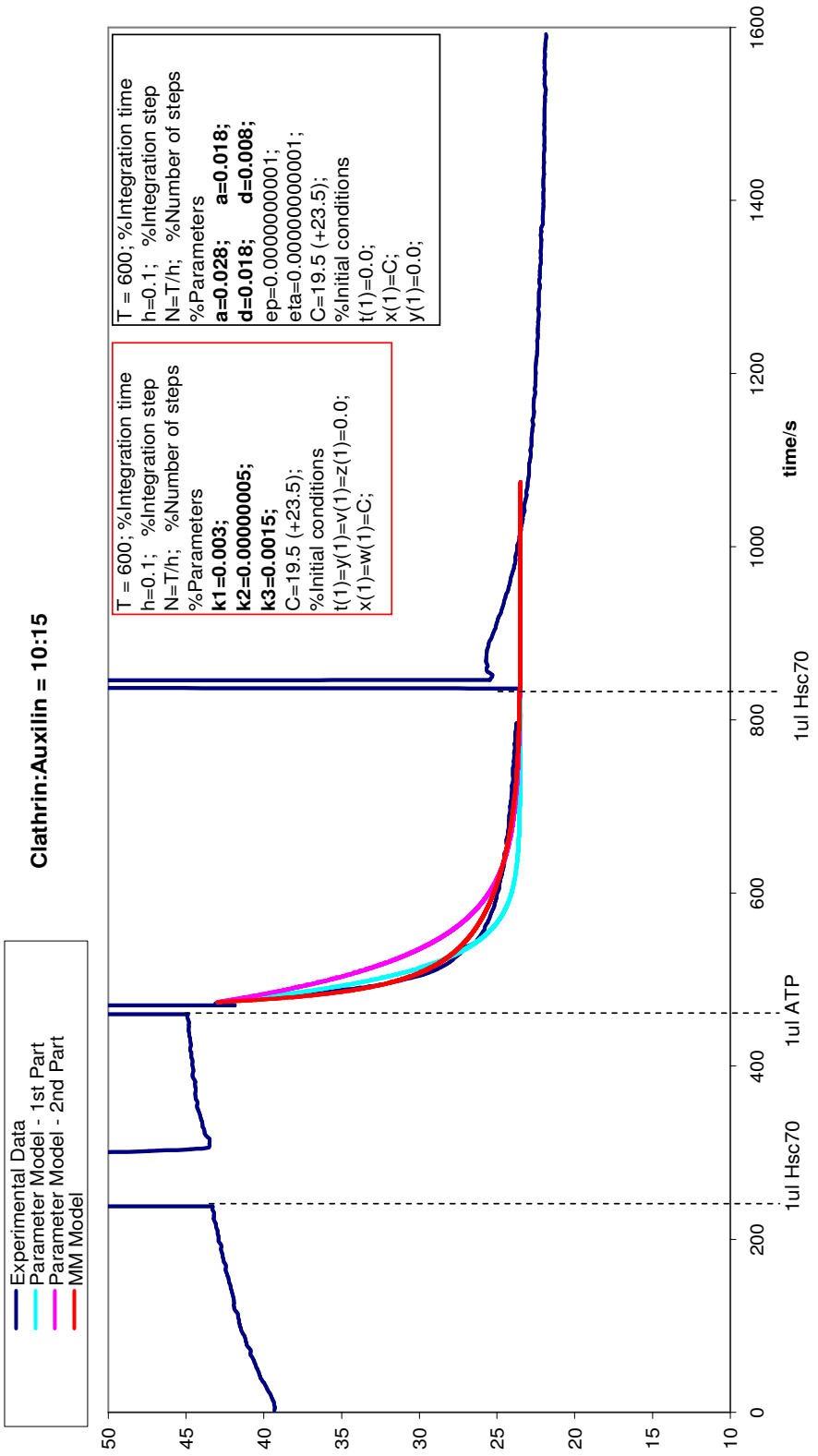
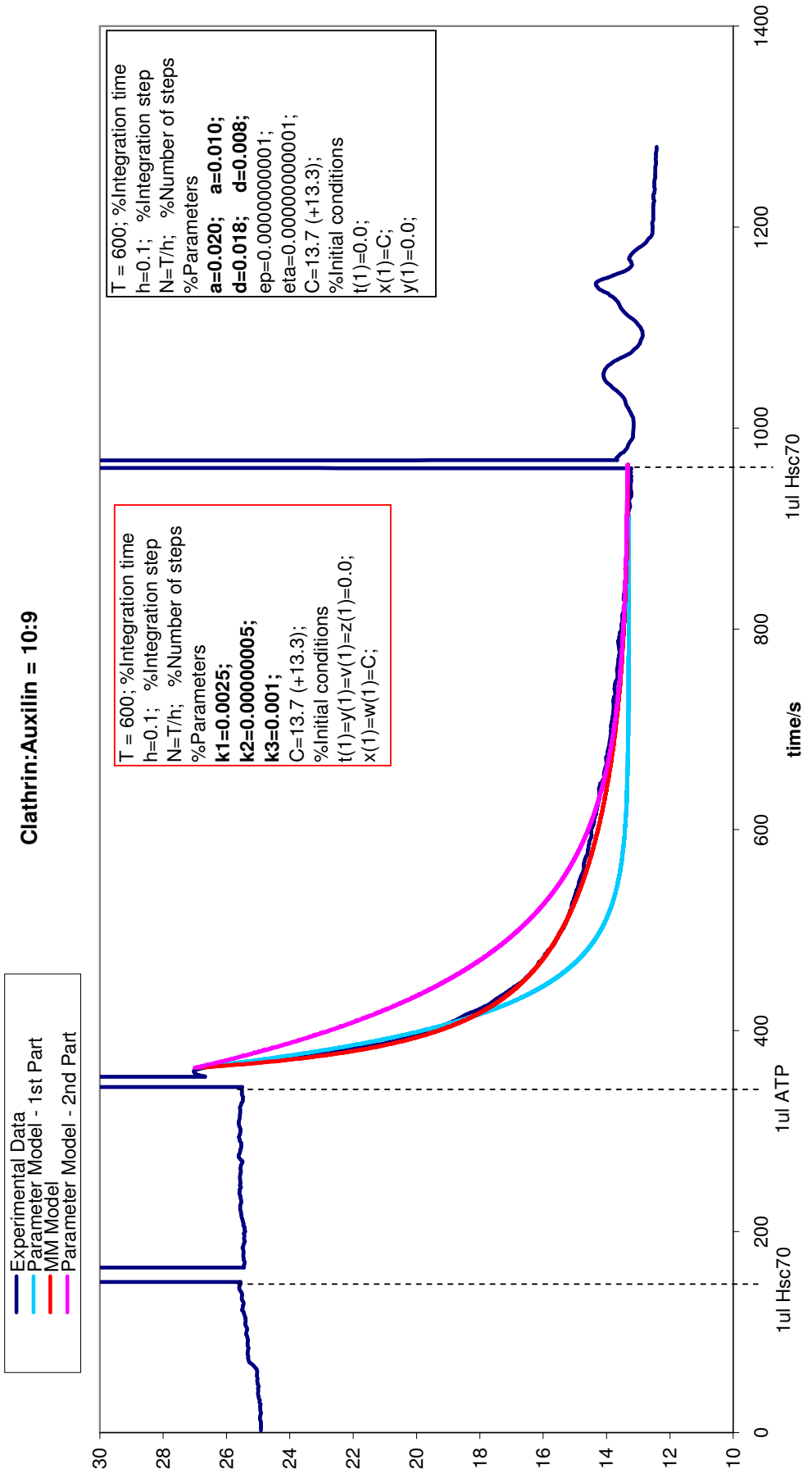
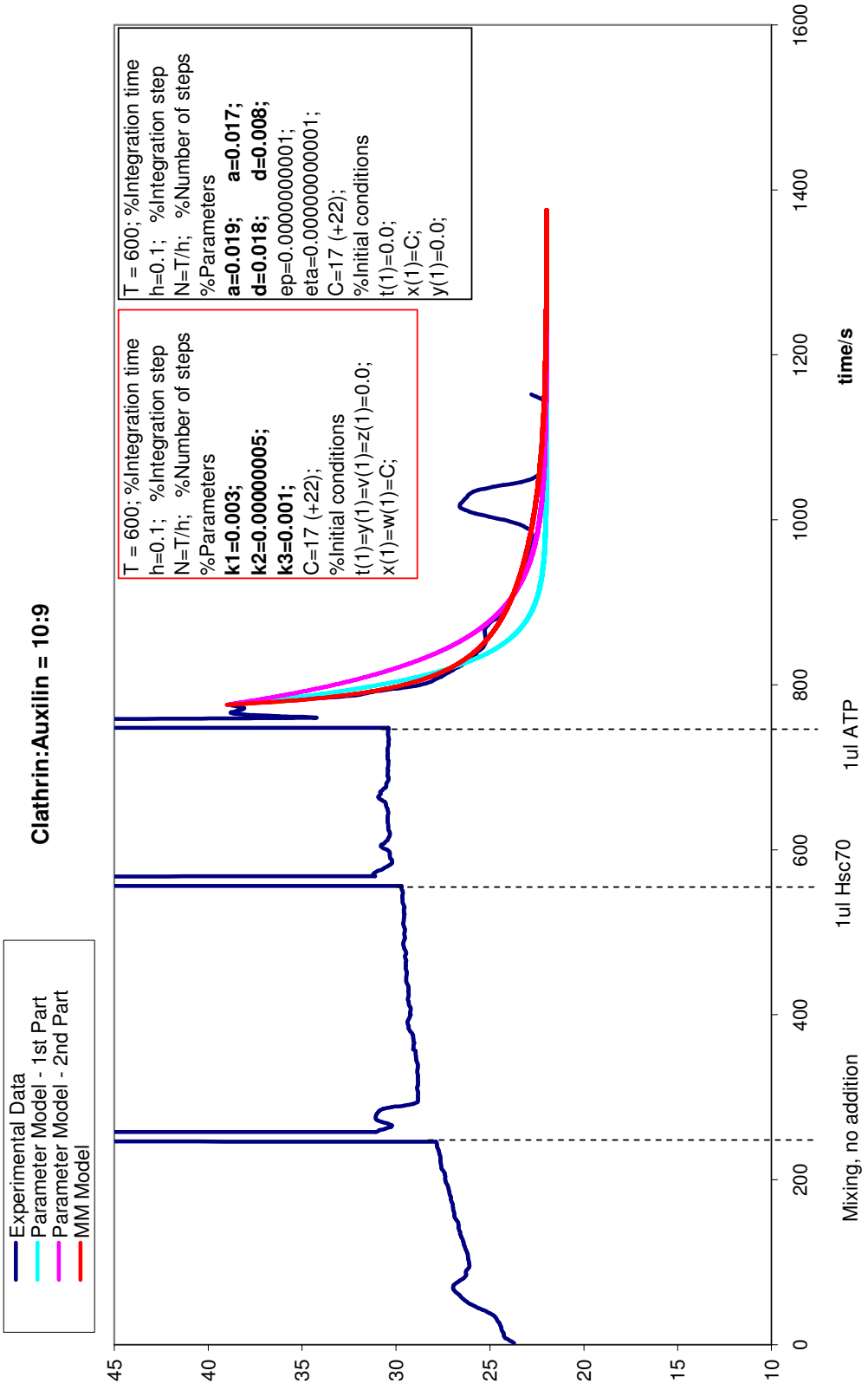
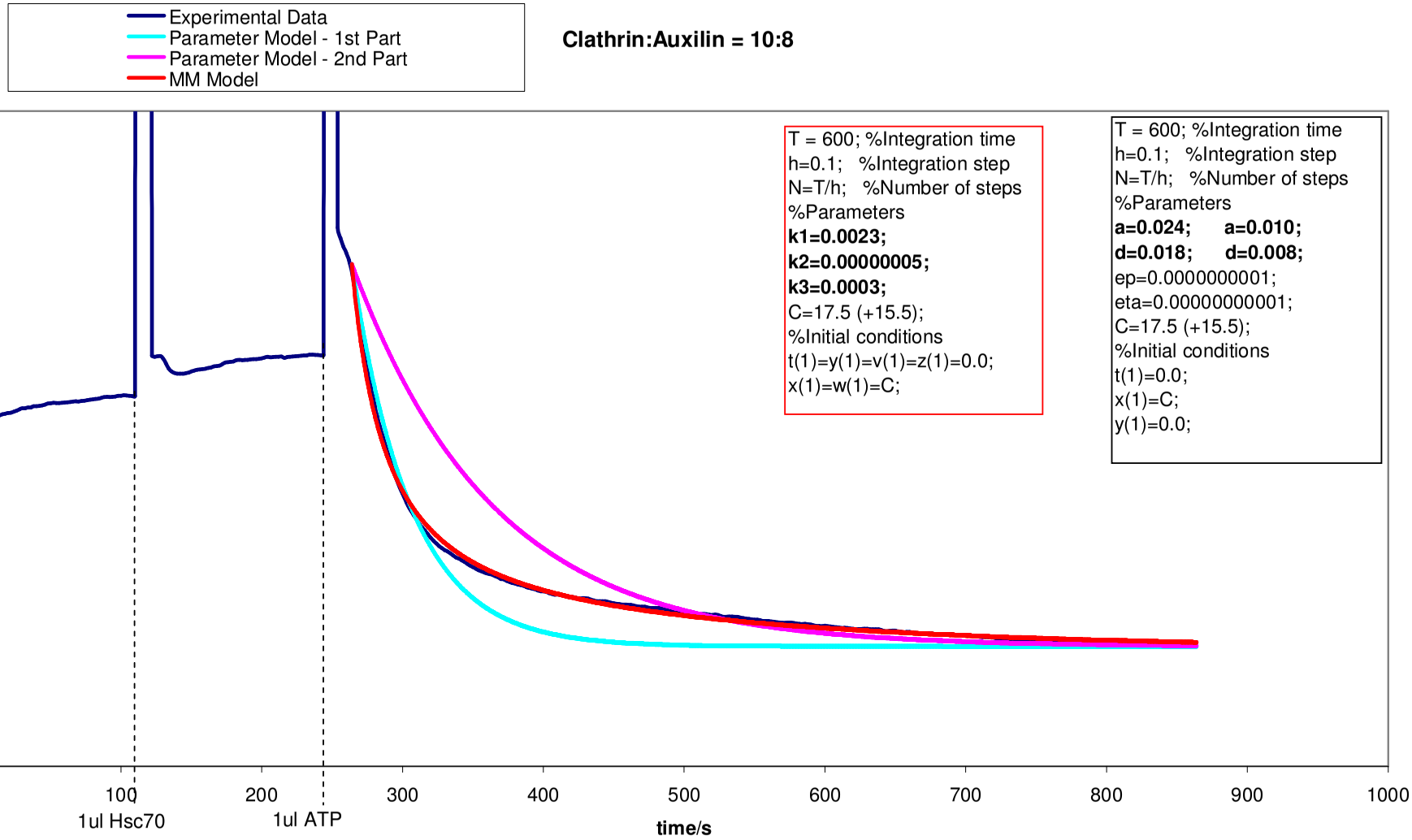
Appendix A

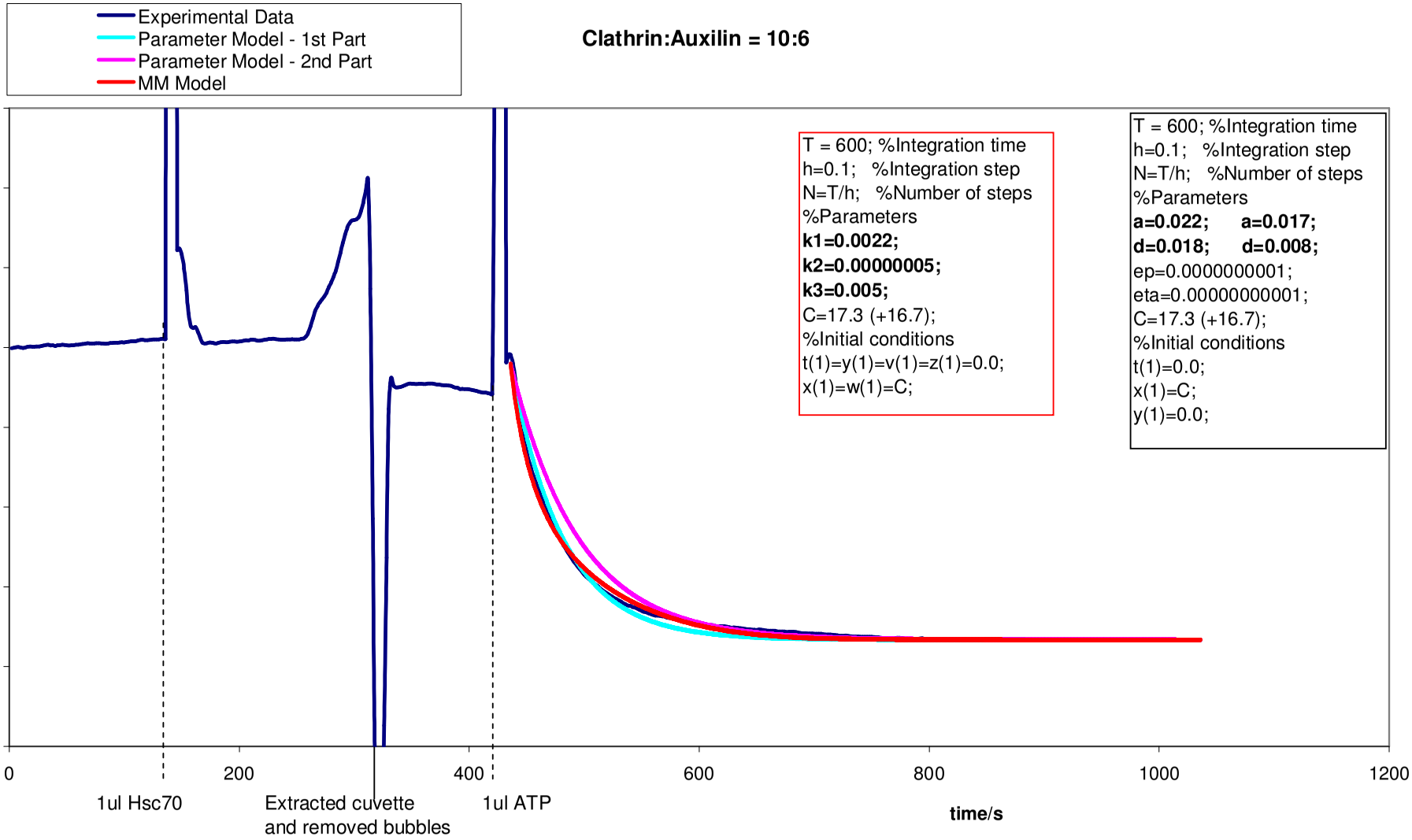
Complete Set of Results

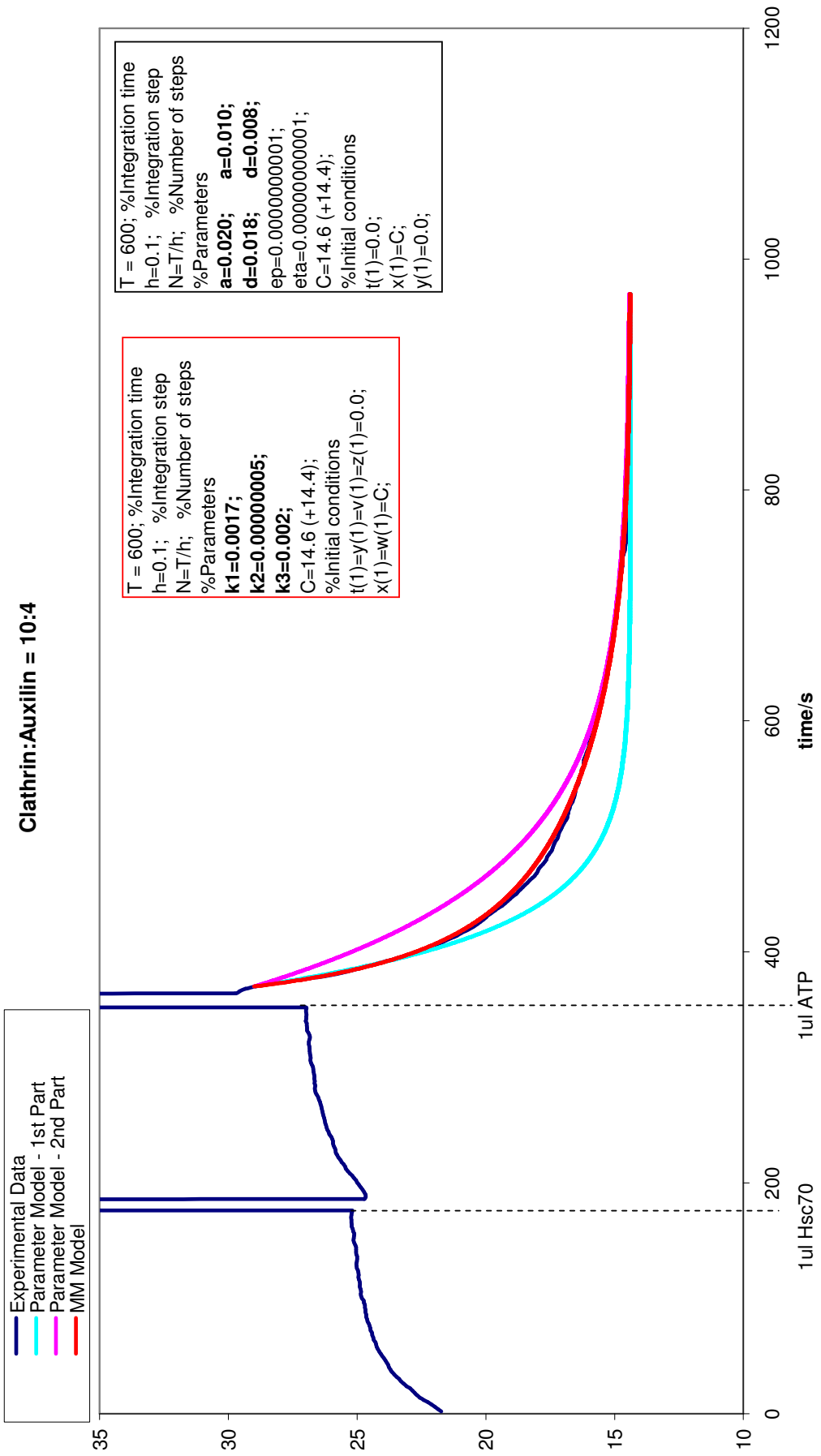


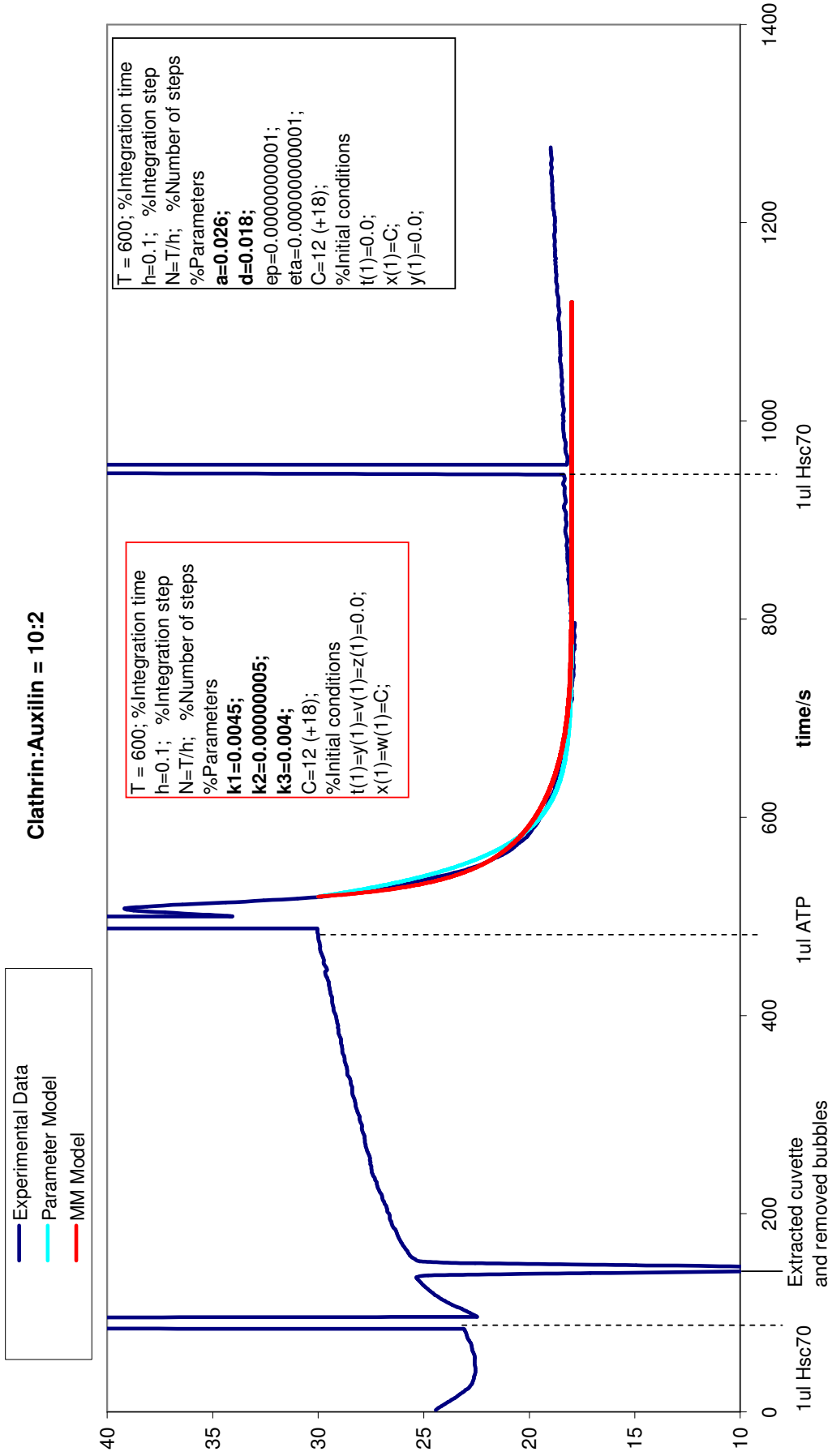


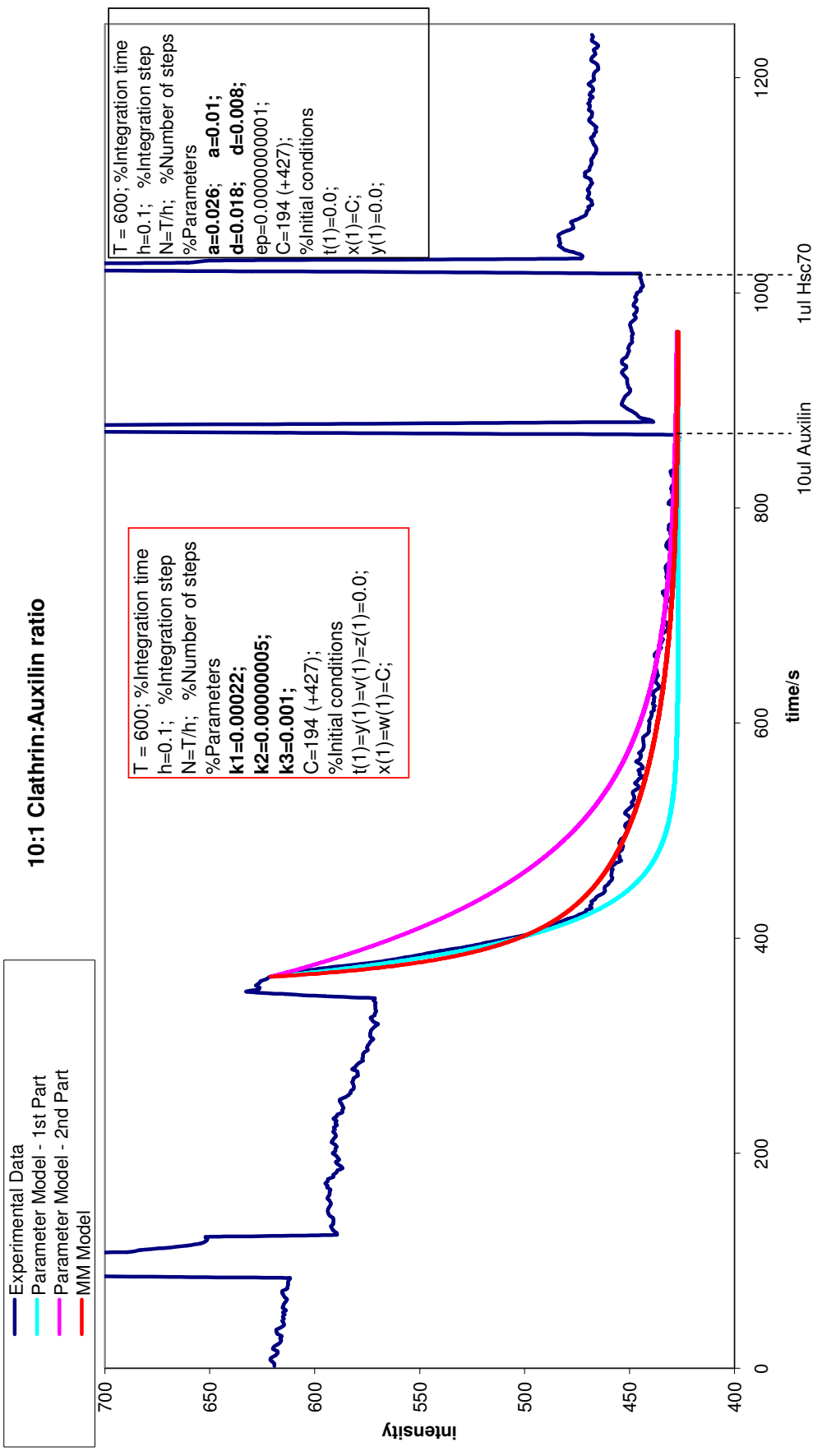


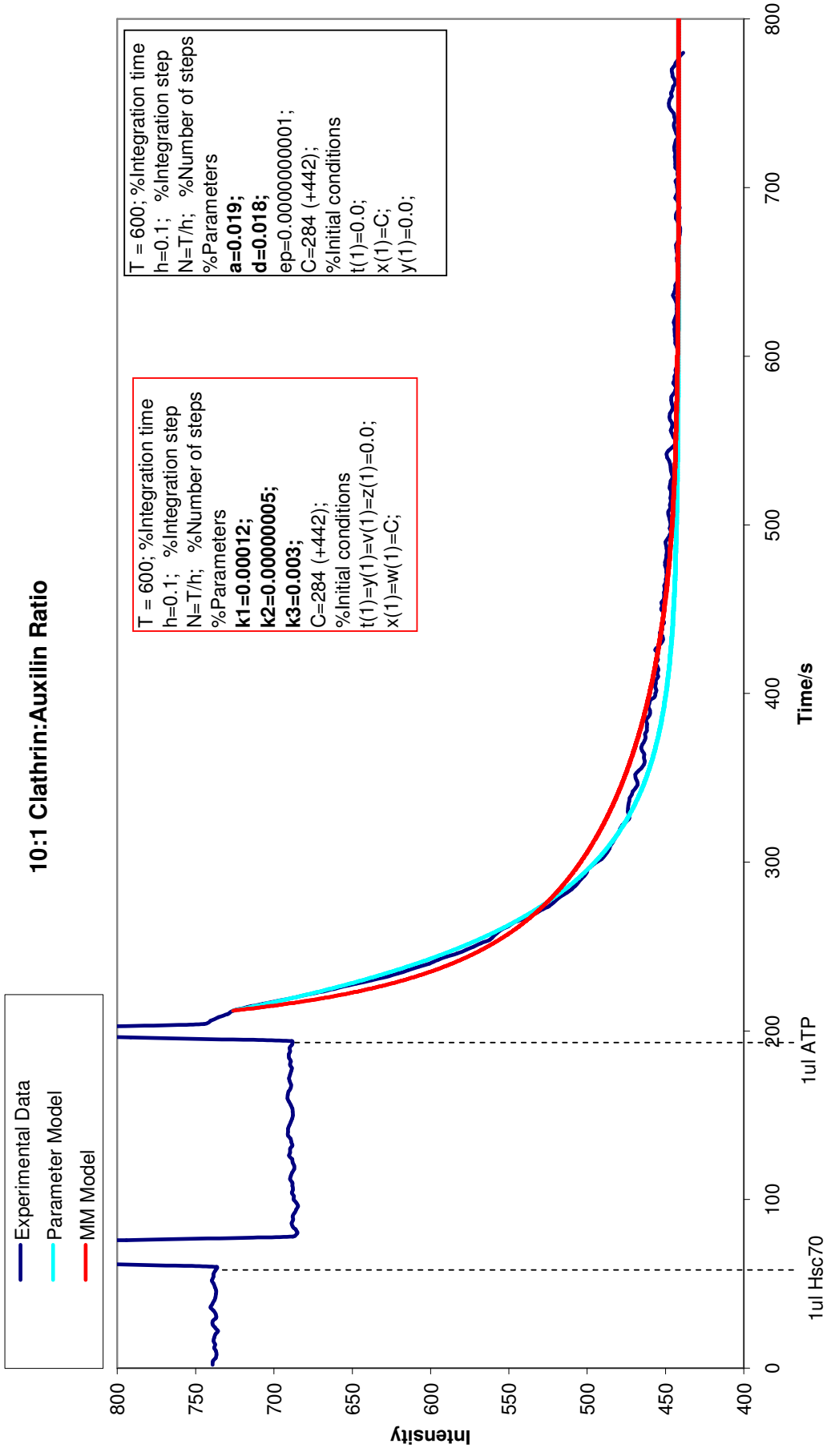


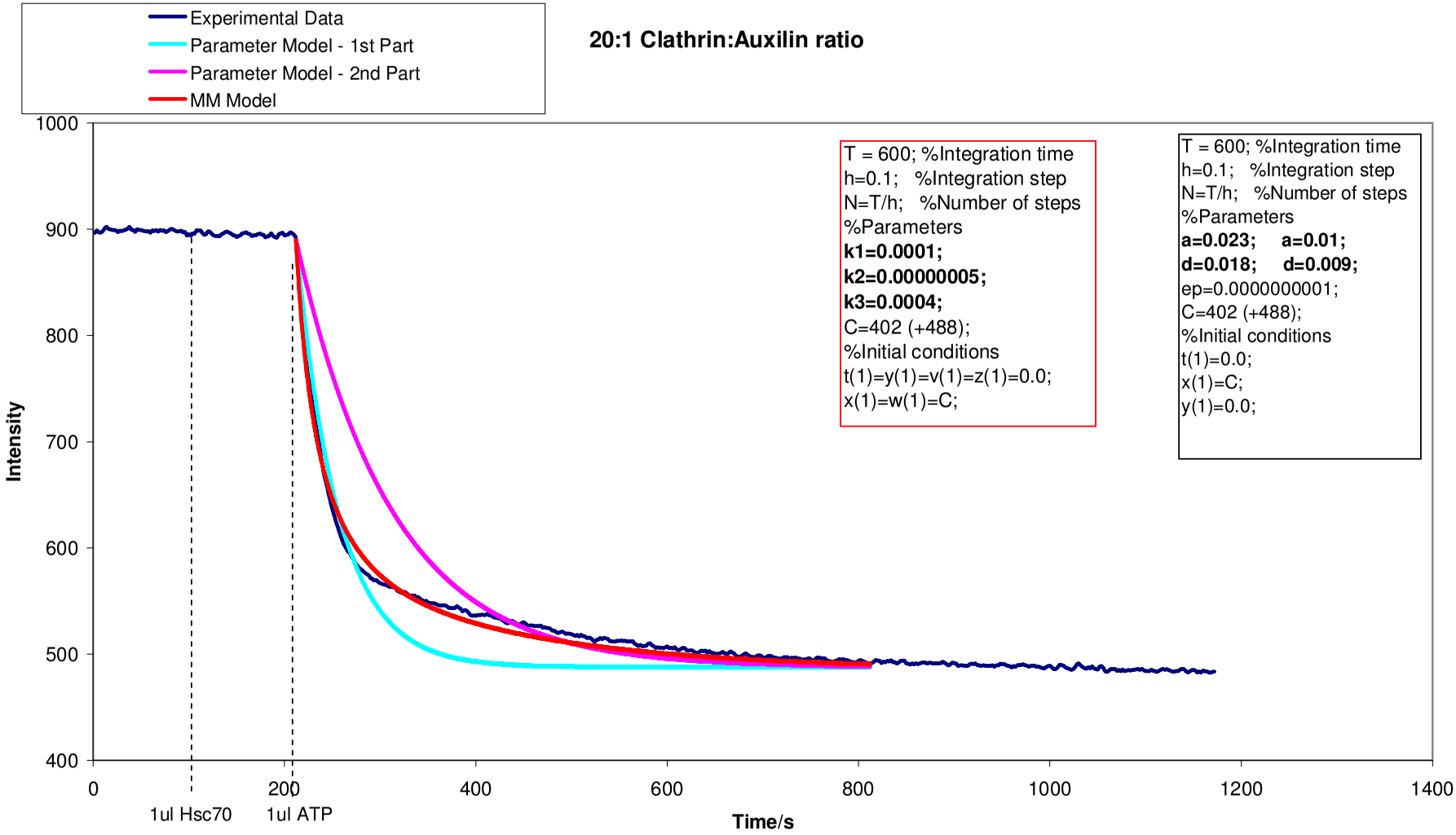


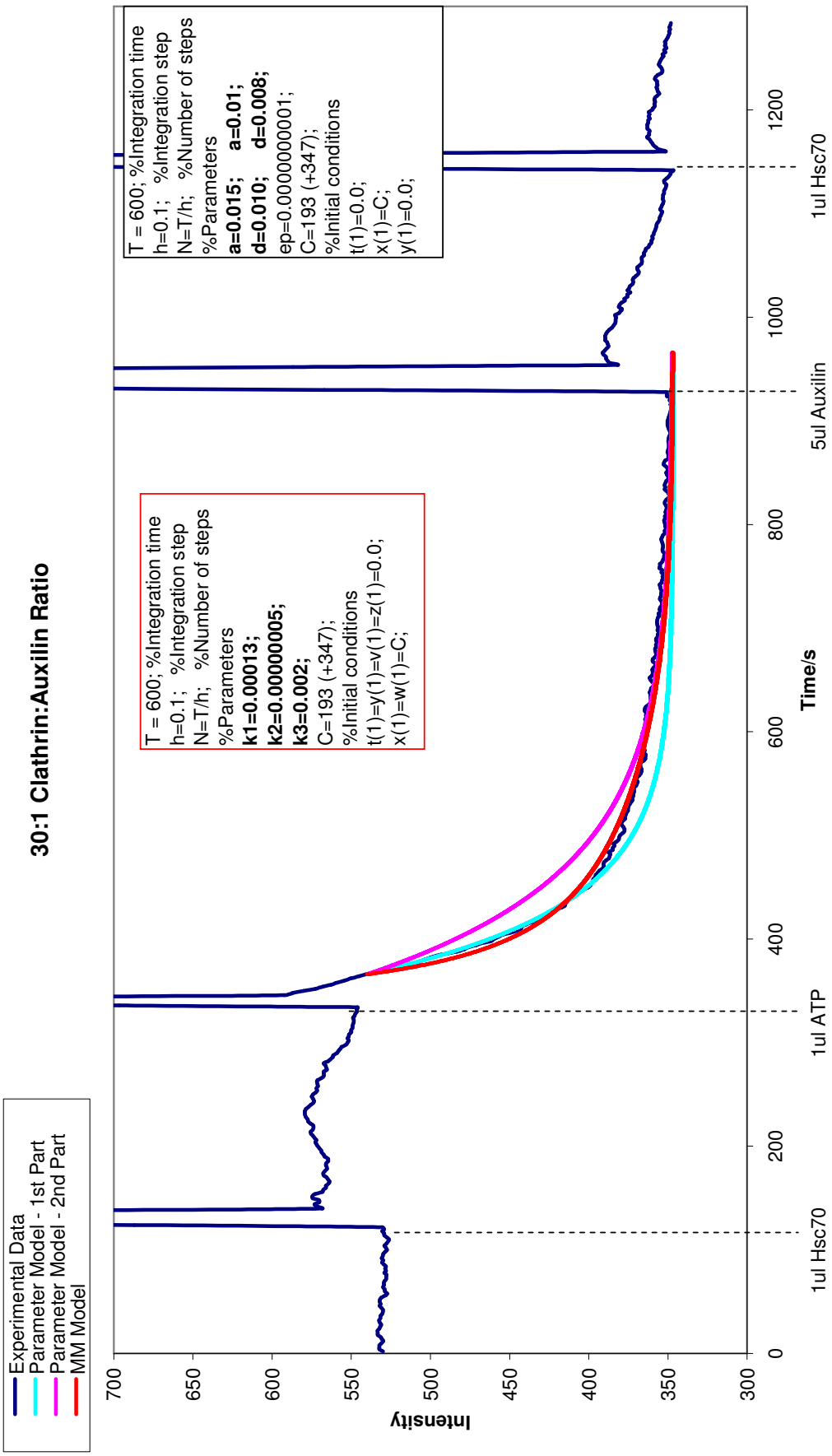


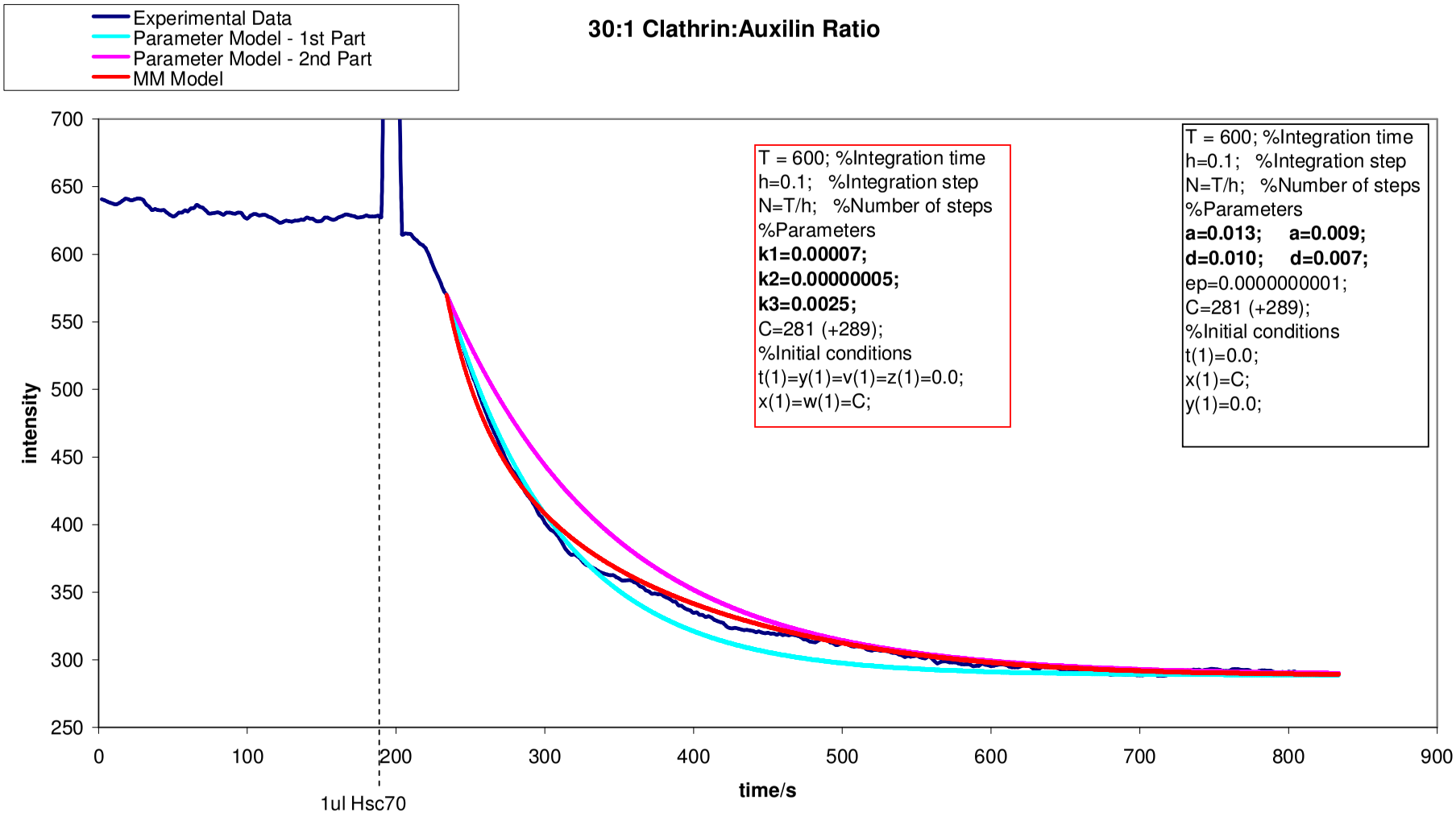


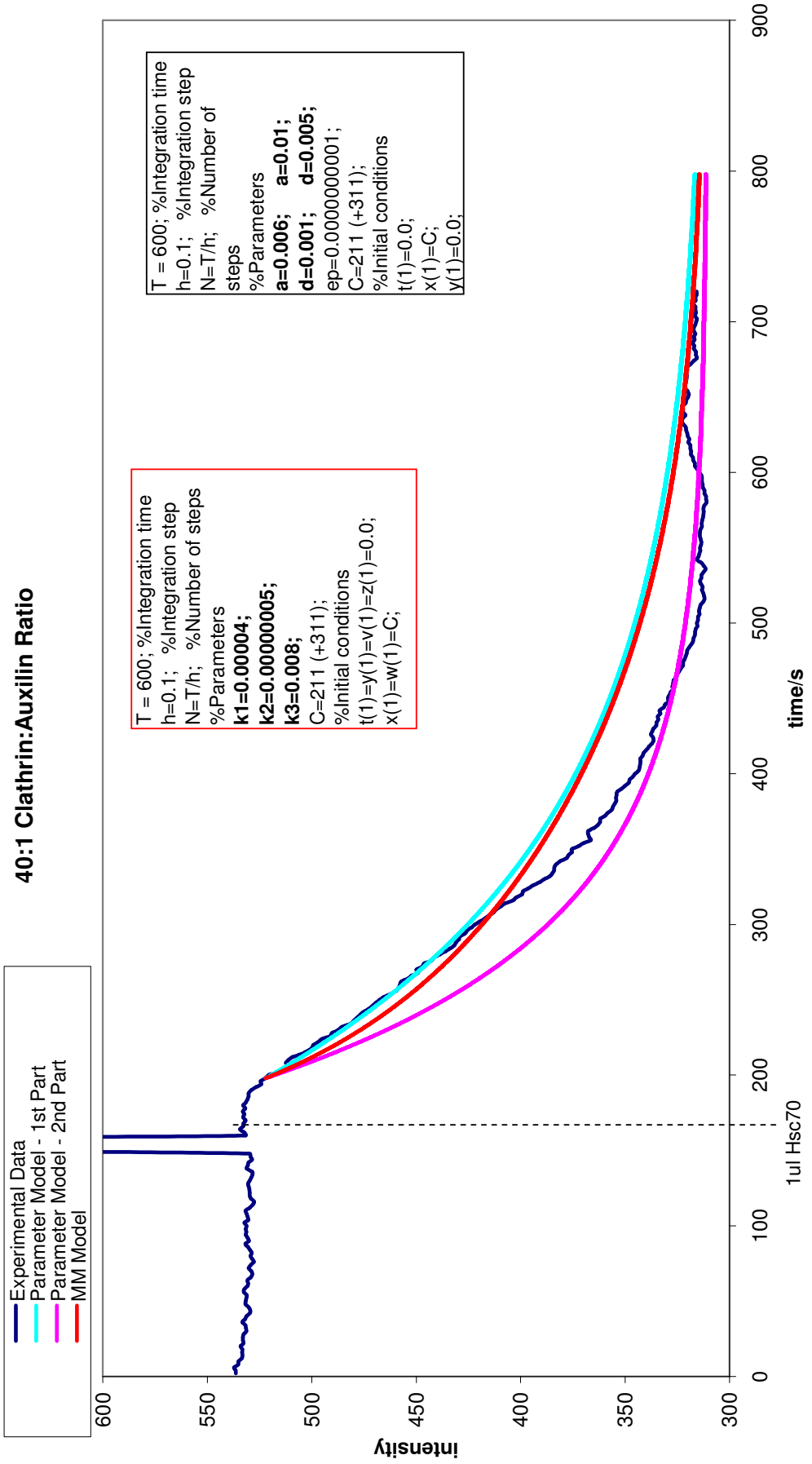


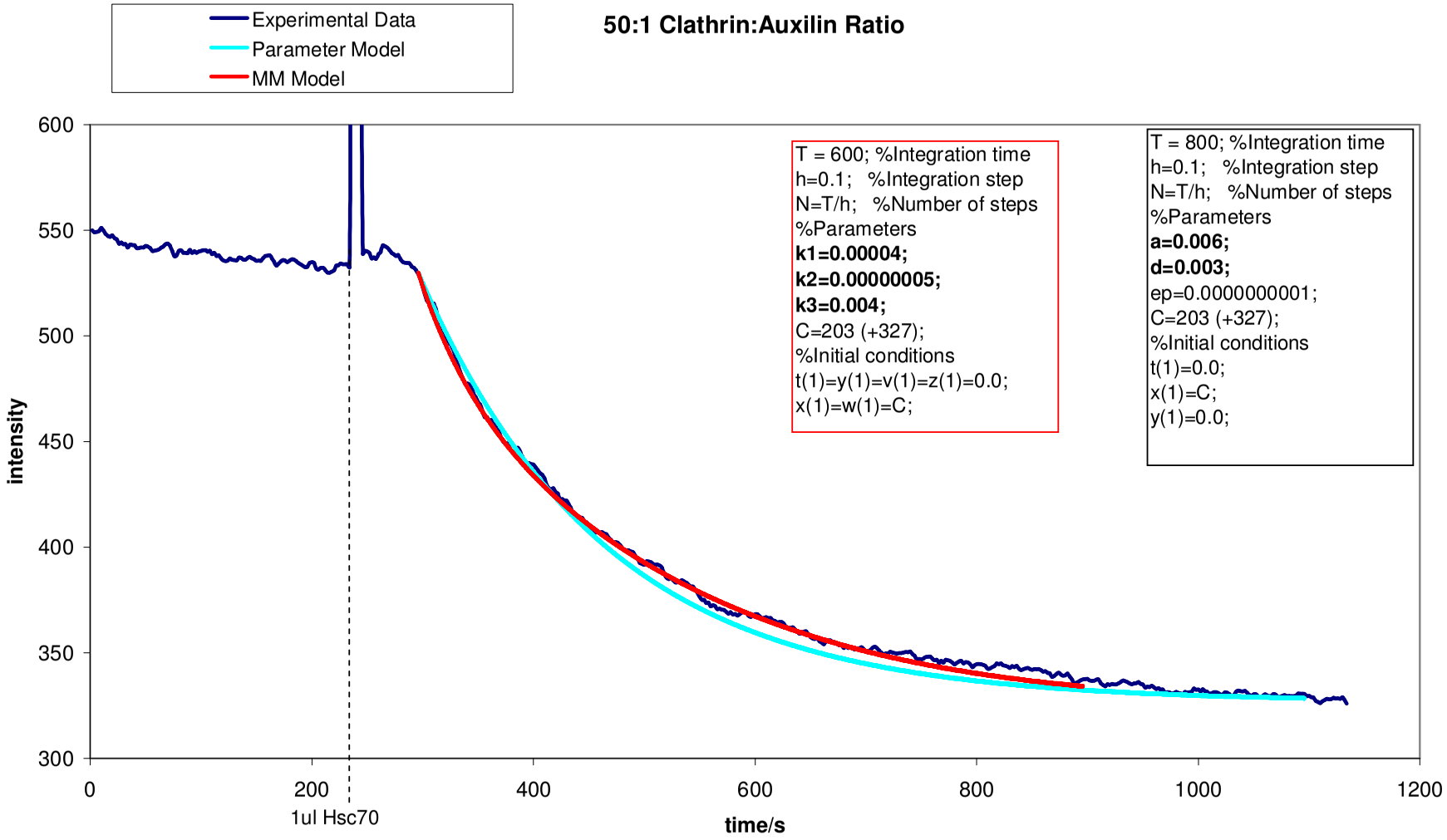












Appendix B

MATLAB Code for the Parameter Model

```
%function Clathrin
%Initialisation
clear;
%clearplot;

T = 800; %Integration time
h=0.1;   %Integration step
N=T/h;   %Number of steps

%Parameters
a=0.006;
d=0.003;           %d<a
ep=0.0000000001;
eta=0.00000000001;        %eta<ep
C=203;


%Initial conditions
t(1)=0.0;
x(1)=C;
y(1)=0.0;
%Integrator. This is a simple Integrator employing the Euler method
for n=1:1:N;

%Integrator
      t(n+1)=n*h;
      x(n+1)=x(n)+h*(-(a)*x(n)+ep*y(n)*(C-x(n)-y(n)));
      y(n+1)=y(n)+h*((d)*x(n)-eta*y(n)*(C-x(n)-y(n)));
%endfor
end

%Text file for Excel
data=transpose([t(1:N);x(1:N)]);
save data.txt data -ascii

%Plotting procedure
figure(2)
```

```
subplot(2,1,2);
plot(t(1:N),x(1:N),'b-')
title('Cage kinetics','FontSize',12)
    xlabel('t','FontSize',12)
    ylabel('x, conc. of cages','FontSize',12)
axis equal
grid on
subplot(2,2,2);
plot(t(1:N),y(1:N),'b-')
title('Triskelion kinetics','FontSize',12)
    xlabel('t','FontSize',12)
    ylabel('y, conc. of triskelions','FontSize',12)
axis equal
grid on
%set title "Cages"
%set xlabel "time"
%subplot(3,1,1)
%plot(t,x)
%mplot(t,x,t,y)
%gset term postscript eps
%gset output "fig-stoch4-mod2.eps"
%replot
%closeplot;
%endfunction
```

MATLAB Code for the Michaelis-Menten Model

```
%function Clathrin
%Initialisation
clear;
%clearplot;

T = 600; %Integration time
h=0.1;   %Integration step
N=T/h;   %Number of steps

%Parameters
k1=0.00004;
k2=0.00000005;
k3=0.004;
C=203;
```

```
%Initial conditions
t(1)=0.0;
x(1)=C;
y(1)=0.0;
v(1)=0.0;
z(1)=0.0;
w(1)=C;
%Integrator. This is a simple Integrator employing the Euler method
for n=1:1:N;

%Integrator
      t(n+1)=n*h;
      x(n+1)=x(n)+h*(k2*z(n)-k1*x(n)*w(n));
    v(n+1)=v(n)+h*k3*z(n);
    z(n+1)=z(n)+h*(k1*x(n)*w(n)-(k2+k3)*z(n));
      y(n+1)=y(n)+h*k3*z(n);
    w(n+1)=w(n)+h*((k2+k3)*z(n)-k1*x(n)*w(n));
%endfor
end

%Text file for Excel
data=transpose([t(1:N);x(1:N)]);
save data.txt data -ascii

%Plotting procedure
figure(2)
subplot(2,1,2);
plot(t(1:N),x(1:N),'b-')
title('Cage kinetics','FontSize',12)
    xlabel('t','FontSize',12)
    ylabel('x, conc. of cages','FontSize',12)
axis equal
grid on
subplot(2,2,2);
plot(t(1:N),y(1:N),'b-')
title('Triskelion kinetics','FontSize',12)
    xlabel('t','FontSize',12)
    ylabel('y, conc. of triskelions','FontSize',12)
axis equal
grid on
```

```
%set title "Cages"
%set xlabel "time"
%subplot(3,1,1)
%plot(t,x)
%mplot(t,x,t,y)
%gset term postscript eps
%gset output "fig-stoch4-mod2.eps"
%replot
%closeplot;
%endfunction
```