

**MODELING THE EFFECT OF AMMONIA
ON THE GLYCOSYLATION PATTERN OF
MONOCLONAL ANTIBODIES PRODUCED
BY CHINESE HAMSTER OVARY CELLS**

by

Joanna Adadevoh

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Honors Bachelor of Chemical Engineering with Distinction

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ABSTRACT

Monoclonal antibodies (mAbs) are therapeutic recombinant proteins which are typically produced industrially by Chinese Hamster Ovary Cells (CHO). In order to approve mAbs for therapeutic purposes, regulatory agencies require manufacturers to assure the bioactivity, purity, and potency of these proteins to consumers. Regulatory agencies now encourage manufacturers to develop strategies to control quality attributes online. One of the most important factors affecting the quality and bioactivity of mAbs is glycosylation – the addition of a carbohydrate chain to a protein. Glycosylation is a post-translational modification which exhibits heterogeneity that arises from variations in site occupancy and structure of attached glycans. As a result of such heterogeneity, glycosylation presents a challenge to manufacturers of mAbs yet an on-line control strategy does not exist for glycosylation. While a number of culture conditions have been found to influence mAb glycoform distribution, in order to develop effective control schemes, it is important to understand the mechanisms underlying how these culture conditions affect glycosylation.

One such culture condition is ammonia levels in culture media. Ammonia has been found to adversely affect cell growth and viability. It is also known to induce heterogeneity in glycosylation by affecting the extent of terminal sialylation, the transfer of a sialic acid residue to a glycoprotein. A few mechanisms have been proposed to describe how ammonia affects sialylation: one involves the reduction of CMP-sialic acid transporter (CMP-SAT) expression level by ammonia; another, the

inhibition of CMP-SAT by an increase in UDP-GlcNAc in the presence of ammonia; and still another, the increase in the trans-Golgi pH causing a decrease in sialylation enzymes. The aim of this study is to develop a model based on these mechanisms in order to understand at a fundamental level the effects of ammonia on glycosylation.

The model developed in this study can be used in conjunction with the Krambeck and Betenbaugh (2005) model to determine the ammonia concentration required in the cell culture to achieve the desired sialic acid content on the mAb. The model approach can also be employed for other culture conditions that affect glycosylation. This model work will aid in the development of a method for on-line quality control of the glycosylation pattern of mAbs.

Chapter 1

INTRODUCTION

1.1 Monoclonal Antibodies

Monoclonal antibodies (mAbs) are proteins that have a high specificity for a target molecule known as an antigen and act as the active component in many therapeutic protein drugs (Saylor, Dadachova, and Casadevall 2009). MAbs are composed of two light and two heavy polypeptide chains which are held together by inter-chain disulfide bonds and non-covalent interactions. As glycoproteins, MAbs usually have oligosaccharide structures, termed glycans, attached to their C_{H2} domains (see Figure 1). The majority of therapeutic monoclonal antibodies are produced as recombinant proteins in Chinese hamster ovary (CHO) cells that are cultivated in bioreactors.

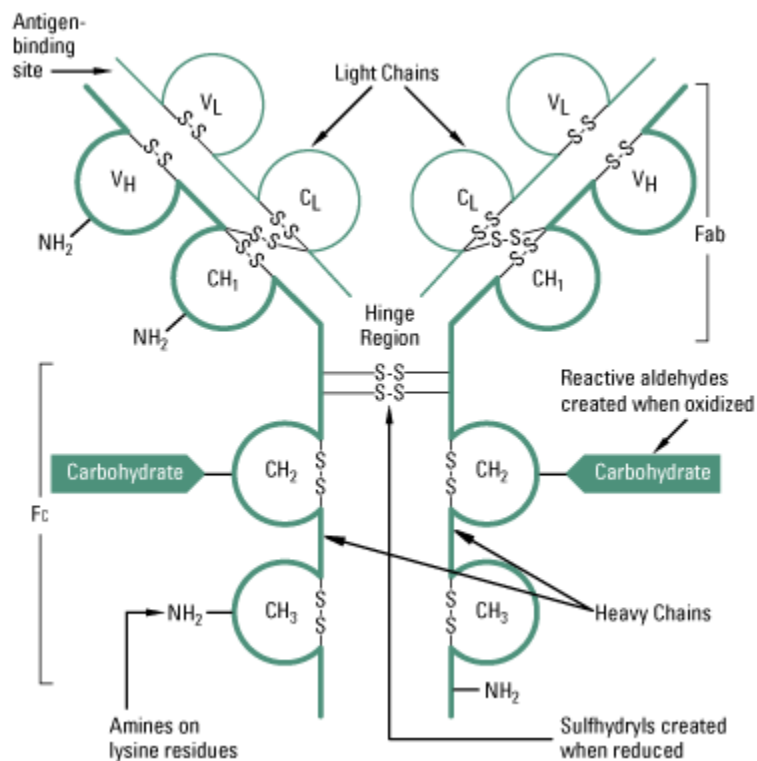


Figure 1: Structure of a Monoclonal Antibody from Thermo Fisher Scientific Inc., 2012. <<http://www.piercenet.com/browse.cfm?fldID=F655EBCB-5056-8A76-4EBE-966C71343C68>>

1.2 Glycosylation – Overview

MAbs undergo glycosylation, a post-translational modification in which a glycan structure is attached to a protein. This cellular process occurs in the endoplasmic reticulum (ER) and the Golgi apparatus of mammalian cells. There are a number of ways in which oligosaccharides are attached to proteins and some of the ways are via an N-glycosidic bond, an O-glycosidic bond, and a Glycophosphatidylinositol anchor. This thesis focuses on glycosylation that occurs via an N-glycosidic bond, mainly because it is the most common type of glycosylation found in biopharmaceuticals and because the glycosylation pattern has a significant

effect on the protein structure and function (Butler, 2006). As shown in Figure 2, the core glycan involved in N-glycosylation is a pentasaccharide consisting of three mannose and two N-acetylglucosamine ($\text{Man}_3\text{GlcNAc}_2$) that is attached via an N-glycosidic bond to the Asparagine residue of an Asparagine-X-Serine/Threonine (Asn-X-Ser/Thr) amino acid sequence where X is any amino acid except Proline (Butler, 2006).

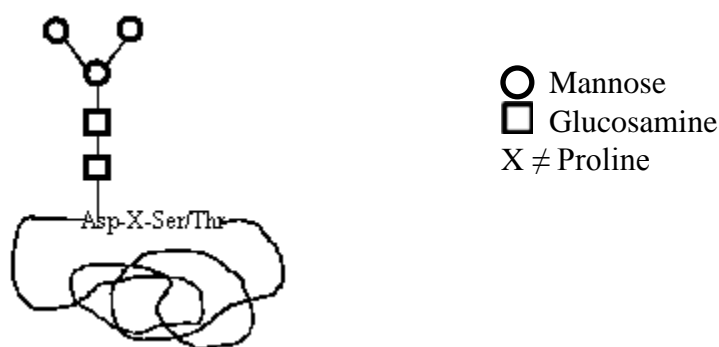


Figure 2: N-Glycan Attached to a MAb

Glycosylation is an important modification of antibodies because it affects the biological properties of mAbs such as pharmacokinetics, secretion, receptor recognition and antigenicity (Butler, 2006). However, the intracellular process of glycosylation is subject to much variability and there is heterogeneity associated with the final glycan structure. The heterogeneity arises when there are variations in the mAb glycosylation profile due to varying site occupancy and structure of attached glycans. Therefore, to assure the safety and efficacy of these products, regulatory agencies require manufacturers to ensure that glycosylation patterns are consistent between batches of mAbs. Currently manufacturers merely test the final product

quality offline and post production. However, regulatory agencies are starting to encourage online quality assurance of these products, but to date no methods exist to monitor or control glycosylation online. To address the need for producing mAbs with consistent glycosylation patterns in the biopharmaceutical industry, the Ogunnaike group aims to develop a strategy for online real-time control of glycosylation in mAbs.

1.3 Glycosylation – Multi-scale Perspective

The approach that the Ogunnaike group proposes for online real-time control of glycosylation in mAbs incorporates the multi-scale nature of glycosylation that consists of micro-scale, meso-scale, and macro-scale levels of impact. Glycosylation, which occurs in the ER and Golgi (micro-scale), is affected by the intracellular processes occurring in CHO cells (meso-scale) which in turn are affected by the culture conditions in the bioreactor (macro-scale). The culture conditions that are monitored in the bioreactor include ammonia concentration, pH, dissolved oxygen content, temperature etc. These process variables are easy to measure and control. For example, the pH of the culture is measured using a pH probe and can be controlled using a buffer solution. The changes in the culture conditions induce changes in the intracellular processes occurring within the cell. For instance, an increase in the pH of the culture could lead to an increase in the pH of the cell cytoplasm. The changes in the meso-scale in turn affect the glycosylation process occurring in the ER and the Golgi. For example, an increase in the pH of the cell cytoplasm would affect the pH in the Golgi which would in turn affect the activity of the glycosylation enzymes. The change in activity of the glycosylation enzymes would affect the glycosylation pattern of the mAbs. Figure 3 illustrates the multi-scale nature of glycosylation.

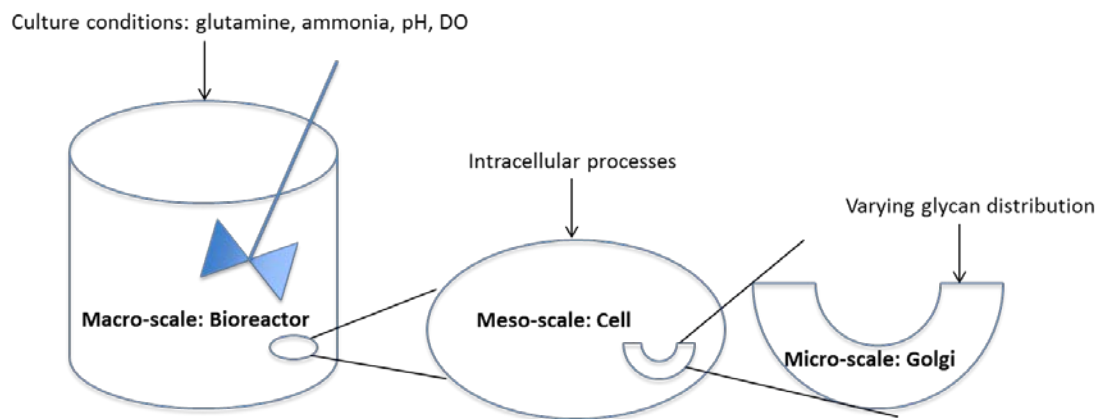


Figure 3: Multi-scale nature of glycosylation. Three scales present: macro-scale, meso-scale, and micro-scale.

To ensure that mAb manufacturers obtain the desired final glycan structure on the antibodies, it is important to develop a method for controlling glycosylation online during mAb production. Unfortunately, the processes occurring in the meso and micro-scales that affect glycosylation are not easy to control. However, as explained above, the cell culture conditions, which are easy to monitor and control, affect the meso and micro-scale processes. A quantitative correlation between the bioreactor process conditions and the processes occurring in the meso and micro-scale is required to develop an effective control strategy for glycosylation. This thesis aims to develop a mathematical model that would link one of the culture process conditions – ammonia – to the meso and micro-scale processes such that ammonia’s effect on glycosylation can be quantified.

1.4 Ammonia

Ammonia (also referred to as ammonium in this thesis) is a toxic byproduct of amino acid metabolism and it is known to adversely affect the growth and viability of

cells in culture (Borys, Linzer and Papoutsakis 1993). A primary source of ammonia in cell culture is glutamine. Although glutamine is an energy source for cells, its metabolism and decomposition lead to the production of ammonia in the culture. The ammonia produced diffuses into the cytosol of the CHO cells and affects some of the intracellular processes occurring in the cell such as the pH of organelles or activity of enzymes. These changes in intracellular processes in turn affect the glycosylation process occurring in the Golgi. For example, studies have shown that ammonia affects glycosylation by decreasing the extent of terminal sialylation which occurs in the Golgi (Andersen and Goochee 1994; Yang and Butler, 2000). A number of mechanisms, which are explained in the following section, have been proposed to explain how ammonia affects sialylation.

1.5 Effect of Ammonia on Protein Sialylation

Sialylation is a type of glycosylation in which a sialic acid is attached to the glycan structure of a glycoprotein. The structure of sialic acid varies between species; the sialic acid associated with humans is N-acetyl-neuraminic acid aka NANA (Butler, 2006). Although most mice have a different sialic acid structure (N-glycolyl-neuraminic acid, NGNA), CHO cells produce mAbs that have mainly NANA which is important for compatibility in humans (Butler, 2006).

Sialylation affects the circulatory half-life of mAbs in the human body. A decrease in the extent of terminal sialylation causes the glycoprotein to be more susceptible to degradation by asialoglycoprotein receptors and this leads to a decrease in the circulatory half-life of the drug.

In the cytosol of the cell, sialic acid is present in the form of a nucleotide donor (CMP-NANA) which is transported to the Golgi apparatus via the CMP-sialic acid

transporter (CMP-SAT). In the trans-Golgi, the sialic acid is transferred from the CMP-NANA molecule to the glycan structure of the mAb via the α -2,3-sialyl transferase enzyme. A number of mechanisms have been proposed to explain how ammonia decreases the extent of terminal sialylation and three of these mechanisms are summarized as follows (Butler, 2006):

- Ammonia decreases the expression of CMP-SAT
- Ammonia is a precursor for UDP-GlcNAc which is a nucleotide that inhibits CMP-SAT
- Ammonia increases the pH of the trans-Golgi which in turn decreases the activity of the α -2,3-sialyl transferase enzyme.

These mechanisms are employed in developing a mathematical model for the effect of ammonia on glycosylation – specifically on the extent of terminal sialylation – and are investigated in greater detail in the following chapters.

1.6 Motivation

The main purpose of this thesis is to develop a mathematical model to investigate the effect of ammonia on the glycosylation pattern of monoclonal antibodies. Developing this mathematical model will be the first step in mapping out the influence of culture conditions on glycosylation following a multi-scaled perspective. With this mathematical model, experimenters will be able to determine how much glutamine should be added to the cell culture in order to achieve a desired sialic acid content in the final glycan distribution of the mAb. The ability to predict the extent of terminal sialylation for a given glutamine or ammonia concentration would help manufacturers decide how much glutamine to add to their culture in order to get their desired sialic acid distribution. A few simplifying assumptions were made in the

development of the model and will be further discussed in subsequent chapters.

However, these assumptions can be relaxed in order to expand the case range to which the model applies. The model approach used for this thesis could also be used for other culture conditions that affect glycosylation. The following chapters are divided into Model Development (Chapter 2), Model Results (Chapter 3) and Conclusion (Chapter 4).

Chapter 2

MODEL DEVELOPMENT

2.1 Introduction to the Model

The mathematical model developed to investigate the effect of ammonium on the glycosylation pattern of monoclonal antibodies is made up of a combination of six sub-models. Each sub-model describes a specific stage in the pathway of ammonium from the cell culture to the Golgi. To begin, the first sub-model is the relationship between the glutamine concentration in the culture and the extracellular ammonium concentration. The ammonium present in the culture arises from the decomposition and cellular metabolism of glutamine. This first sub-model is important because it will allow users to predict the quantity of glutamine required to obtain an extracellular ammonium concentration that would yield the desired sialic acid distribution once the main model is completely developed. The second sub-model gives the correlation between the extracellular and intracellular ammonium concentrations. Once the intracellular ammonium concentration is computed, its effect on intracellular processes can also be determined. In particular, this thesis focuses on the following intracellular processes (Butler, 2006):

- 1) Reduction in the expression level of CMP-SAT
- 2) Increase in the intracellular UDP-GlcNAc concentration
- 3) Inhibition of CMP-NANA transport by UDP-GlcNAc
- 4) Increase in the pH of the trans-Golgi and its effect on enzyme activity

Each of these intracellular processes has a sub-model. All the sub-models are explained in greater detail in the following sections of this chapter. To sum up the approach to the model development, glutamine is decomposed or metabolized to form ammonium. The ammonium diffuses into the cell and affects some of the intracellular processes including UDP-GlcNAc expression levels, CMP-SAT expression levels and activity, and trans-Golgi pH. These intracellular processes in turn affect the extent of terminal sialylation. A schematic to explain the model approach is displayed in Figure 4.

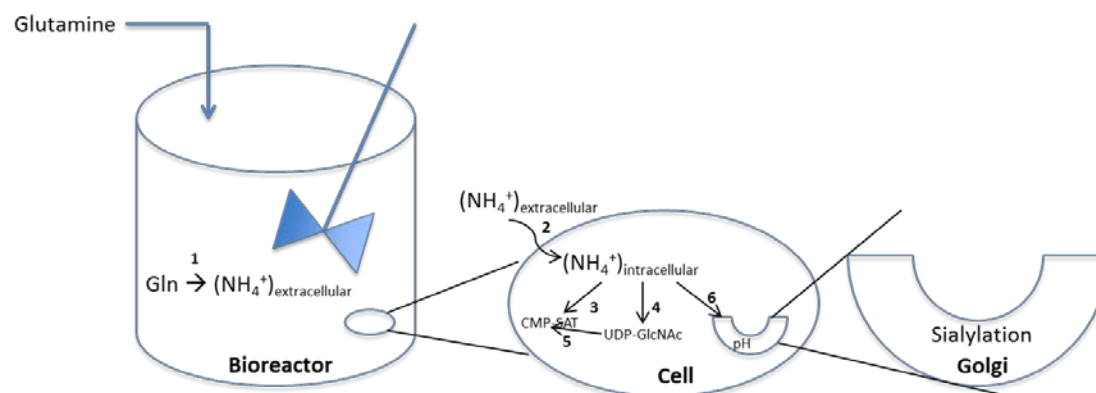


Figure 4: Schematic of Approach to Model Development. Each sub-model is labeled 1 to 6 on the diagram.

It is important to note that the graphs illustrated in the following sections of Chapter 2 are based on the experimental results of some researchers – whose names will be explicitly stated – and on how their experimental results aid the model development.

2.2 Sub-Model 1: Glutamine to Extracellular Ammonium

Glutamine, when present in the culture, diffuses through the cell membrane into the mitochondrial matrix where it is catabolized by a phosphate-dependent glutaminase to form glutamate and a molecule of ammonium (Schneider *et al.*, 1996). The glutamate is further degraded by glutamate dehydrogenase to form another molecule of ammonium. The molecules of ammonium present in the mitochondrion form an equilibrium with ammonia based on the pH of the matrix:

where k is the glutamine decomposition rate constant and $[Gln]$ is the glutamine concentration. k depends on the pH of the media and is given by

transport. The ammonia, on the other hand, diffuses from the cytosol to the culture because it has a higher permeability through the cell membrane. Hence, there is a continuous flux of ammonium/ammonia in and out of the cell membrane. Wu, Ray, and Shuler (1993) developed a model for the flux of ammonium into the cell membrane as shown:

No literature value was found for

initial concentration of 10mM NH_4^+ . Other than the difference in their ammonium concentrations, the cultures were exposed to the same conditions. The ammonium concentration in each culture was measured at different culture times. At the end of the culture time, the cells were harvested and the supernatant and pellets were preserved at -20 and -80°C, respectively, for subsequent gene expression analysis. The expression level of the CMP-SAT gene was measured using real time quantitative reverse transcription (QRT) polymerase chain reaction (PCR). The results that are relevant to the development of sub-model 3 are displayed in Figure 5.

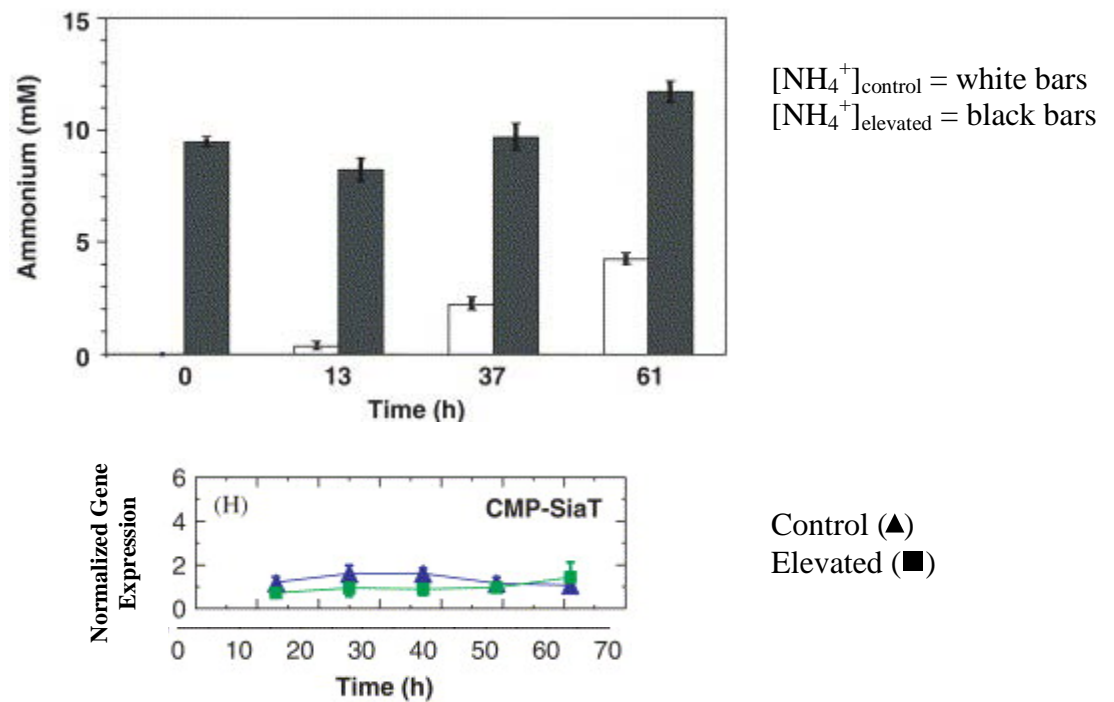


Figure 5: Figures reproduced from Chen and Harcum (2006)

These results show that though the gene expression of the CMP-SAT (also known as CMP-SiaT in the Chen and Harcum paper) does not vary with culture time, it does decrease with an increase in ammonium concentration in the culture. This is consistent with one of the previously proposed mechanisms by which ammonium affects sialylation.

From these experimental results, a plot of normalized CMP-SAT gene expression versus ammonium concentration in the culture was made for the case of elevated ammonium. A linear equation was fit to the plot using Minitab® to obtain a direct relationship between ammonium and the gene expression level of CMP-SAT. The plot obtained and the corresponding linear equation are shown in Figure 6.

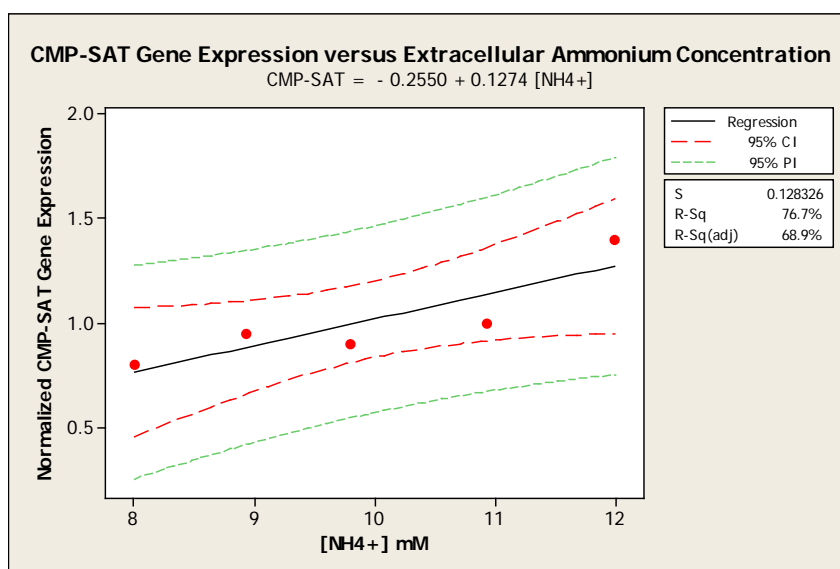


Figure 6: Normalized Gene Expression of CMP-SAT versus Extracellular Ammonium Concentration.

Figure 6 shows an upward sloping curve which implies that as the ammonium concentration increases, the CMP-SAT gene expression increases. However, this is inconsistent with the experimental results in Figure 5 and the mechanisms proposed by previous studies. The trend of the graph, however, can be explained. If two CHO cell cultures have different concentrations of ammonium, the culture with the lower concentration of ammonium will have a higher CMP-SAT expression. This relationship is what is observed in Figure 5. Figure 6, however, shows the trend of CMP-SAT with ammonium for one culture and does not compare to another culture with a different ammonium concentration. Hence, it is possible for the CMP-SAT expression in a culture with elevated ammonium to increase during the culture period, and still be relatively lower when compared to another culture of lower ammonium concentration. The red dotted line represents the confidence interval and the blue dotted line represents the prediction interval of the graph. The p-value associated with the graph in Figure 6 is 0.052 implying that the results are statistically relevant. Figure 5 and 6 are effective for explaining the effect of ammonium on CMP-SAT but they will not be used in the model development because they represent data for the gene expression, not protein concentration, of CMP-SAT. This analysis of the effect of ammonia on CMP-SAT was performed in order to obtain a comprehensive understanding of the role of ammonium on glycosylation. However, it was not included in the model. For model calculations, the CMP-SAT concentration was assumed to be constant.

2.4.2 Sub-Model 4: Increase in the Intracellular UDP-GlcNAc Concentration

To develop a sub-model for the effect of ammonium on the intracellular concentration of UDP-GlcNAc, a study performed by Gawlitzek *et al.* (1999) was

analyzed. In the study, ^{15}N from ammonium was incorporated into the glycan structure of a mAb produced by CHO cells. The cells were initially cultured in a 10 l bioreactor with a working volume of 8.5 l for 4 days. During the 4 days, the cells were not subject to any ammonium ions or glutamine except those present in the culture medium. On the 4th day, the cells were harvested and resuspended in 4 media types: (1) glutamine-free + 13 mM NaCl, (2) glutamine-free + 13 mM $^{15}\text{NH}_4\text{Cl}$, (3) glutamine containing + 13 mM NaCl, (4) glutamine-containing + 13 mM $^{15}\text{NH}_4\text{Cl}$. Each media type was duplicated resulting in 8 flasks of CHO culture. The cultures were harvested on the 10th day. The ammonium concentration and intracellular uridine-5'-diphospho-N-acetyl hexosamine (UDP-GNAc) pool in each culture were measured each day. UDP-GNAc is the sum of the UDP-glucosamine (UDP-GlcNAc) and UDP-galactosamine (UDP-GalNAc) content present in the cell. The results from Gawlitzek *et al.* that are relevant to the development of sub-model 4 are presented in Figure 7.

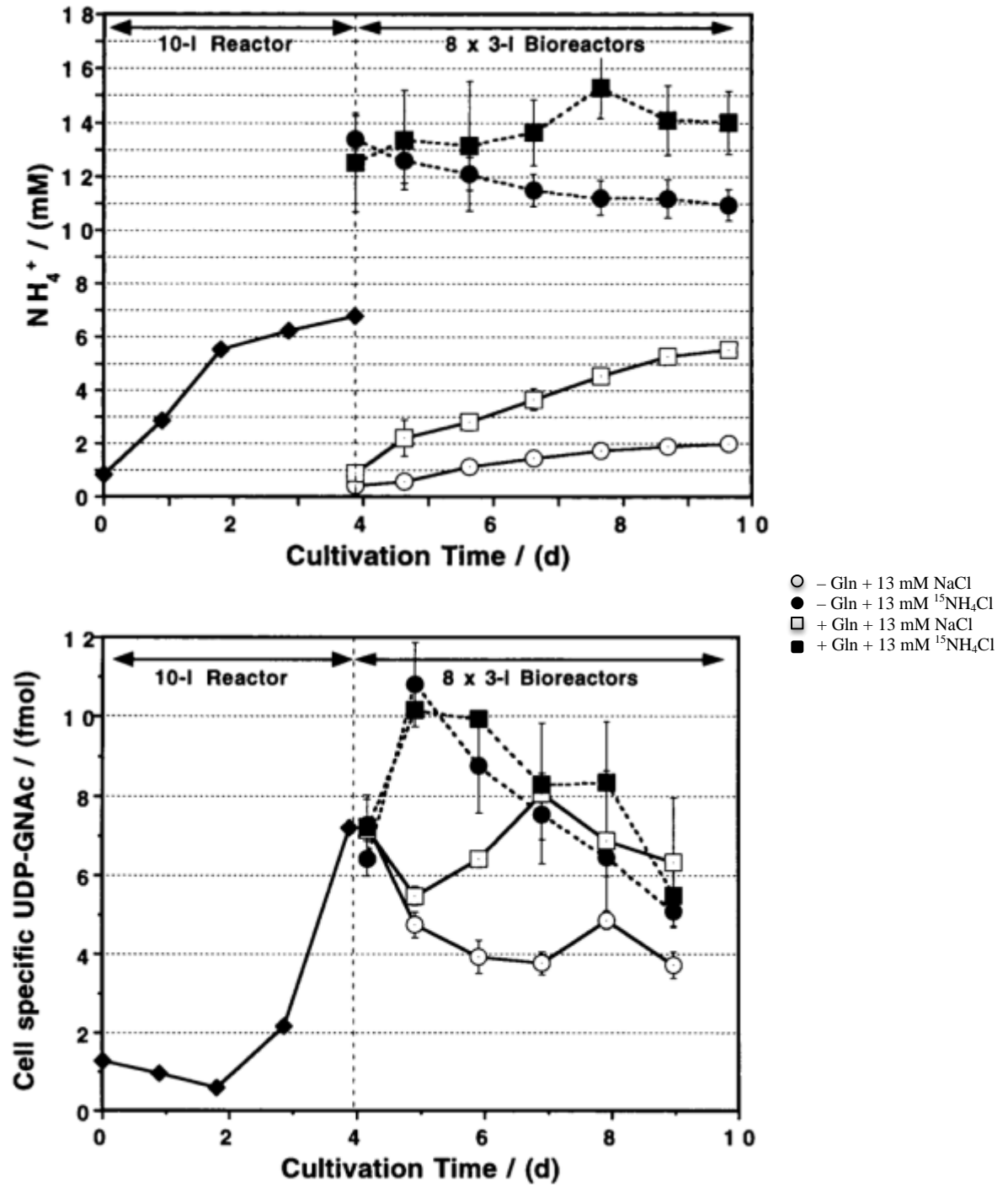


Figure 7: Plots reproduced from Gawlitzek *et al.* (1999)

The plots on Figure 7 show the trends in extracellular ammonium concentration and cell specific UDP-GNAc content with culture time. From these results, the graph of cell specific UDP-GlcNAc versus extracellular ammonium concentration was plotted for the first four culture days. It was assumed that the UDP-GlcNAc content was half of the reported UDP-GNAc. This assumption was based on the knowledge that UDP-GlcNAc is in equilibrium with UDP-GalNAc. Figure 8 shows the plot of the experimental values and the exponential model fit with equation

The model compares well with the last three experimental data points. However, the first two experimental points do not fit well to the model because they do not follow the exponential relationship. Based on this discrepancy between the experimental and model curve, it was assumed that the first two measurements of UDP-GlcNAc were taken during the cell growth lag phase when the cells were focused on adapting to the culture environment rather than on growing or producing UDP-GlcNAc. This is a valid assumption because the first two UDP-GlcNAc measurements were taken before day 2 of cell culturing. In light of this, a second model was developed to determine the effect of ammonium on UDP-GlcNAc. In this second model, it was assumed that before day 2, the cells have a constant UDP-GlcNAc content and that after day 2, the UDP-GlcNAc content varies exponentially with ammonium. It was also projected that after a certain culture time, the UDP-GlcNAc content will reach a saturation point where no more UDP-GlcNAc will be produced regardless of the extracellular ammonium concentration. This projection was based on the knowledge that cells usually reach a stationary and, eventually, death phase where the cells channel their remaining culture nutrients towards their viability rather than productivity. Based on these assumptions and projections, the UDP-GlcNAc cell content followed saturation kinetics with respect to extracellular concentration. A graph of the modified model of the effect of ammonium on UDP-GlcNAc is shown in Figure 9. A graph of the experimental results obtained from Gawlitzek *et al.* (1999) is also shown in Figure 9.

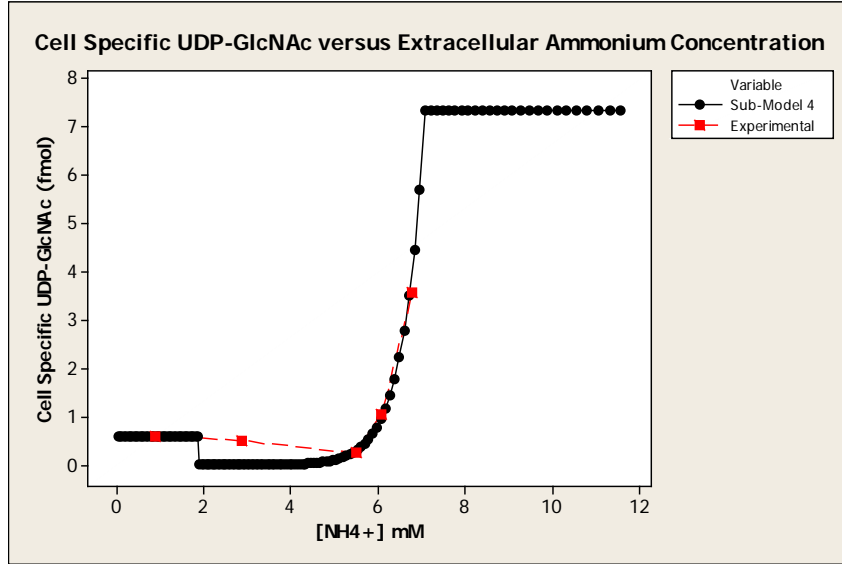


Figure 9: Sub-Model 4 for the Effect of Ammonium on UDP-GlcNAc

The model shows a decrease in cell specific UDP-GlcNAc at an ammonium concentration of 2 mM and then a gradual increase until it reaches a saturation point. This decrease in UDP-GlcNAc is also seen in the experimental results (Figure 8) and probably represents the stage in the cell culture where no UDP-GlcNAc is being produced but the amount initially present is decreasing because it is being used for intracellular processes such as being transported into the Golgi apparatus for glycosylation reactions (Krambeck and Betenbaugh, 2005).

2.4.3 Sub-Model 5: CMP-NANA Transport Inhibition by UDP-GlcNAc

UDP-GlcNAc, when in excess in the cell, leads to the inhibition of CMP-SAT that results in a decrease in the transport of CMP-NANA from the cytosol to the Golgi. In the previous section of this Chapter, the effect of ammonium on the expression level of UDP-GlcNAc was evaluated. Knowing the expression level of

UDP-GlcNAc, it is possible to determine the rate of transport of CMP-NANA into the Golgi. CMP-SAT activity follows Michaelis-Menten kinetics; hence this rate expression was employed to determine the transport rate. CMP-SAT was taken as the enzyme, CMP-NANA as the substrate, and UDP-GlcNAc as the inhibitor. No other inhibitors of CMP-SAT were taken into account. The resulting Michaelis-Menten equation is shown in equation 8:

cell cytosol, it equilibrates with ammonia and H^+ ion. Ammonia, which has a high permeability, diffuses through the membrane to the Golgi where it is in equilibrium with ammonium as given by the following reaction

Hence the saturation constant of

The ODEs were solved using the ODE 15s solver in MATLAB. The pH was derived from the model knowing that $\text{pH} = -\log [\text{H}^+]$. Table 2 lists the literature sources from which the model parameters were obtained. No literature values were found for

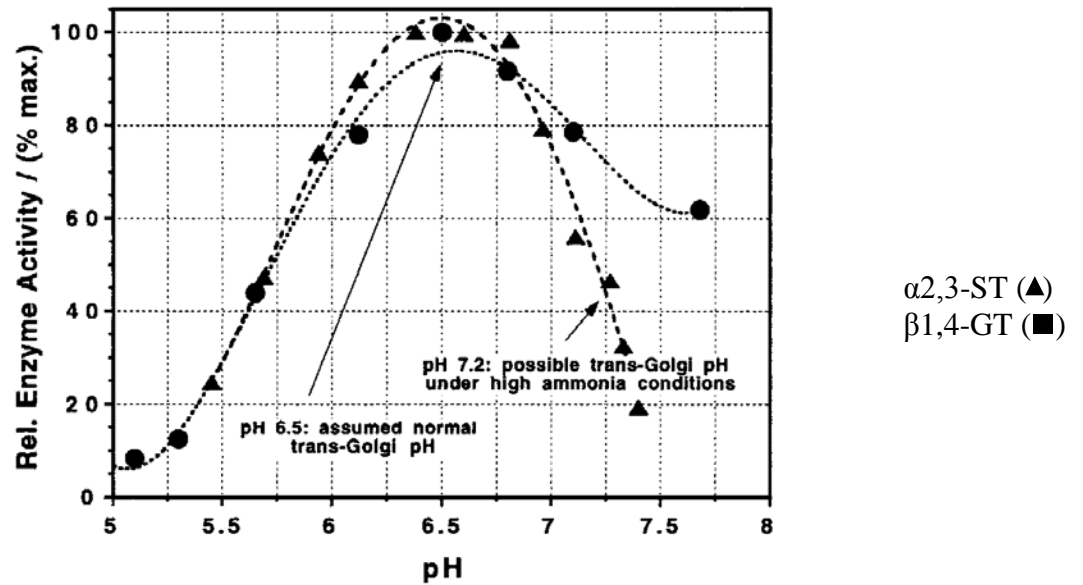


Figure 10: Experimental Graph of the Relative Activity of $\alpha 2,3$ -ST and $\beta 1,4$ -GT as a Function of pH Obtained from Gawlitzek *et al.* (2000)

The graph shows the relative enzyme activity for both $\alpha 2,3$ -ST and $\beta 1,4$ -galactosyltransferase ($\beta 1,4$ -GT) enzymes but this thesis is only concerned with the results for $\alpha 2,3$ -ST. The result of this study was consistent with previous studies that showed that the normal pH of the trans-Golgi is 6.5 and that the activity of the enzyme decreases with increasing pH above the optimum (Andersen and Goochee, 1995).

A Michaelis-Menten formulation was used to develop a model for the $\alpha 2,3$ -ST enzyme activity as a function of pH (named sub-model 6b). The following assumptions were made in developing the model

- 1) $\alpha 2,3$ -ST is an ionizing enzyme and depends on the pH of the Golgi to function.

This means that $\alpha 2,3$ -ST becomes active when it binds with an H^+ ion. This assumption was made in order to incorporate the effect of the H^+ ion on the activity of the enzyme.

- 2) α 2,3-ST has two active sites where the CMP-NANA and the glycan structure of the protein bind.
- 3) Other sugar molecules compete with the substrates for the occupancy of the active sites.

The derivation of the model is shown in Appendix A. The resulting sub-model 6b is shown in equation 15:

Table 3: Literature Sources for Parameter Values Used in the α 2,3-ST Enzyme Activity Model

Model Parameter	Parameter Value	Literature Source
	484 min ⁻¹	Krambeck and Betenbaugh, 2005
	1 μ M	Krambeck and Betenbaugh, 2005
	950 μ M	Jimenez del Val <i>et al.</i> , 2011
	57 μ M	Krambeck and Betenbaugh, 2005
	0.8 μ M	Chiaramonte <i>et al.</i> , 2001
	260 μ M	Krambeck and Betenbaugh, 2005
	1200 μ M	Jimenez del Val <i>et al.</i> , 2011

The values K_1 , K_2 , and

- 3) Relationship between extracellular ammonium concentration and the expression level of CMP-SAT.
- 4) Relationship between extracellular ammonium concentration and UDP-GlcNAc concentration.
- 5) Correlation between UDP-GlcNAc concentration and CMP-NANA transport.
- 6) Correlation between extracellular ammonium concentration and the pH of the trans-Golgi.

With sub-models 1 to 6 developed, they are combined to form the main model which computes the effect of ammonia on the extent of terminal sialylation.

Chapter 3

MODEL RESULTS AND DISCUSSION

After developing the six sub-models which make up the mathematical model that computes the effect of ammonium on the glycosylation pattern of mAbs, the results of the model were obtained and analyzed. Chapter 3 focuses on the results and discussion of the sub-models and how these results help to explain the effect of ammonia on the glycosylation of mAbs produced by CHO cells. The sub-models are listed below for reference:

1. Sub-model 1: Glutamine \rightarrow NH_4^+ extracellular
2. Sub-model 2: NH_4^+ extracellular \rightarrow NH_4^+ intracellular
3. Sub-model 3: NH_4^+ extracellular \rightarrow CMP-SAT
4. Sub-model 4: NH_4^+ extracellular \rightarrow UDP-GlcNAc
5. Sub-model 5: UDP-GlcNAc \rightarrow Transport of CMP-NANA
6. Sub-model 6: NH_4^+ extracellular \rightarrow trans-Golgi pH \rightarrow α 2,3-ST activity

3.1 Extracellular and Intracellular Ammonium Concentration

Sub-model 1 (equation 5) and sub-model 2 (equation 6) that were previously discussed in Chapter 2 were employed in this section. The model equations were used to determine the ammonium concentration in the CHO cell culture and cytosol with a known glutamine concentration of 20 mM and no initial ammonium concentration. The model results displayed in Figure 12 are for CHO cells seeded at a density of $4\text{E}+4$ cells/mL and at a temperature of 37°C for 144 hours.

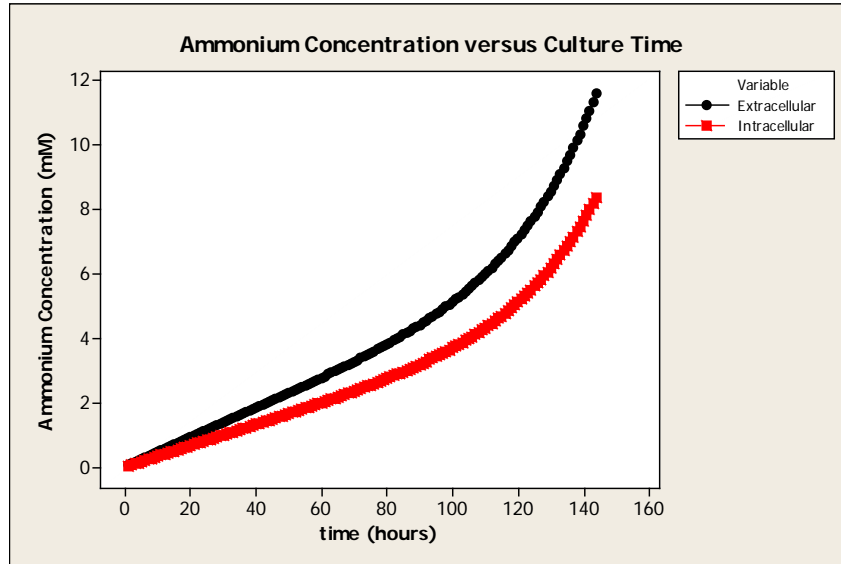


Figure 12: Model Results for Extracellular and Intracellular Ammonium Concentrations as a Function of Culture Time. For cells seeded at $4E+4$ cells/mL with a glutamine concentration of 20 mM

Figure 12 shows that the intracellular ammonium concentration is always less than the extracellular concentration. The model values are biologically reasonable and can be compared to the experimental values from Chen and Harcum (2006) that are shown in Figure 5. The study shows that for the control culture (no extra ammonium added to the culture), the extracellular ammonium concentration at 61 hours was 4 mM which is similar to the model concentration of 3 mM.

The models were also used to determine the extracellular and intracellular ammonium concentrations at 80 hours of culture time for different initial glutamine concentrations. Figure 13 shows the graph of this simulation.

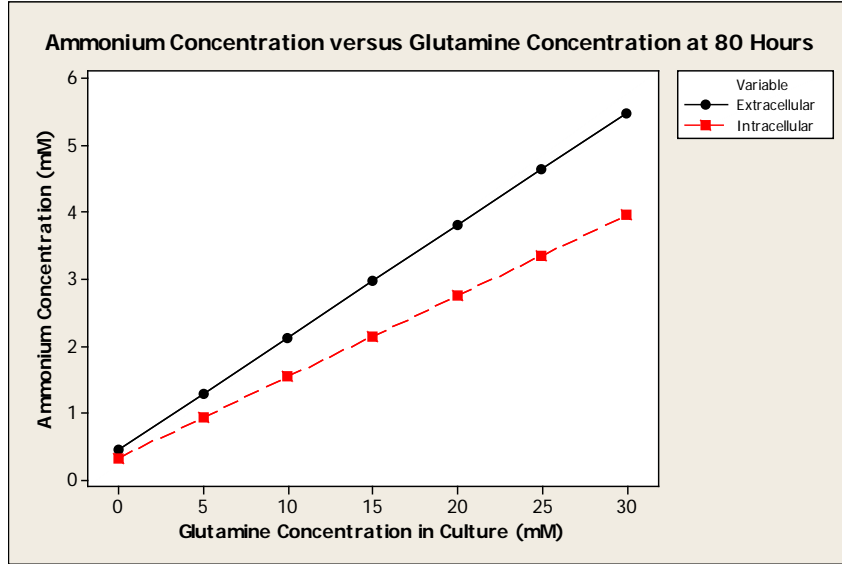


Figure 13: Ammonium Concentrations at 80 Hours of Culture Time for Varying Initial Glutamine Concentrations. Cells seeded at $4E+4$ cells/mL

Figure 13 shows that an increase in initial glutamine concentration would lead to an increase in both intracellular and extracellular ammonium concentration as expected. It also shows again that the intracellular ammonium concentration is less than the extracellular ammonium concentration. Both trends observed have a linear upward sloping curve.

3.2 Effect of Extracellular Ammonium on UDP-Glucosamine

Sub-model 4 (Figure 9) can be used to calculate the UDP-GlcNAc content in the cell from a known extracellular ammonium concentration.

With the UDP-GlcNAc content or concentration known, and using sub-model 5 (equation 8), the inhibiting effect of the nucleotide-sugar on the rate of transport of CMP-NANA from the cytosol to the Golgi can be investigated. It is expected that the rate of transport would decrease as UDP-GlcNAc binds competitively with the active

site of CMP-SAT. Figure 14 shows this expected trend. As the concentration of UDP-GlcNAc in the cytosol increases due to an increase in the ammonium concentration in the cell, the rate of transport decreases.

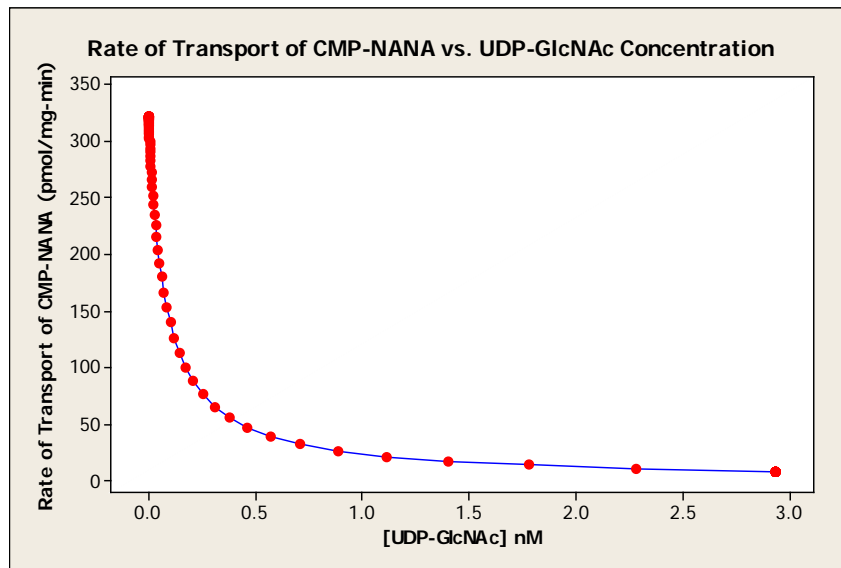


Figure 14: Inhibiting Effect of UDP-GlcNAc on Transport of CMP-NANA into the Golgi

Figure 14 shows that the rate of transport of CMP-NANA decreases rapidly with a little increase in UDP-GlcNAc. At a UDP-GlcNAc concentration of 1 nM, the rate of transport of CMP-NANA is almost at 0 pmol/mg-min. This result implies that the effect of the increase of UDP-GlcNAc on the transport of CMP-NANA is significant.

3.3 Effect of Extracellular Ammonium on the pH of the trans-Golgi and on the Activity of the Alpha2,3-Sialyl Transferase Enzyme

An increase in intracellular ammonium concentration decreases the activity of the α 2,3-ST enzyme by increasing the pH of the trans-Golgi above the optimum pH. Therefore, in order to investigate the decrease in activity of the enzyme, the pH of the trans-Golgi has to be determined. Sub-model 6a (equation 10) was used to compute the hydrogen ion concentration, and consequently the pH, in the trans-Golgi. Figure 15 shows the model results of pH versus extracellular ammonium concentration over the course of a CHO cell culture seeded at a density of $4E+4$ cells/mL with 20 mM glutamine and total culture time of 144 hours.

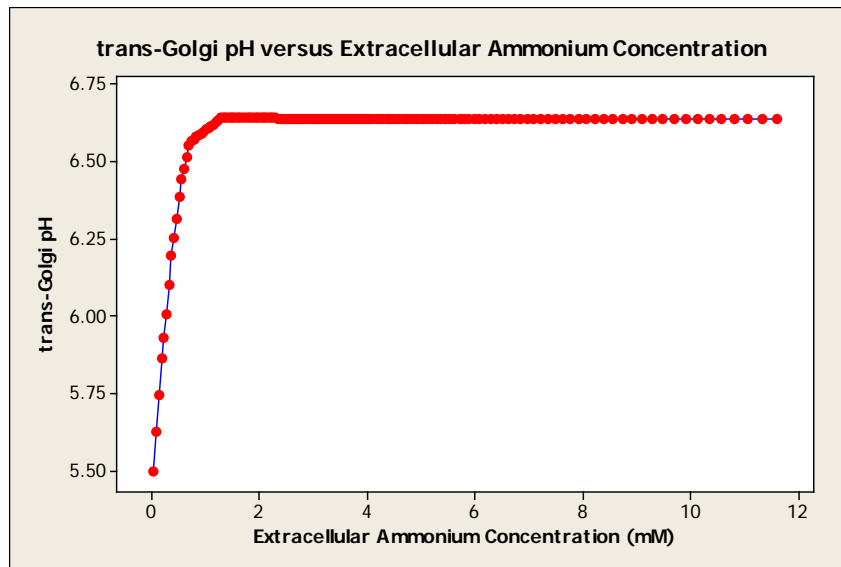


Figure 15: Hydrogen ion Concentration in the Golgi as a Function of Intracellular Ammonium Concentration

As can be seen in the graph in Figure 15, the pH of the trans-Golgi increases with an increase in extracellular ammonium concentration (as predicted in literature)

until it reaches a steady state at a pH of 6.63. The model result shows that initial increase in extracellular ammonium concentration causes a significant rise in the trans-Golgi pH. The rise in trans-Golgi pH above the optimum pH would lead to a drop in the α 2,3-ST enzyme activity. Sub-model 6b was used to investigate this relationship between pH and α 2,3-ST enzyme activity. Figure 16 shows the trend observed.

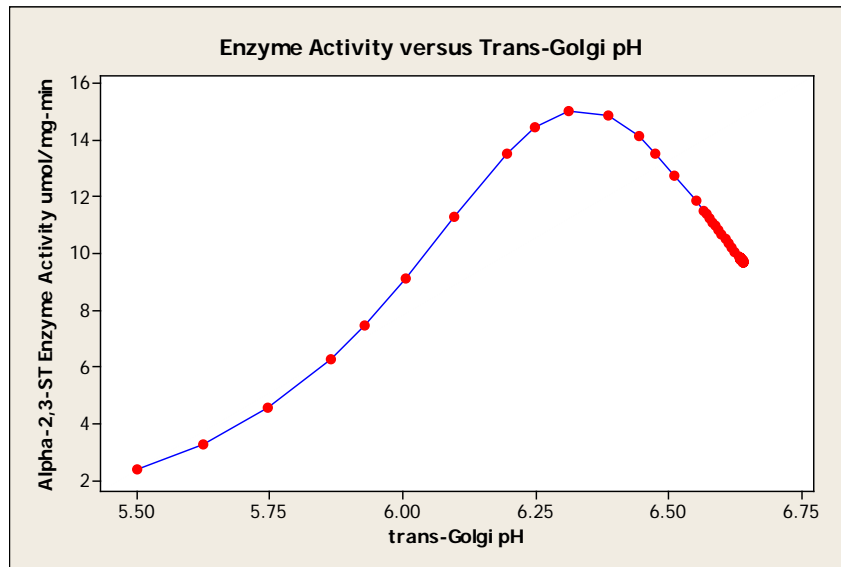


Figure 16: Alpha-2,3-ST Enzyme Activity as a Function of Trans-Golgi pH

Figure 16 shows that the maximum enzyme activity is at 15 μ mol/mg-min and occurs at a pH of 6.30. The figure shows that as the trans-Golgi pH increases, the enzyme activity initially increases until it reaches a maximum, and thereafter decreases. This model result differs from literature in terms of the enzyme's optimum pH. Literature reports the optimum pH as 6.5 (Gawlitzeck *et al.*, 2000; Andersen and Goochee, 1995). Figure 17 is shown below to further compare between the model results and the results obtained from Gawlitzeck *et al.* (2000).

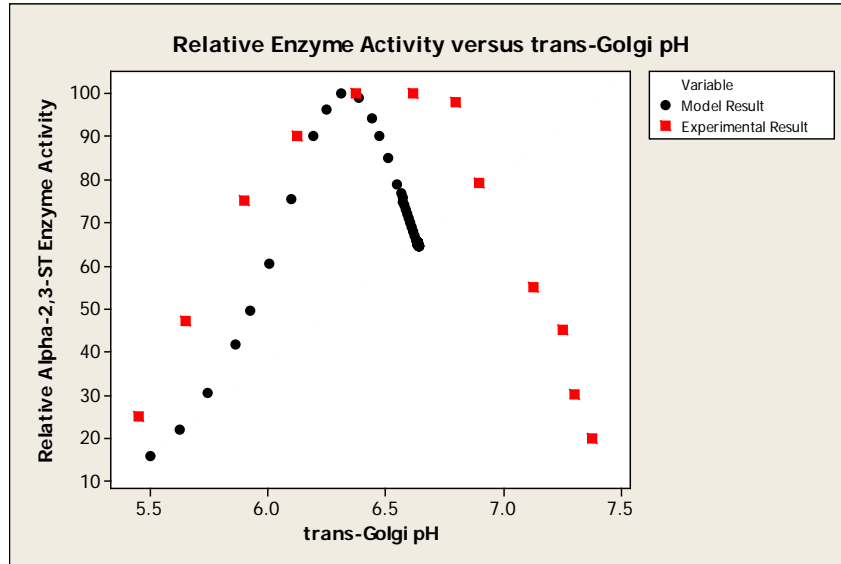


Figure 17: Model and Experimental Results for Relative Alpha-2,3-ST Enzyme Activity versus Trans-Golgi pH

The model result displayed in Figure 17 follows the general trend of the experimental results obtained from Gawlitzek *et al.*, 2000. The main differences between the model and experimental results are the optimum trans-Golgi pH and the trans-Golgi pH range over which the enzyme remains active. These differences probably exist due to the approximate parameter values used in the development of the model, specifically the values of

3.4 Effect of Extracellular Ammonium on CMP-SAT and Alpha-2,3-ST

With the different sub-models developed, it is easy to directly determine the effect of extracellular ammonium on the rate of transport of CMP-sialic acid from the cytosol to the Golgi. It is also easy to determine the relationship between extracellular ammonium concentration and alpha-2,3-ST activity. Figures 18 and 19 show the previously stated relationships respectively.

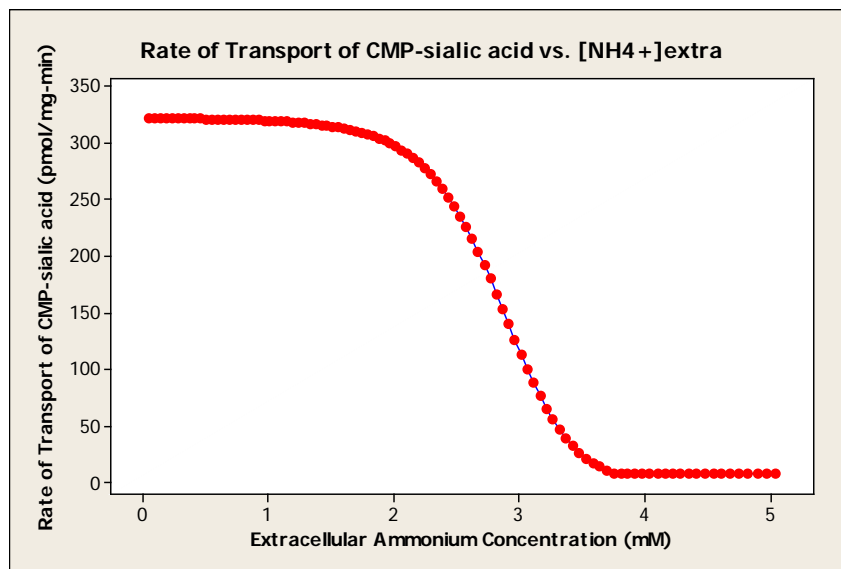


Figure 18: Rate of CMP-sialic acid Transport as a Function of Extracellular Ammonium Concentration

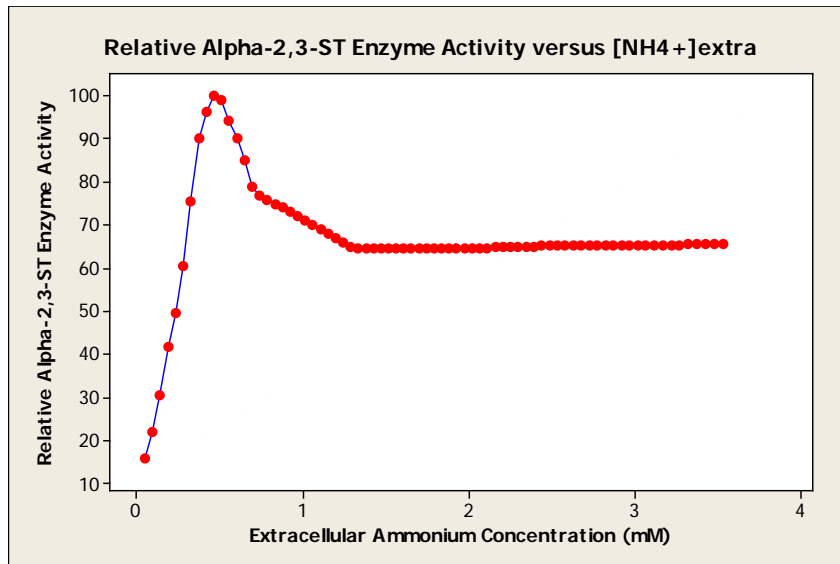


Figure 19: Relative Activity of Alpha-2,3-ST as a Function of Extracellular Ammonium Concentration

Figure 18 shows an initial lag in CMP-sialic acid transport as extracellular ammonium concentration increases up till approximately 1.8 mM. Thereafter, a steady decrease in the transport is observed until an ammonium concentration of 3.7 mM is reached. At this point, the rate of transport is negligible. A possible explanation for this trend is that at low extracellular ammonium concentrations, the flux of ammonium into the cell is not high such that the intracellular ammonium is not enough to produce an excess of UDP-GlcNAc. Without an excess of UDP-GlcNAc, CMP-SAT will not be inhibited and the transport of CMP-sialic acid will remain at a high rate.

Figure 19 shows a steady increase in the relative α 2,3-ST enzyme activity until a maximum is reached; this is followed by a decrease in relative enzyme activity until a saturation point is reached at approximately 1.4 mM extracellular ammonium. This result seems to imply that at a certain point, the α 2,3-ST enzyme activity is not

affected by the extracellular ammonium concentration. The validity of this result can be checked by performing experiments to see how extracellular ammonium concentration affects α 2,3-ST enzyme activity.

The model developed in this thesis provides a novel method for directly mapping extracellular ammonium concentration to CMP-sialic acid transport and α 2,3-ST enzyme activity.

3.5 Summary

This chapter presented and examined the results obtained from the different sub-models that make up the mathematical model for the effect of ammonia on glycosylation. The model results generated generally have a similar trend as the results seen in literature. However, some inconsistencies between the model and experimental results are probably due to approximate parameter values used for developing the model. Although not implemented in this thesis, the model results can be linked up with the Krambeck and Betenbaugh (2005) model to compute the sialic acid content of the mAb as a function of glutamine or ammonium concentration in the culture. When the link between this thesis work and the Krambeck model is made, experimenters will be able determine how much glutamine to add to the culture to achieve the desired sialic acid content.

Chapter 4

CONCLUSION

Ammonia, one of the CHO cell culture conditions, affects the glycosylation pattern of mAbs by decreasing the extent of terminal sialylation. Some mechanisms were proposed to explain the effect of ammonia on glycosylation. These mechanisms are as follows (Butler, 2006):

- Ammonia decreases the expression of CMP-SAT
- Ammonia is a precursor for UDP-GlcNAc which is a nucleotide that inhibits CMP-SAT
- Ammonia increases the pH of the trans-Golgi which in turn decreases the activity of the α -2,3-sialyl transferase enzyme.

The mechanisms stated above were incorporated in the development of a mathematical model to explain the effect of ammonium on glycosylation following a multi-scale perspective. First, it was important to map out how the ammonium is introduced into the cell culture and eventually into the cell cytoplasm. Then, the function of the ammonium in the intracellular processes of the cell was explored, taking into consideration the proposed mechanisms. Finally, the effect of the intracellular processes on glycosylation was investigated. The mathematical model is made up of six sub-models which are listed as:

1. Sub-model 1: Glutamine \rightarrow NH_4^+ _{extracellular}
2. Sub-model 2: NH_4^+ _{extracellular} \rightarrow NH_4^+ _{intracellular}
3. Sub-model 3: NH_4^+ _{extracellular} \rightarrow CMP-SAT

4. Sub-model 4: $\text{NH}_4^+_{\text{extracellular}} \rightarrow \text{UDP-GlcNAc}$
5. Sub-model 5: $\text{UDP-GlcNAc} \rightarrow \text{Transport of CMP-NANA}$
6. Sub-model 6: $\text{NH}_4^+_{\text{extracellular}} \rightarrow \text{trans-Golgi pH} \rightarrow \alpha 2,3\text{-ST activity}$

From the results obtained from the sub-models, it was found that the UDP-GlcNAc content in the cell – which is increased by the presence of ammonium – had a significant effect on the transport of CMP-NANA from the cell cytosol to the Golgi apparatus thereby decreasing the amount of sialic acid available for sialylation in the Golgi. It was also found that the presence of ammonium significantly affects the pH of the trans-Golgi by rapidly increasing the pH until it reaches a steady-state value. This increase in pH in turn affects the activity of the $\alpha 2,3\text{-ST}$ enzyme. When the pH is above the optimum value, the enzyme activity decreases leading to a decrease in sialylation. Coupling ammonium's effect on CMP-SAT and $\alpha 2,3\text{-ST}$ would lead to a considerable decrease in the sialic acid content of mAbs.

Based on comparison of model results with literature, it can be assumed that the model results are consistent with literature to a reasonable extent. However, in the future, experiments can be performed to support the validity of the model presented. For example, CHO cells can be cultured with the seeding density, culture temperature, glutamine concentration, and other culture parameters known. The glutamine and resulting extracellular and intracellular ammonium concentrations would be monitored over culture time. Similarly, the CMP-SAT and UDP-GlcNAc expression levels, the trans-Golgi pH, and $\alpha 2,3\text{-ST}$ activity in the cell would be measured. The known culture parameters can be inputted to the model to obtain model results. The model results can be compared to the experimental values to analyze how closely the model fits the experimental results.

By linking the model developed in this thesis with the Krambeck and Betenbaugh (2005) model, it would be possible to determine the sialic acid content of mAbs given the glutamine or ammonium concentration. This model approach can be adopted for other process variables that affect the glycan pattern of mAbs. This work will aid in developing a method for real-time quality control of glycosylation of mAbs.

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Appendix A

MODEL DERIVATIONS

A.1 Sialylation Rate Equation

Consider the following reaction in which a sialic acid molecule is attached to a polysaccharide in a reversible manner

Appendix B

RELEVANT GRAPHS

The cell specific content of UDP-GlcNAc as a function of initial glutamine concentration in the culture at a culture time of 80 hours.

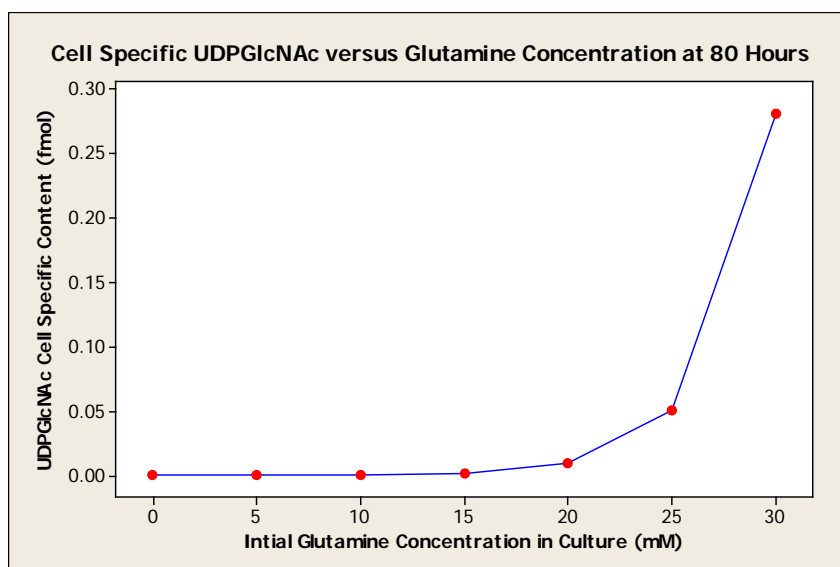


Figure B1: Cell Specific UDP-GlcNAc as a Function of Glutamine Concentration at 80 Hours of Culture Time.

The trend in hydrogen ion concentration in the cytosol of the CHO cell as a function of culture time.

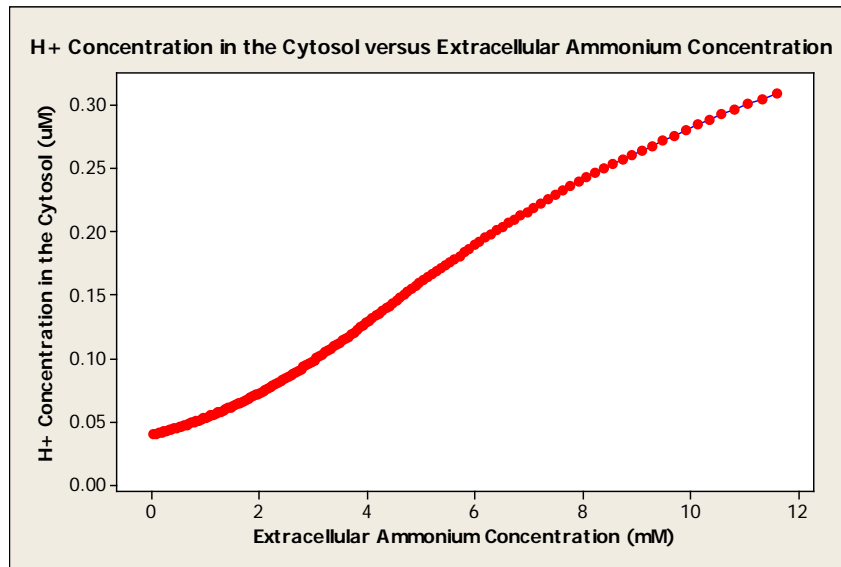


Figure B2: Hydrogen Ion Concentration in the Cytosol versus Culture Time