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Studies on the Relationship Between Metabolism and Control of \(N\)-linked Glycosylation in CHO Cells

A dissertation submitted to Trinity College, University of Dublin, in candidature for the degree of Doctor of Philosophy

by

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School of Biochemistry and Immunology
Trinity College Dublin

July 2010
Declaration

I certify that none of the work presented in this thesis has been submitted for any degree at this or at any other University. I declare that this thesis is entirely my own work. I agree that the library may lend or copy this thesis upon request.
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I would like to give thanks to Dr. Gavin Davey for the opportunity to work on this topic and project, and for his patience and support along the way. Another word of thanks to Professor Keith Tipton, for always having time for a chat, regardless of the topic, and for the good humour you bring to every conversation. Credit and praise Dr. Teun van de Laar for instilling in me a sense of curiosity, enthusiasm and excitement for all cell culture results, regardless of how unexpected they are (not to mention the invaluable technical help and support). To Dr. Corné Stroop, many thanks for the education in N-linked glycosylation and chocolate! I am indebted to Dr. Wout van Grunsven for the freedom and assistance I was afforded while working in SSCC&D in MSD, Oss, the Netherlands.

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Summary

The process of post-translational attachment of oligosaccharide structures to asparagine residues in nascent polypeptides is termed N-linked glycosylation. These glycan structures can influence the folding, stability, solubility, antigenicity, activity and rate of clearance of a glycoprotein from the bloodstream. Cellular metabolism for the generation of energy and biosynthetic intermediates is intrinsically linked to the process of N-linked glycosylation. Consequently, the mechanisms by which altered metabolism of Chinese hamster ovary cells (CHO) affected the N-linked glycosylation of recombinantly expressed human chorionic gonadotrophin (HCG) was explored in a batch and continuous mode of cultivation. The structures of the N-linked glycans attached to HCG were assessed with the use of IEF and HPLC, and, cellular metabolism was assessed by examining rates of consumption and production of metabolites and using metabolic flux analysis. The mechanism of any changes in glycosylation was determined by assaying the intracellular content of nucleotides and sugar nucleotides.

High concentrations of ammonium can have negative impacts on cell growth, metabolism and glycosylation. Since the chemical degradation and intracellular catabolism of glutamine produces ammonium, varying the initial concentration of glutamine in the culture medium was explored as a method to reduce these effects. A concentration range of 0mM to 20mM glutamine was tested during a five-day batch culture. Significant differences were noted when the cells were cultivated at 0mM glutamine; the rate of glucose consumption, lactate production and growth rate were reduced, and the culture pH was increased. The rate of production of HCG was improved on day 5 of culture. From day 1 of culture, a decrease in the sialylation, antennarity and fucosylation of HCG was observed while the proportion of neutral structures increased. However, due to the characteristic changing cultivation conditions during batch culture, the changes in N-linked glycosylation of HCG could not be confidently attributed to the lack of glutamine. Use of L-alanine-L-glutamine (GlutaMAX-I) as a glutamine replacement strategy reduced the ammonium concentration and increased the viable cell number, but caused no significant change to the glycosylation of HCG.

The mechanisms by which glutamine causes these changes in N-linked glycosylation were subsequently explored in continuous mode of culture; in this case, the culture conditions stabilise after a period of time (a steady state is defined). By varying the glutamine
concentration between 0mM and 8mM glutamine four steady states were examined. A reduction in sialylation, antennarity and fucosylation of the N-linked glycans attached to HCG was accompanied by an increase in the proportion of neutral structures at steady state 4, where the glycolytic flux was reduced and glutamine was not supplied to culture medium. These glycosylation changes were accompanied by a significant decrease in the intracellular content of UDP-GlcNAc. Since this sugar nucleotide is a substrate for the glycosyltransferase enzymes that catalyse the addition of new glycan branches, and is precursor for the synthesis of sialic acids, the glycosylation changes can be directly linked to this change.

The effect of varying the cell growth rate on the cellular metabolism and glycosylation of HCG was examined by altering the rate of dilution of culture medium in a continuous culture. Four steady states were examined by varying the dilution rate between 0.2/d and 0.5/d. At a low dilution rate, the flux through glycolysis and the TCA cycle was reduced, while flux to alanine and aspartate and biomass was similarly decreased. The intracellular content of nucleotides and sugar nucleotides was also reduced, and this led to a decrease in the sialylation, fucosylation and antennarity of HCG, while the proportion of neutral structures increased. At steady state 3 (the second instance of a dilution rate of 0.2/d), a significant increase in the productivity of HCG occurred alongside a change in the metabolism of amino acids. Despite the increase in intracellular nucleotides and sugar nucleotides, the glycosylation of HCG was consistent with that observed at steady state 1 (also operated at a dilution rate of 0.2/d). This difference in metabolism at these two steady states (with the same feed and dilution rate) is termed steady state multiplicity.

The effect of the extended time in culture on the growth, metabolic and productivity characteristics of this cell line was also examined. At the end of each continuous culture, between 62 and 91 generations (or population doublings) had accumulated, and an aliquot of these cells were inoculated in batch shake flasks for five days. An increase in cell growth and metabolism was noted in the early days of these high generation number batch cultures, but these values reduced to control levels thereafter. The total and volumetric productivity of HCG was also decreased.
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<td>Amino acids</td>
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<tr>
<td>2-AB</td>
<td>2-Aminobenzamide</td>
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<td>Sialidase A</td>
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</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BKF</td>
<td>Bovine kidney fucosidase</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BTG</td>
<td>Bovine testes galactosidase</td>
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<td>Chinese hamster ovary cells</td>
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<td>CMP-N-acetyl neuraminic acid</td>
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<td>GPI</td>
<td>Glucosamine-6-phosphate isomerase</td>
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<td>GPS</td>
<td>Glucosamine-6-phosphate synthetase</td>
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<td>Histidine</td>
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<td>Jack bean mannosidase</td>
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<td>Kilodaltons</td>
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<td>KH₂PO₄</td>
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<td>Potassium hydroxide</td>
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<td>ΔL/ΔG</td>
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<td>NAD⁺</td>
<td>β-Nicotinamide adenine dinucleotide</td>
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<tr>
<td>NADH</td>
<td>Reduced form of β-nicotinamide adenine dinucleotide</td>
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<td>NADPH</td>
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<td>Sodium dihydrogen phosphate</td>
</tr>
<tr>
<td>NaN₂PO₄</td>
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<td>NH₃</td>
<td>Ammonia</td>
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<td>viii</td>
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<td>Symbol</td>
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<td>OAA</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>pCO$_2$</td>
<td>Partial pressure of carbon dioxide</td>
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<td>Phe</td>
<td>Phenylalanine</td>
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<tr>
<td>pI</td>
<td>Isoelectric point</td>
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<td>Pi</td>
<td>Orthophosphate ion</td>
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<td>PNGase F</td>
<td>Peptide N-glycosidase F</td>
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<tr>
<td>pO$_2$</td>
<td>Partial pressure of oxygen</td>
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<td>Pro</td>
<td>Proline</td>
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<tr>
<td>q</td>
<td>Rate of consumption or production</td>
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<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<tr>
<td>Ser</td>
<td>Serine</td>
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<td>SuccCoA</td>
<td>Succinyl-Co-A</td>
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<tr>
<td>TCA cycle</td>
<td>Tricarboxylic acid cycle / Krebs cycle / Citric acid cycle</td>
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<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
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<tr>
<td>tPA</td>
<td>Tissue plasminogen activator</td>
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<tr>
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<tr>
<td>Val</td>
<td>Valine</td>
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<tr>
<td>VC</td>
<td>Viable cell</td>
</tr>
<tr>
<td>Vh</td>
<td>Volt hours</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume per unit volume</td>
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<tr>
<td>W</td>
<td>Watts</td>
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<tr>
<td>w/v</td>
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General Introduction
1.1 The process of N-linked glycosylation

1.1.1 Sugars involved in glycosylation

An N-linked glycoprotein is composed of a protein that is covalently linked to a chain of monosaccharides. The sugar constituent, which can also be termed as the glycan or oligosaccharide moiety, is held together by glycosidic linkages.

The seven key monosaccharides that occur within the N-linked glycans in mammalian eukaryotic cells are; glucose, mannose, galactose, fucose, N-acetylgalactosamine, N-acetylgalactosamine and N-acetylneuraminic acid (abbreviated to Glc, Man, Gal, Fuc, GlcNAc, GalNAc and NeuAc, respectively, Figure 1.1 A). Most of the sugars are present in nature in the D conformation as cyclic hemiacetals or hemiketals. All of these sugars are structurally linked; epimerisation of glucose at C2 or C4 leads to the formation of mannose and galactose, respectively. Replacement of the 2 OH group in galactose and glucose with 2-acetamido forms N-acetylgalactosamine and N-acetylglucosamine, respectively. Fucose is a mirror image of galactose, but with the loss of the 6 OH group. Consequently, fucose is in the unusual state of the L conformation. The most common form of sialic acid in humans is N-acetylneuraminic acid (sometimes termed Neu5Ac or simply NeuAc), although different forms predominate in other species. NeuAc is formed by the condensation of activated forms of pyruvate with N-acetylmannosamine. This is then further converted to Neu5Ac. This nine-carbon sugar acid is usually found in a six-member ring conformation that is formed by joining the carbonyl group at C2 and the 6-hydroxyl group in a hemiketals. There are several unique substituents extending from various positions on the ring. Each of these structures is usually found in a pyranose ring conformation within glycoconjugates, which is illustrated in Figure 1.1 A.

These monosaccharides can be linked together with a glycosidic bond, where the hemiacetal group attached to the ring of one monosaccharide condenses with the hydroxyl group of another monosaccharide to form an acetal group. One water molecule is eliminated in this reaction, as illustrated by the β1-4 glycosidic linkage of galactose and glucose to form lactose in Figure 1.1 B. Two types of stereochemistry can exist for glycosidic bonds – a β linkage is formed when bonds are made in the same plane as the monosaccharide conformation (i.e. equatorial bonds to the pyranose ring). An α linkage is formed by reaction of the axial bonds of the pyranose ring (perpendicular to the plane of the ring structure). The resulting disaccharide retains the ability to form another glycosidic linkage at the reducing end.
Figure 1.1 Common monosaccharides that occur in N-linked glycans and the glycosidic linkage.

A) The pyranose ring formation of the seven monosaccharides used commonly in mammalian N-linked glycoproteins (Varki et al., 2009). B) The formation of a glycosidic bond by condensation of the hemiacetal group of galactose with the hydroxyl group of glucose forms lactose, while a reducing end is retained for the formation of further glycosidic linkages (Taylor and Drickamer, 2004).
1.1.2 Formation and activation of monosaccharides

The sugars incorporated into glycoproteins are derived from three main sources. Firstly, external sugars can be transported into the cell, either by an energy-independent facilitated diffusion transporter (e.g. glucose transporters), or by energy-dependent ion-coupled transporters (e.g. sodium glucose transporters). Secondly, there is evidence that significant amounts of carbohydrates are salvaged from other degraded glycoproteins, since lysosomal carriers for neutral hexoses, N-acetylated amino sugars and acidic hexoses have been identified. This allows the monosaccharides to exit the degradation pathways of the lysosome to gain access to the cytosol, where the biosynthetic enzymes are located. Additionally, there are a variety of enzymes and pathways that exist to interconvert the existing monosaccharides into the required sugars. These major pathways are shown in Figure 1.2 A (which also shows the close link of this pathway to glycolysis and the TCA cycle).

The monosaccharides need to be activated to a sugar nucleotide in order to be added to the growing glycan chain. This occurs by activation of the sugar with a kinase, followed by the transfer of the NDP to the sugar-phosphate structure by a nucleotidyltransferase (Figure 1.2 B, reaction 1). Alternatively, the activated donors can be generated from another existing activated sugar nucleotide, as illustrated in Figure 1.2 B (reactions 2 and 3). Glucose, galactose, N-acetylglucosamine and N-acetylgalactosamine form UDP activated sugars, while mannose and fucose form GDP sugar nucleotides. Sialic acids form a specific CMP-NeuAc sugar nucleotide.

Once these activated sugars have been formed in the cytosol, they need to be transported to the endoplasmic reticulum (ER) or Golgi. This is achieved via the use of energy-independent sugar nucleotide antiporters that deliver the NDP-sugar into the lumen of these organelles, with the accompanying exit of NMPs, which were derived from the use of these nucleotide diphosphates. As a result, the rate of sugar nucleotide utilisation is normally coupled to the rate of import, although a pool of transported but unused sugar nucleotides can exist in the Golgi. Return of the nucleotide monophosphates to the cytosol also makes them available for re-activation, making it a highly efficient salvage system for the precursors. The antiporters are organelle specific, and usually correspond to the location of known transferase enzymes that catalyse the transfer of sugars to the growing oligosaccharide chain. The $K_m$ of the transporters ranges from 1 to 10 $\mu$M (Varki et al., 1999).
Figure 1.2 The formation and interconversion of sugar nucleotides.

A) The biosynthesis and interconversion of monosaccharides for the formation of N-linked glycans. The relative contribution of each substrate under physiological conditions is unknown. Donors are shown in red shadowed rectangles, monosaccharides as blue shadowed rectangles. The link between glycolysis, the TCA cycle and the formation of monosaccharide donors is illustrated. The influence of glutamine and ammonium on the formation of UDP-GlcNAc is shown, as is the eventual synthesis of CMP-NeuAc from UDP-GlcNAc. The total amount of UDP-GlcNAc and UDP-GalNAc is known as the UDP-GNAc pool. B) Activated sugar donors are formed by the sequential action of a kinase and nucleotidyltransferase (reaction 1). Alternatively, they can be generated from existing activated sugars, such as the interconversion of UDP-GlcNAc and UDP-GalNAc by UDP-N-acetylglucosamine-4-epimerase (reaction 2). Nucleotide exchange reactions can also occur to form rare sugars such as ADP-Glc, which are more common in plants and microbes (reaction 3). (Varki et al., 1999).
1.1.3 Synthesis of oligosaccharide precursor

The first step in production of all N-glycans involves synthesis of a lipid-linked oligosaccharide (Figure 1.3 A). The precursor consists of the polyisoprenol lipid dolichol linked to 14 specific monosaccharides by a pyrophosphate linkage (Dol-P-P-Glc₃Man₉GlcNAc₂).

Firstly, N-acetylglucosamine-1-phosphate is transferred from UDP-N-acetylglucosamine to dolichol-P by GlcNAc-1-phosphotransferase. Subsequently, one N-acetylglucosamine and five mannose residues are added to this structure in a distinct conformation by the action of GlcNAc transferase and mannosyltransferases, respectively. This structure is then flipped from the cytoplasmic face of the endoplasmic reticulum membrane bilayer to the luminal face. In yeast, this translocation is catalysed by the action of Rft1p, an ATP-independent, bi-directional, membrane-spanning flippase (Helenius et al., 2002).

Once this structure faces the inside of the endoplasmic reticulum, four more mannose residues and two glucose residues are added to the chain from dolichol-P donors. These donors are synthesised on the cytoplasmic face of the endoplasmic reticulum by reaction of UDP-Glc or GDP-Man with dolichol-P, and they are also translocated across the membrane. All the monosaccharides for this process are derived from dolichol-P donors, and cleavage of the saccharide-phosphate bond yields the energy for synthesis of the glycosidic linkages.

1.1.4 Transfer of oligosaccharide precursor to the nascent polypeptide

The enzyme complex oligosaccharyl transferase (OST) catalyses the co-translational transfer of this precursor oligosaccharide to a nascent polypeptide (Figure 1.3 B). The enzyme complex is located in the lumen of the endoplasmic reticulum, and the yeast form is known to be composed of at least nine non-identical transmembrane subunits. The complex catalyses the addition of Glc₃Man₉GlcNAc₂ to the asparagine residue within the consensus sequence Asn-X-Ser/Thr, where X can be any amino acid except proline. Cleavage of the glycan phosphate bond provides the energy for creation of the GlcNAc-asparagine linkage. Dolichol phosphate is regenerated at this stage by the cleavage of one of the phosphate groups from dolichol-PP by a phosphatase.
Figure 1.3 Formation and attachment of the lipid-linked oligosaccharide precursor to a nascent polypeptide.

A) Synthesis of the lipid-linked oligosaccharide precursor Dol-P-P-Glc$_3$Man$_9$GlcNAc$_2$, occurs on both the cytoplasmic and luminal sides of the endoplasmic reticulum. B) The multi-subunit enzyme complex oligosaccharyltransferase catalyses the transfer of the lipid precursor to the nascent protein as it is being synthesised by the ribosome (Taylor and Drickamer, 2004).
1.1.4 Processing of the oligosaccharide

Following transfer to the polypeptide, the oligosaccharide is subject to a series of processing steps (Figure 1.4 A). These initial steps are conserved throughout vertebrates.

The first modification of the Glc$_3$Man$_9$GlcNAc$_2$ structure occurs by the action of glucosidases I and II that rapidly remove two glucose molecules, followed by removal of a third a few minutes later. This removal process is important for the retention of the protein within the endoplasmic reticulum until the protein is properly folded. The chaperones calnexin and calreticulin will bind to specific improperly folded proteins, while the enzyme $\alpha$-glucosyltransferase re-glucosylates the oligosaccharide chain to allow time either for the protein to become properly folded, or to be deglucosylated fully and eventually degraded.

As this structure migrates from the endoplasmic reticulum to the cis-Golgi, it can either be targeted to the lysosome, or subject to the action of a mannosidase. Modification of the glycan by action of GlcNAc phosphotransferase at a mannose residue, and eventual removal of the GlcNAc to yield Man-6-P targets the protein to the lysosome, by a largely unknown process. If the protein is not directed to the lysosome, $\alpha$-mannosidase acts to cleave mannose residues.

From this point onwards, a variety of pathways exist which will result in the formation of one of the three types of N-linked glycan structures – high mannose, complex or hybrid (Figure 1.4 B). High mannose type glycans result from a lack of addition of different sugars to the basic GlcNAc$_2$Man$_5$ structure as the glycoprotein travels through the Golgi – although the number of mannose residues may vary. A hybrid structure is formed when one mannose branch is substituted with other sugars while the remaining branch retains its mannose residues. A complex N-linked glycan can be obtained by substituting both the $\alpha$-3 and $\alpha$-6 linked core mannose residues with GlcNAc residues, and subsequently extending these chains with other sugars. Complex oligosaccharides are the most commonly found structures in vertebrates.

The formation of either hybrid or complex structures begins with modification of GlcNAc$_2$Man$_5$ (which results from cleavage of Glc$_3$Man$_9$GlcNAc$_2$ in the Golgi by a number of mannosidases, Figure 1.5).
Figure 1.4 Processing of N-linked glycoproteins and the formation of high mannose, hybrid and complex structures.

A) Processing of the Glc$_3$Man$_9$GlcNAc$_2$ structure occurs via the sequential action of a range of glycosidases and transferases as the glycoprotein passes through the endoplasmic reticulum and Golgi. Firstly, three glucose residues are removed by the action of glucosidases I and II in the ER. A number of mannose residues are cleaved by mannosidases before addition of GlcNAc residues on either branch arm (or both). These structures can then be extended with a range of other structures including galactose and sialic acids by activity of galactosyltransferase and sialyltransferase enzymes, respectively.  B) Differential processing of the N-linked glycans results in formation of high mannose, hybrid and complex glycans which differ in the type of substitution to the core mannose residues (Taylor and Drickamer, 2004).
GlcNAc transferase I (GlcNAcT-I or GnT I) acts first to add GlcNAc to Man₃GlcNAc₂ in a β,1-2 linkage. If this structure becomes a substrate for the action of GlcNAc transferase III, a bisecting GlcNAc residue will be added to the core mannose residue. A hybrid structure results from this reaction since α-mannosidase II cannot act on this glycan. However, if α-mannosidase II cleaves the two remaining α-1,3 and α1,6 linked mannose residues, the extent of glycan branching can be increased by the subsequent action of GlcNAc transferases II, IV and V. These enzymes result in the formation of complex bi-, tri- and tetra-antennary structures, respectively. Additionally, once these branches have been initiated, the addition of bisecting GlcNAc residues is possible. However, elongation of this bisecting GlcNAc will only occur if the branch initiated by GlcNAc transferase II is missing. GlcNAc transferase VI, IX and VB can result in the formation of more highly branched structures, although this has only been observed in birds and fish. A schematic of these branching steps is illustrated in Figure 1.5.

No bisecting GlcNAc residues are found in the N-linked glycans of CHO cells, due to the transcriptional repression of GlcNAc transferase III, which results in no detectable enzyme activity (Stanley et al., 2005). CHO cells, which are derived from ovary cells of Chinese hamsters, are commonly used for the production of recombinant proteins due to the favourable growth characteristics and similarities in glycosylation.

With the exception of the GlcNAc residues added by GlcNAc transferase III, the oligosaccharides can be further extended from this point with other monosaccharides such as galactose or sialic acids. This requires the action of galactosyltransferase and sialyltransferase enzymes. The sialic acids attached to N-linked glycans in CHO cells are usually in an α-2,3 linkage (Lee et al., 1989). In contrast, human glycoproteins generally carry both α-2,3 and α-2,6 linked sialic acids (Bragonzi et al., 2000). Engineering of cell lines may be required in the future due to the immune functions mediated through this α-2,6 linked sialic acid structure (Hedlund et al., 2008; Crespo et al., 2009).

Core fucosylation is a common modification that occurs after processing in the medial Golgi compartment. This involves the addition of fucose in an α-1,6 linkage to the GlcNAc that is directly linked to the asparagine residue of the glycoprotein. There are 13 members of the fucosyltransferase family, but only isoform VII is capable of forming this linkage. Core fucosylation is not found on high mannose structures since the prior action of GlcNAc transferase I is necessary for enzyme activity.
Figure 1.5 The formation of hybrid and complex N-linked glycoproteins.

GlcNAc T I begins the conversion of a high mannose type glycan by the catalysis of the addition of GlcNAc to the structure Asn-GlcNAc$_2$-Man$_5$. GlcNAcT–III can add a bisecting GlcNAc to the core mannose residue at this stage. However, further branching of the glycan structure is prevented, meaning a hybrid structure will result. Action of α-mannosidase II after the action of GlcNAcT-I removes the α1,3 and α1,6 mannose residues and the N-glycans subsequently become substrates for GlcNAcT-IV and -V. This will produce tri- and tetra-antennary glycans. The addition of bisecting GlcNAc residues can occur after the branching process has begun by the action of GlcNAcT-II, -IV and -V. However, the bisecting chain will only be elongated if the branch added by GlcNAcT-II is not modified. Further branching to hepta-antennary structures has been found to occur in birds and fish by the action of GlcNAc transferase VI, IX and VB. These structures can be further modified by the addition of further monosaccharides such as fucose, galactose or sialic acid by the action of fucosyltransferase, galactosyltransferase and sialyltransferase enzymes, respectively. Symbols: green circles; mannose, blue squares; N-acetylglucosamine (Varki et al., 2009).
The structure of the glycans attached at each N-glycosylation site within any given protein may be different, a concept which is termed microheterogeneity. This may occur due to various factors such as differences in sugar nucleotide metabolism, protein transport rates in the Golgi or the localisation and expression of transferases. Macroheterogeneity, which occurs when not every possible glycosylation site is occupied, is another common phenomenon. This can also be described as a variation in site occupancy.

The structure of the N-linked glycans affects the conformation, solubility, antigenicity and recognition properties of the glycoprotein itself. Defects in the pathway for the synthesis of N-linked glycans have been shown to lead to congenital disorders of glycosylation (CDGs). Forty different types of CDGs have currently been described, although this autosomal recessive disorder occurs rarely. Most patients have psychomotor retardation with variable neuromuscular involvement in addition to hormonal abnormalities and changes in blood coagulation. The severity of the disease varies ranging from slight mental retardation to multi-organ dysfunctions, which is often associated with infantile lethality (Haeuptle and Hennet, 2009).

1.2 Human chorionic gonadotrophin as a model glycoprotein

1.2.1 Structural features of HCG

This work involves the use of a CHO cell line that overexpresses the glycoprotein human chorionic gonadotrophin (HCG). Human chorionic gonadotrophin (HCG) is part of the glycoprotein hormone family, which also includes follicle stimulating hormone (FSH), leutinising hormone (LH) and thyroid stimulating hormone (TSH). All of these hormones are heterodimeric in structure, consisting of an α subunit that is non-covalently associated with a β subunit. In a given species, the α subunits are all identical, and the β subunits are homologous. Both subunits are required for activity of the hormone, though the β subunit determines activity. The shared α subunit is transcribed from a single gene on chromosome 6q21.1-23 in humans, with a resulting polypeptide of 92 amino acids. The α subunit has two N-linked oligosaccharides at Asn 52 and 78.

HCGβ contains 145 amino acids, and is glycosylated at six positions in the peptide chain. N-linked oligosaccharides occur at Asn 13 and 30, while O-linked oligosaccharides are found in the so-called carboxy terminal peptide (CTP) domain at positions Ser 121, 127, 133 and 138. In humans, a family of at least six genes that are clustered on chromosome 19q13.3 encodes the β subunit.
The molecular weight of HCG as determined by MALDI-TOF MS is 37.5 kDa, although the calculated weight of a 'typical' molecule would be closer to 39 kDa. This illustrates that the carbohydrate chains used to calculate the theoretical weight are on average larger than what is found. HCGα and β subunits were determined to have molecular weights of 14000 and 23500 according to mass spectroscopy analysis (Acevedo, 2002; Stenman et al., 2006). One third of the total mass of the glycoprotein is contributed by the glycans.

HCG was the first “pregnancy hormone” to be discovered in 1928, although its crystal structure was not elucidated until 1994. Structural analysis revealed that both subunits were stabilised through a cysteine knot formed from three disulphide bonds (Figure 1.6). The heterodimer is formed and secured by a portion of the β subunit wrapping around the α subunit in a seatbelt fashion (Lapthorn et al., 1994).

LH and HCG have similar biological properties, and this is reflected in the 96% sequence identity of their β subunits. The HCG peptide backbone is identical to LH, but contains an additional 24 amino acids. The two molecules differ significantly in their sugar moieties, and thus have radically different half-lives. Both molecules effect their action through the same G-protein coupled receptor. The receptor is composed of seven transmembrane domains, a short C-terminal domain and a large extracellular domain (Srisuparp et al., 2001).

1.2.2 Glycosylation of HCG

The first investigations into the N-linked glycan structures attached to urinary forms of HCG were carried out in 1979 (Endo et al., 1979; Kessler et al., 1979). Results showed that a biantennary, galactosylated and potentially disialylated structure predominated, with the added possibility of core fucosylation (Figure 1.7). Endo et al also identified a hybrid structure where the α1-3 mannose arm was substituted with a GlcNAc, Gal and NeuAc residue. With the advent of new technologies for determining the structures of N-linked glycans, such as ion-pair RP-HPLC-ESI-MS, many additional publications have described the glycosylation of HCG. These same structures were found with the use of these new techniques, and have indicated that these are the predominant N-linked glycan structures attached to HCG (Weisshaar et al., 1991; Liu and Bowers, 1997; Kobata and Takeuchi, 1999; Gervais et al., 2003; Toll et al., 2006).
Figure 1.6 Schematic of the three dimensional structure of HCG.

A) The assembly of the α and β subunits to form the heterodimer. The polypeptide chain of the α subunit is shown in red, and the β in green, each divided into the three loop regions. The black region of the β subunit illustrates the seatbelt region. Yellow bars, thick blue lines and thin blue lines denote the cysteine knots on both subunits, the seatbelt disulfide bonds and the small loop disulfide bond of the β-seatbelt, respectively. Other disulfide bonds are shown with grey lines. The amino acid numbers are also illustrated. B) Spatial representation of the αβ assembly showing how the carboxy terminus peptide on the α subunit is stabilised by the seatbelt loop of the β subunit (de Medeiros and Norman, 2009).
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<th>N-linked glycans</th>
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<td><img src="image2" alt="O-linked glycans" /></td>
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**Figure 1.7 The N- and O-linked glycans typically attached to HCG.**

Letters indicate the initial letter of the first author of each relevant publication (Endo *et al.*, 1979; Kessler *et al.*, 1979; Weisshaar *et al.*, 1991; Liu and Bowers, 1997; Gervais *et al.*, 2003; Toll *et al.*, 2006). The glycans attached to a recombinant form of HCG expressed in a CHO cell line are described in the paper by Gervais *et al.* The remaining papers are based on data from urinary forms of HCG. The legend for the symbols used is shown on the right hand side. N-linked glycans were drawn with the use of GlycoForm (Mc Donald *et al.*, 2010).
Additionally, the glycosylation of HCG expressed recombinantly in CHO cells has also been explored. The glycans were released from HCG by hydrazinolysis, separated by HPLC and identified by mass spectrometry. A small proportion of tri-antennary structures with variable sialylation were identified, as well as one tri-sialylated, tetra-antennary glycan (Gervais et al., 2003). The glycosylation of urinary HCG has been determined by a similar method, and a much smaller number of triantennary species were identified (Toll et al., 2006). This suggests that the method of production or sourcing of HCG may alter the N-glycan antennarity.

In first 3 to 4 weeks of pregnancy, a hyperglycosylated and thus larger, more bioactive type of HCG is produced. In contrast to normal HCG, all the O-glycans on this glycoprotein have a tetrasaccharide core structure. This type of HCG is responsible for the implantation of the fetus, and levels decline soon after this time. In malignant trophoblastic diseases, such as a choriocarcinoma, the hyperglycosylated variant of HCG predominates and is responsible for the invasive activity of the tumour cells (de Medeiros and Norman, 2009).

1.2.3 Functions of the glycan chains in HCG

The impact of the glycans on the folding of HCG was explored via recombinant expression in E. coli, a system that lacks eukaryotic-type glycosylation. In these cases, the subunits were found to fold incorrectly and as a result, dimerisation did not occur (Strickland et al., 1985; Huth et al., 1994). Site directed mutagenesis of the N-glycosylation sites of the α-subunit in CHO cells showed that the deletion of the Asn-52 residue caused a reduction in the secretion levels of the dimer, indicating that glycosylation at this particular residue is important for folding and assembly. Additionally, the glycans at Asn-78 site contribute to the stability of the dimer. (Matzuk and Boime, 1988)

The influence of sialylation on the half-life of HCG within circulation has been recognised since 1971. Digestion of HCG with neuraminidase was found to reduce the half life from 50 minutes to 1 minute in the rat (Van Hall et al., 1971). It is likely that the hepatic asialoglycoprotein receptor is responsible for this clearance from circulation, since it mediates endocytosis and degradation of a wide range of glycoproteins lacking terminal sialic acids (Stockell Hartree and Renwick, 1992; Weigel and Yik, 2002). The O-glycans attached to the β subunit also play an important role in the half-life of HCG. Removal of this “carboxy terminal peptide” region has no impact on the activity, but the half life
decreases dramatically (Kalyan and Bahl, 1983). As a result, various techniques have been used to engineer this sequence into other proteins such as EPO and FSH to increase their half life and consequently reduce the frequency of administration of these therapeutics (Lee et al., 2006; Fauser et al., 2009).

The interaction of HCG with its receptor and subsequent transduction of a signal is greatly influenced by the glycans. It has long been established that removal of sialic acid residues will increase the affinity of the protein for its receptor, while also resulting in a 50% decrease in the stimulation of the intracellular cAMP cascade. Removal of all the sugars attached to HCG increases the affinity even more, while reducing the activity to almost zero. Desialylation of EPO has been found to increase receptor affinity due to the reduction in charge or electrostatic attraction, which suggests a similar mechanism for this protein (Darling et al., 2002). This affinity – activity relationship is modulated through the glycans at Asn-52 on the α-subunit, meaning this sugar chain, and this subunit are crucial for activity (Thotakura and Blithe, 1995). Crystallisation studies of HCG have shown that the Asn-52 sugar chain residue is located close to the receptor-binding domain of the protein, and a region homologous to the soybean lectin has been found in the human HCG receptor (McFarland et al., 1989; Lapthorn et al., 1994).

1.2.2 The function of HCG in the developing embryo

Fertilisation of an ovum occurs with fusion of a spermatozoa and an oocyte. This ovum then develops through the 2 cell, 4 cell, 8 cell and morula stages to form a blastocyst. The blastocyst is composed of three distinct regions—the blastocyst cavity, the inner cell mass and the trophoblast layer (Figure 1.8A). The inner cell mass differentiates to form a fetus, while the trophoblast layer helps the developing fetus to attach to the uterine lining and forms the placenta.

The blastocyst orientates itself so that the inner cell mass is located next to the endometrium, and the external zona pellucida is lost as blastocyst attachment occurs. The invasive properties of the trophoblast cells allow them to direct blastocyst implantation. Some trophoblast cells fuse with maternal cells to form the synctiotrophoblast layer, while others retain their cellularity to become cytotrophoblasts, which can be the source of further trophoblastic cells. These differentiating cell layers are illustrated in Figure 1.8B. The synctiotrophoblast cells secrete many placental hormones, including HCG.
A) On day five of gestation, the developing fetus is termed a blastocyst. At this stage three distinct regions have formed – the trophoblast layer, the inner cell mass and the blastocyst cavity. The inner cell mass differentiates to form a fetus, while the trophoblast layer helps the developing fetus to attach to the uterine lining and forms the placenta. Taken from http://stemcellresearchblog.com/wp-content/uploads/2009/03/blastocyst.png. B) Once the blastocyst has attached and implanted into the endometrium wall, the inner cell mass begins the process of differentiation to form a fetus. The synctiotrophoblast layer is formed through fusion of trophoblast cells with maternal cells, and is the site of HCG production. Taken from http://missinglink.ucsf.edu/lm/IDS_101_embryology_basics/blastocyst_and_implantation.htm.
When an ovum has not been fertilised, the corpus luteum (the region in the ovary where the last follicle was released) eventually degenerates causing a decrease in the levels of progesterone, oestrogen and androgens. This reduction in hormone levels results in sloughing of the uterine layers and menstruation. The function of HCG in the developing embryo is to maintain the activity of the corpus luteum so that key hormone levels are sustained and the pregnancy alongside. Once the placenta becomes sufficiently developed at about 60 to 70 days gestation, it will assume this role and HCG levels will decline (Srisuparp et al., 2001).

HCG can be detected in the cell membranes of embryonic, fetal and sperm cells at every stage of development. The presence of terminal sialic acid residues on the oligosaccharide chains confers HCG with a strong negative charge, and as a result, cells from the immune system cannot approach the growing embryo and sperm cells. Consequently, the sperm cells, embryo and fetus are immunologically inert and will survive in the mother, despite being 'non-self' (Acevedo, 2002; Kayisli et al., 2003).

HCG is secreted in a pulsatile fashion with peak levels detected at approximately 7 am each day, and these decrease throughout the day. It can be detected on day 7 of in vitro culture of blastocysts, by laboratory tests at day 10, and in urine by day 14 of gestation. HCG levels rise in an exponential fashion in the first trimester of pregnancy, with concentrations almost doubling each day to a peak around weeks 7 to 9. They decline after this until week 20, and will remain low and constant up to term. Most home pregnancy kits are based on the detection of HCG in the urine, and HCG is thought to contribute to the nausea associated with early pregnancy (Keay et al., 2004).

Most circulating HCG is metabolised by the liver, with 20% excreted through the kidney. The kidney causes degradation of HCG to result in subunit dissociation and formation of a variety of nicked forms, with the most abundant form termed HCGβcf. This β core fragment cleavage product consists of residues 6-40 and 55-92 of the β subunit disulfide linked to one another (Stenman et al., 2006).

1.2.4 Other functions of HCG
HCG is used during a wide range of assisted reproduction technologies, such as in-vitro fertilisation (IVF). In these instances, HCG induces final oocyte maturation in the ovary and causes production of testosterone in the testes. In normal situations, LH carries out this
task, but HCG is used as a surrogate in these processes due to the high sequence identity of the two glycoprotein hormones, and since purified LH is not typically available in high enough doses. HCG was initially purified from the urine of pregnant women, but the advent of DNA technologies means that it is now also produced in a recombinant form using CHO cells (Gervais et al., 2003). This recombinant form has been shown to work as effectively as the urinary form, although fewer immunoreactivity issues were detected. These reactions were generally caused by the HCGβcf (Keay et al., 2004).

Anti-HCG antibodies have been tested at phase II clinical trials as a potential birth control vaccine. By administering antibodies to HCG, ovulation and hormonal production patterns are normal, but the blastocyst fails to implant in the endometrium. The vaccine was highly effective, fully reversible and caused no side effects. However, only 60 – 80% of women produced antibody titres above the protective threshold value; a birth control vaccine needs to be effective in 90-95% of recipients to be acceptable. Consequently, further work is required for this potential use (Kaz et al., 2005).

HCG has been investigated as a potential agent to prevent infection with HIV / AIDS. The replication of HIV and the synthesis of reverse transcriptase and other genes and proteins have been shown to be suppressed by HCG in a lymphocyte model. Transmission of the virus was also prevented. The rapid progression of the disease and premature death of murine HIV model pups was also prevented with the use of HCG (Rahman and Rao, 2009). However, further studies are necessary to fully establish the preventative role of HCG in HIV infection.

1.3 Cell metabolism

The in vitro cultivation of a cell population is possible by the provision of an appropriate culture medium and physical conditions. The cells metabolise nutrients within the medium in order to provide the energy for cell growth, and to produce all the biosynthetic intermediates required to make new cellular constituents and recombinant protein, if necessary. The key metabolic pathways in cultured mammalian cells are; glycolysis, the TCA cycle, the pentose phosphate pathway, oxidative phosphorylation and amino acid metabolism (some of which are shown in Figure 1.9). Since these metabolic routes take place in different cellular compartments, efficient interlinking requires the use of transporters for important substrates and products of key reactions.
Most nutrients are supplied in culture medium at concentrations that exceed metabolic needs, for example, glucose is generally supplied to cultured cells at approximately 30mM. At these concentrations, glucose transporters work at their maximal rate, which results in a high glycolytic flux. The end product of glycolysis is pyruvate, however, a large amount of lactate is also produced. Many reasons have been proposed for this production of lactate, although the idea quoted most often is that this reaction regenerates NAD\(^+\) which is necessary for glycolysis to continue (Ozturk and Hu, 2006). Glycolysis occurs in the cytosol and provides energy in the form of 2 ATP molecules. However, the high flux through glycolysis means that this pathway is an important method for energy generation in cultured cells. Additionally, glycolysis is necessary for the provision of precursors for the formation of glycoproteins (UDP-Gal, UDP-GalNAc, UDP-Gluc, UDP-GlcNAc and CMP-NeuAc, see Figure 1.2A), ribose and NADPH (from glucose-6-phosphate and via the pentose phosphate pathway) and lipids for the formation of cell membranes (from dihydroxyacetone phosphate, Figure 1.9). Gluconeogenesis is not an active metabolic pathway in cultured cells.

Pyruvate is transported into the mitochondrion where it is firstly converted to acetyl-CoA, which fuels the TCA cycle. Acetyl-CoA can be regenerated in the cytosol from citrate, which further synthesises fatty acids for incorporation into lipid bilayers. Amino acid metabolism also occurs around the TCA cycle. Amino acid biosynthesis is generally not necessary in cultured cells, since high concentrations are provided in the culture medium. However, the interconversion of amino acids and their subsequent metabolism is an important source of TCA cycle intermediates. The conversion of glutamine to glutamate and \(\alpha\)-ketoglutarate is a particularly relevant feature of cells in culture. A high rate of glutamine consumption is observed in cells in culture, which results in high ammonium concentrations that are toxic (see Section 1.5 for more details). Additionally, NADH and FADH\(_2\) are generated during the TCA cycle. These reductive intermediates are used in the process of oxidative phosphorylation to synthesise ATP. This process occurs on the matrix side of the mitochondrial inner membrane.

1.4 Modes of cell culture

Batch cultivation is often used for the \textit{in vitro} culture of cells. In this set-up, a population of cells is inoculated with a set volume of culture medium for a defined period of time. No further addition or removal of culture medium is made during this time, and the entire culture is harvested at a set end point. The viable cell density increases rapidly in the early
Glucose is transported into a cell before the process of glycolysis generates NADPH and ribose via the pentose phosphate pathway at glucose-6-phosphate, lipids from dihydroxyacetone phosphate and pyruvate. Within the mitochondrion, pyruvate is converted to acetyl-CoA to fuel the TCA cycle (Krebs cycle). This provides the energy intermediates NADH and FADH$_2$, which generate ATP by the process of oxidative phosphorylation (not shown). The formation of phospholipids from citrate in the TCA cycle also occurs. The conversion of glutamine to glutamate and α-ketoglutarate is important in cultured cells (Taken from http://www.scielo.br/img/fbpe/bjembr/v36n2/html/4678i01.htm on 28/06/10).
stages of culture, as nutrient concentrations are high and concentrations of inhibitory compounds are low. This is caused by a high, stable growth rate. The production of recombinant protein also increases during this time. However, at some point during culture, the growth rate begins to decrease due to the accumulation of inhibitory compounds, or the decrease in concentration of important substrates. Consequently, the number of viable cells and recombinant protein levels decrease. As a result, the changing culture conditions during batch culture result in altered rates of growth and metabolism at different time points (Figure 1.10 A). Despite these issues, this method of culture is simple and reliable, and is often used. However, controlled feeding of nutrients in a batch culture can minimise the formation of toxic metabolites. This so-called “fed-batch” culture results in the production of higher amounts of recombinant protein, and extends total culture time from a few days to weeks.

In 1950, Jacques Monod (in Paris) and Aaron Novick and Leo Szilard (in Chicago) independently developed the chemostat as a method for the continuous cultivation of bacterial cells (Monod, 1950; Novick and Szilard, 1950). They described how limiting a single nutrient in the culture medium controlled the growth rate. Eight years later, fibroblast cells were successfully cultivated using this method of cultivation (Graff and McCarty, 1958). The cells are initially cultivated in a batch mode for the first few days of culture. After this time, a continuous flow of fresh medium into the bioreactor is matched by the same out-flow of cell suspension, which results in a constant culture volume (Figure 1.10 B, diagram on right). After the total volume has been replaced five times with new culture medium (also termed ‘residence time’) a steady state is attained. At this point, the cell number and concentrations of nutrients, by-products and recombinant protein are stable. Consequently, cells are cultured at stable cultivation conditions (Figure 1.10 B, illustration on left). The relationship between the flow rate of medium (in ml/d), the calculated dilution rate (in d⁻¹ or /d) and the length of time required for the total volume to be replaced with new medium (in days) is described in Section 2.9.3. Once the rate of dilution of culture medium is within the range of the growth rate of the cells (to ensure adequate cell growth), the viable cell number and protein production is comparable to that observed in batch cultivation. Bioreactors are commonly used for this type of culture to control process parameters such as pH, pO₂ and pCO₂. Today, many tools are available to study cellular processes at the genomic and proteomic level. With the use of a continuous cultivation for these studies, the defined, stable culture conditions can provide a way to gather reproducible information from a cell population (Hoskisson and Hobbs, 2005).
A) Batch culture

B) Continuous culture

Figure 1.10 Illustration of the differences between batch and continuous culture.

A) In batch culture, cells are inoculated with an aliquot of medium, and no further additions or withdrawals will occur during the culture time (image on the right). This causes the viable cell number to increase to a maximal value during the culture time, and subsequently decreasing, while the growth rate and concentrations of product and nutrients also changes (as illustrated by the orange arrows). In a continuous culture (B), cells are inoculated in a bioreactor and cultivated in a batch mode for 3 days. At this point, fresh medium is perfused into the bioreactor at the same rate as culture is removed (image on the right). The culture will eventually reach a steady state, where the cell number, growth rate and concentration of product and nutrients stabilise (as represented by the orange arrow). Consequently, continuous culture is an excellent mode for the study of the responses of cells to various stimuli, since the effects of changing culture conditions can be neglected.
1.5 The effect of glutamine on cultured cells

1.5.1 The role of glutamine in cell culture

Harry Eagle initially discovered in 1956 that glutamine, along with arginine, cystine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine and valine were essential for the growth of a fibroblast and a HeLa cell line (Eagle et al., 1956). As a result, glutamine is routinely supplied to growing cells today. In fact, some cell lines use glutamine as a major energy source (Reitzer et al., 1979; Neermann and Wagner, 1996). Glutamine feeds into the TCA cycle at α-ketoglutarate, the key reactions for which are shown in Figure 1.11. Glutamine is firstly deamidated to glutamate by the action of glutaminase, a reaction that releases ammonium into the cytosol. Glutamine synthetase catalyses the ATP-dependent reverse reaction. This glutamate is then subject to one of two fates. Glutamate dehydrogenase can directly deaminate glutamate to α-ketoglutarate, releasing another ammonium molecule, and resulting in the generation of 27 ATP molecules. Alternatively, glutamate can be transaminated to an amino acid such as alanine, which maintains the amino group within an amino acid, while also producing α-ketoglutarate and 9 ATP molecules (Cruz et al., 1999). The difference in the energetic efficiency of these pathways is due to the fact that the transamination pathway consumes a pyruvate molecule in order to produce alanine, which is an inefficient use of pyruvate and glutamine. These reactions are an important hub for the provision and use of glutamine in the TCA cycle, or elsewhere in the cell.

However, glutamine also has an important role in the formation of purine and pyrimidine nucleotides, nicotinamide coenzymes and in the pathway for formation of activated sugars. The formation of all of these structures utilises the amido group of glutamine in key reactions. A simplified overview of the reaction networks for the synthesis of AMP, GMP, UTP and CTP is shown in Figure 1.12 (abbreviations are detailed elsewhere). The synthesis of the purine structures, AMP and GMP requires the formation of IMP. The synthesis of this molecule begins with the transfer of two phosphate groups from ATP to the initial substrate α-D-ribose-6-phosphate. The subsequent steps in the pathway utilise glutamine as well as glycine and aspartate in order to produce IMP. AMP and GMP are formed from IMP after two further distinct reactions, consuming another molecule of aspartate and glutamine in the process. The first reaction in the synthesis of the pyrimidine ribonucleotides, UTP and CTP use glutamine as substrate (Figure 1.12). Carbamoyl phosphate synthetase II catalyses the reaction of two ATP molecules, bicarbonate and water with glutamine to form carbamoyl phosphate (CP) (Campbell et al., 2008).
Glutamine is firstly deaminated to glutamate by glutaminase, releasing one ammonium molecule. Glutamate can then be deaminated to α-ketoglutarate by glutamate dehydrogenase, which yields another ammonium molecule. However, cells in culture preferentially transaminate glutamate to other amino acids such as alanine, which prevents the amino group from being released (Street et al., 1993). The amido group is given in red, while the amino group is shown in blue.
Glutamine is required as a substrate for the conversion of nicotinate adenine dinucleotide to nicotinamide adenine dinucleotide (NAD\(^+\)) in a transamidation reaction catalysed by NAD\(^+\) synthetase.
The next step in this pathway consumes aspartate, and four further reactions occur before UMP is formed. UTP is synthesised from UMP by the sequential actions of nucleoside monophosphate kinase and nucleoside diphosphate kinase. CTP synthetase then catalyses the glutamine-dependent conversion of UTP to CTP.

Glutamine is also required for the synthesis of NAD$^+$ and NADP$^+$. The nicotinamide moiety of these coenzymes is derived from dietary nicotinamide, nicotinic acid or through the degradation of tryptophan. NAD$^+$ synthetase uses glutamine, ATP and water to transamidate nicotinate adenine dinucleotide to nicotinamide adenine dinucleotide (NAD$^+$). Alternately, NAD$^+$ can be synthesised in two reactions from nicotinamide. NAD$^+$ is converted to NADP$^+$ by the action of NAD$^+$ kinase (Figure 1.13).

Glutamine has also been found to act as a substrate for the synthesis of glucosamine-6-phosphate from fructose-6-phosphate by the action of GPS. This reaction is a prelude to the formation of UDP-GlcNAc and UDP-GalNAc, the activated donors that are used for the incorporation of GlcNAc and GalNAc into N-linked glycan structures. This is discussed in more detail in Section 1.5.8.

Additionally, the synthesis of glutamine from glutamate by glutamine synthetase has been used as a tool for the selection of recombinant cell clones. The activity of this enzyme is not sufficient for the survival for a number of cell lines, while endogenous activity in other cell lines can be inhibited with use of methotrexate. The inclusion of the gene of interest in an expression vector containing glutamine synthetase results in selection of recombinant clones, when cells are grown in glutamine-free medium (Brown et al., 1992). This system has been shown to result in increased productivity of NSO cell lines, as marketed by Lonza (Rance et al., 2006).

### 1.5.2 Chemical degradation of glutamine

One key aspect of glutamine chemistry is its chemical degradation to pyrrolidone carboxylic acid and ammonium (Figure 1.14). This reaction rate is increased at high temperatures and pH (Ozturk and Palsson, 1990a). With the routine incubation of cell culture and medium at 37°C, this has a significant effect. The decrease in glutamine concentration can be as high as 62.5% over 7 days (in DMEM medium supplemented with 8mM glutamine and incubated at 37°C, SAFC Biosciences technical bulletin). However, some aspects of the kinetics of degradation were explored for this work, and a 20%
decrease in concentration after 5 days was found in CD-CHO medium (all other conditions identical, Figure 2.2). The toxicity of pyrrolidone carboxylic acid has been explored, and concentrations of up to 20 mM were shown to have no effect on cells, and the compound itself was not metabolised (Stoll, 1995).

Much investigation has taken place into the response of cells to ammonium. In vivo, the enzymes of the urea cycle detoxify excess ammonium for excretion in the liver. However, no such system exists in cultured cells. Experiments with $^{15}$N have shown that the fate of the amino and amido nitrogen of glutamine in cultivated CHO cells is quite different. The amido group is released into the culture as ammonium by the action of glutaminase to form glutamate from glutamine. The amino group of glutamate is then found in alanine, aspartate and glycine, indicating that transamination is preferred, rather than direct oxidative deamination to α-ketoglutarate. Although transamination generates less ATP than deamination, it results in the conservation of the amino group within another amino acid, and as such, is considered to be another mechanism of ammonium detoxification (Street et al., 1993). However, it is not as efficient as the urea cycle, and ammonium accumulates rapidly to become toxic to the cells.

1.5.3 Effects of ammonium on pH

A pH-dependent equilibrium exists between ammonium ($\text{NH}_3$) and ammonium ions ($\text{NH}_4^+$). The two compounds are usually considered to be in equilibrium due to the high rate of forward and reverse reactions. However, at physiological pH (the pH at which most mammalian cell culture is carried out), 99% of the ammonia/ammonium ion content is in the form $\text{NH}_4^+$.

$$\text{NH}_3 + \text{H}^+ \rightleftharpoons \text{NH}_4^+$$

$\text{NH}_3$ is a small uncharged, lipophilic molecule that easily diffuses across membranes, following its gradient of chemical potential, (also known as the partial pressure of $\text{NH}_3$). Any small amount of $\text{NH}_3$ present will rapidly diffuse across membranes to equilibrate any transmembrane gradient and the pH equilibrium is restored rapidly by protonation to $\text{NH}_4^+$ within this compartment. Low pH compartments generally have low partial pressures of $\text{NH}_3$, resulting in a flow of $\text{NH}_3$ across the membrane into the compartment, until equilibrium is attained which means an increase in pH of the compartment. This reaction therefore results in functional disturbance of the various low pH organelles, such as the lysosome. The charge on $\text{NH}_4^+$ results in a very slow diffusion rate, and it will most likely pass through membranes either by facilitated diffusion or active transport. Since this
The chemical decomposition of L-glutamine produces ammonium and pyrrolidone carboxylic acid. The rate of reaction is increased at high temperature and pH.
occurs through use of ionic transporters (i.e. Na⁺K⁺-ATPase, Na⁺K⁺2Cl⁻ co-transporter) it will perturb the normal transmembrane gradients of these ions. This constrains ionic gradients all over the cell, and energy will be consumed in returning these to normal. The increased energy requirement decreases cell proliferation (Schneider et al., 1996). The effect of ammonium formation by glutamine degradation in the mitochondria is different to ammonium supplied to the cell (i.e. through chemical degradation of glutamine in medium, or supplementation of NH₄Cl to growing cells). Mitochondrial catabolism of glutamine produces ammonium and the NH₃ easily and rapidly diffuses out of the mitochondria and cytosol, leaving H⁺ ions behind and increasing the pH in both compartments. Externally added ammonium transiently increases the pH of the cytoplasm due to rapid diffusion of NH₃ into the cell, followed by acidification due to the slower transport of NH₄. Diffusion of NH₃ into the mitochondria and other organelles then causes a decrease in the pH of these compartments (Figure 1.15, (Schmeider et al., 1996)).

1.5.4 Effects of ammonium on growth rate

Supplementation of cells in culture with NH₄Cl has led to the observation that elevated ammonium concentrations decrease the cell growth rate (Kurano et al., 1990; Mc Queen and Bailey, 1990; Ozturk et al., 1992; Yang and Butler, 2000a). Concentrations as low as 0.6 mM were found to cause this effect in transformed fibroblast cells (Schneider et al., 1996). However there is a large variation in the sensitivity of individual cell lines to ammonium. The concentration of ammonium required to decrease the viable cell concentration by half, the IC₅₀, was quoted as 8 mM and 33 mM for CHO cells by two separate papers (Kurano et al., 1990; Yang and Butler, 2000b). And, within a continuous culture (chemostat) the growth rate of CHO cells was not found to alter with ammonium concentrations ranging from 0 to 7.5 mM (Hansen and Emborg, 1994).

As mentioned previously, the active transport of ammonium ions perturbs the transmembrane gradients of a variety of ions, and the energy that is used up in returning the cells to the normal state causes a decrease in cell proliferation (Schneider et al., 1996). The reasons for this decrease in growth rate have also been explored at the genomic level. Downregulation of the genes for anaphase-promoting complex, Eif5a2 (a sensor for polyamine levels, its depletion has been shown to inhibit cell growth) and KIAA1091 (a modulator of cell motility and communication) were found when 10 mM NH₄Cl was supplemented to CHO cells for 2 days. These results are consistent with a decrease in growth rate (Chen and Harcum, 2007).
Figure 1.15 The effect of ammonium on pH gradients.

Red arrows indicate the direction of change of the pH from normal. Ammonium produced externally or supplied to the cell culture medium decreases the cytosolic pH but increases pH outside the cell and in the mitochondrion, Golgi, and other subcellular organelles. Mitochondrion-derived ammonium causes similar effects; with the exception that mitochondrial pH is decreased.
1.5.5 Effects of ammonium on metabolism

Increasing ammonium concentrations has been observed to cause an increase in the calculated metabolic energy production of the cells (Ozturk et al., 1992). This means that rates of glucose and glutamine consumption, as well as production rates of lactate and ammonium were increased (Ozturk et al., 1992; Yang and Butler, 2000a; Yang and Butler, 2000b; Yang and Butler, 2002). This trend was also noted for the production of amino acids, with the largest increases found for alanine and glutamate (Ozturk et al., 1992; Yang and Butler, 2000b). The rationale for the increased amino acid production is explained in Section 1.5.2.

Ozturk et al investigated the metabolic changes in hybridoma cells at varying ammonium concentrations. However, they could not directly relate the changes in metabolism to the variations in pH caused by elevated ammonium concentrations (Ozturk et al., 1992). On a genomic level, upregulation of ATP synthase (subunit C) and mitofusion 1 (Mfn1) has been observed at elevated ammonium concentrations. ATP synthase is the protein that is responsible for production of ATP in oxidative phosphorylation. Mfn1 regulates mitochondrial fusion and morphology, and overexpression has been found to result in large mitochondrion with abnormal internal structures, which may indicate an increased energy production capacity. Both these findings are consistent with the increased energy metabolism of cells at elevated ammonium concentrations (Chen and Harcum, 2007).

1.5.6 Effects of ammonium on productivity

The relationship between elevated ammonium concentrations and the production of recombinant proteins has not yet been fully clarified. The secreted IgG content of a murine hybridoma cell line was decreased at high ammonium concentrations, but since this was accompanied by a decrease in growth rate, the specific productivity was unaffected (Ozturk et al., 1992). However, cultivation of CHO cells with 0 to 40 mM NH₄Cl for 4 days resulted in a significant increase in the EPO content above 5 mM. Despite the reduction in growth rate at elevated ammonium concentrations, the specific productivity of EPO was also significantly increased above concentrations of 5 mM NH₄Cl (Yang and Butler, 2000b). This same cell strain was also grown with 2, 4 or 20 mM glutamine, and the increasing ammonium concentrations caused a decrease in EPO content, most significantly at 20 mM glutamine. Once again, despite the reduced growth rate at 20 mM glutamine, the specific productivity of EPO was highest at 20 mM glutamine (Yang and Butler, 2000a).
1.5.7 Effects of ammonium on glycosylation

Research into the impact of elevated ammonium concentrations on N-linked glycosylation has yielded some clear trends. A reduction in terminal sialylation and galactosylation has been observed (Thorens and Vassalli, 1986; Andersen and Goochee, 1995; Rijcken et al., 1995a; Gawlitzek et al., 1998; Grammatikos et al., 1998; Gawlitzek et al., 2000; Yang and Butler, 2000b; Yang and Butler, 2000c; Yang and Butler, 2002; Gillmeister et al., 2009). This is accompanied by an increase in the heterogeneity of the glycoprotein, due to the increased range of the average pi (Yang and Butler, 2000b; Yang and Butler, 2002). The antennarity of the glycans is also affected; some cases see a decrease in antennarity (Yang and Butler, 2000c; Yang and Butler, 2002) while others detect an increase in antennarity at elevated ammonium concentrations (Gawlitzek et al., 1998; Grammatikos et al., 1998).

1.5.8 The mechanisms by which ammonium impacts glycosylation

1.5.8.1 Glycosylation gene expression

The expression of glycosylation genes in CHO cells in response to 10 mM NH₄Cl has been explored using RT-PCR. The endoplasmic reticulum localised genes associated with early N-linked glycosylation steps were found to be insensitive to ammonium. However, the initial expression of the UDP-Gal transporter was increased, while expression of the CMP-NeuAc transporter, UDP-glucose pyrophosphorylase, α2,3-sialyltransferase and β1,4-galactosyltransferase was decreased. This indicates that the reduction in sialylation at increased ammonium concentrations was brought about by decreased transport of sialic acids and sialylation rates, while the reduction in galactosylation is caused by decreased galactosyltransferase activity as a result of impaired gene expression (Chen and Harcum, 2006). However, the mRNA levels of β1,4-galactosyltransferase and α2,3-sialyltransferase (also in CHO cells) were found to be unaffected for a period of five days after the addition of 13 mM NH₄Cl (Gawlitzek et al., 2000). This indicates that the initial changes in gene expression may not lead to sustained changes in mRNA levels, suggesting that enzyme levels themselves are also unaffected.

1.5.8.2 pH

The anti-malarial drug, chloroquine, has been used to shed light on the impact of ammonium on glycosylation through its effects on pH. As noted above, ammonium causes an increase in the pH of intracellular compartments such as lysosomes and Golgi. Chloroquine mimics this effect. A direct observation of the increase in lysosomal pH with NH₄Cl addition has been carried out (Gillmeister et al., 2009).
The first experiments in 1986 showed that sialylation of secreted Ig α chains by plasma cells was completely abolished with the addition of 10 mM NH₄Cl or chloroquine (Thorens and Vassalli, 1986). Recent work with CHO cells has also shown that the reduction in sialylation effected by ammonium can be replicated by use of chloroquine (Andersen and Goochee, 1994; Gawlitzek et al., 2000). However, in another case also using CHO cells, the glycosylation effect could not mimicked (Yang and Butler, 2000b). Increasing the extracellular pH has been found to amplify effects on glycosylation (Borys et al., 1993).

The increased Golgi pH may also impact on the activity and localisation of glycosylation enzymes. Extraction of the membranes of plasma cells has shown that the pH optimum for galactosyltransferase and sialyltransferase activity is in the range 5.8 and 7.0 (Thorens and Vassalli, 1986). A sharp pH 6.5 optimum was observed for α2,3-sialyltransferase and β1,4-galactosyltransferase in CHO cell lysates. They demonstrated that the activities decreased by 50 to 60% at pH 7.0 to 7.2, which is the likely pH of the trans-Golgi in the presence of 10 to 15 mM ammonium (Gawlitzek et al., 2000). Neutralisation of the pH of the secretory pathway in HeLa cells with 25mM NH₄Cl causes a redistribution of GalNAc transferase 2, β1,2-GlcNAc transferase 2 and β1,4-galactosyltransferase. These enzymes normally reside in the Golgi stack, medial/trans Golgi and the trans Golgi network, respectively, but under these conditions they all relocalised to endosomal-type vesicles (Axelsson et al., 2001). Further work on the topic in COS-7 cells showed that increasing the Golgi pH with chloroquine by 0.2 pH units mislocalised α-2,3 sialyltransferase into endosomal compartments, causing impaired α-2,3 sialylation of a reporter glycoprotein. A greater pH increase was needed to impair other glycosylation steps such as β-1,4 galactosylation (Rivinoja et al., 2009).

1.5.8.3 UDP-GNAc pool

Another mechanism by which ammonium influences N-linked glycosylation was explored by a group led by Roland Wagner in the 1990’s. They initially discovered that UDP-GNAc sugars (UDP-GlcNAc and UDP-GalNAc) were elevated at the end of the exponential growth phase of batch and perfusion cultures of BHK cells. Since this accumulation coincided with the decrease in growth rate, they firstly proposed that the UDP-GNAc sugars themselves were inhibiting cell growth (Ryll and Wagner, 1992). Further exploration showed that this increase in UDP-GNAc could be effected in many cell lines by supplementation with NH₄Cl. A dramatic 18-fold increase in this pool was seen within
24 hours of a 15 mM addition to BHK cells, while CHO cells demonstrated an 8-fold increase. This dose-dependent relationship was not caused by an increase in osmolality. Accumulation of the pool required the consumption of glucose, while glutamine stimulated the action. Elevated culture pH increased the effect, and it was shown that long term adaptation to high ammonium concentrations could occur in a hybridoma cell line, though BHK cells remained sensitive after many months (Ryll et al., 1994). The authors suggest that a cell-line dependent change in the activity of glucosamine-6-phosphate isomerase, which uses excess ammonium as a substrate for the production of glucosamine-6-phosphate, resulted in altered tolerance of elevated ammonium concentrations.

The work of Rijcken et al into the effects of increased sugar nucleotide concentrations and their incorporation into the glycoproteins of isolated rat hepatocytes provided some key insights. They found that artificially increasing the UDP pool (and its derivatives) by addition of either uridine or cytidine increased the incorporation of N-acetyl hexose sugars into secreted and cell-associated glycoconjugates, as well as resulting in a reduction in sialylation. They suggested that increased levels of HexNAc decreased sialylation due to impaired transport of CMP-NeuAc across the Golgi membrane. They also found evidence for the feedback inhibition of UDP-GlcNAc-2-epimerase by CMP-NeuAc. It is of note that a similar increase in the UTP pool was observed, irrespective of whether uridine or cytidine was used to effect this change (indicating the tight regulation of CTP synthesis). There was a smaller increase in UDP-sugar levels than for UTP, and a three-fold increase in CTP did not lead to a increase in CMP-NeuAc levels (Rijcken et al., 1995b).

An overview of the pathway of UDP-GNAc formation is presented in Figure 1.16. This figure illustrates that the initial step in this network is the conversion of glucose-6-phosphate to fructose-6-phosphate. This reaction forms a part of glycolysis and is catalysed by phosphoglucone isomerase. The fructose-6-phosphate is then aminated to glucosamine-6-phosphate, which is subsequently acetylated to N-acetylglucosamine-6-phosphate. Acetyl glucosamine phosphomutase converts this to N-acetylglucosamine-1-phosphate. OMP can be sequentially converted to UMP, UDP and UTP before reacting with N-acetylglucosamine-1-phosphate to form UDP-N-acetylglucosamine. This is easily interconverted to UDP-N-acetylglactosamine, forming the UDP-GNAc pool. Inhibitors of this pathway have been used to show the influence of key components on glycosylation. Adenosine (Ado) depletes the UTP pool, and as a result decreases the UDP-GNAc pool.
Figure 1.16 The pathway of UDP-GNAc formation.

Glucose-6-phosphate (Glc-6-P) is converted to fructose-6-phosphate (Frc-6-P) by phosphoglucone isomerase, an enzyme of glycolysis. This is acetylated to glucosamine-6-phosphate (GlcN-6-P) and subsequently converted to glucosamine-1-phosphate (GlcN-1P). OMP is converted to UMP, UDP and UTP before these two structures combine to UDP-N-acetylglucosamine (UDP-GlcNAc). This is easily interconverted to UDP-N-acetylgalactosamine (UDP-GalNAc), forming the UDP-GNAc pool. The elements shown in green illustrate the changes that occur at elevated ammonium concentrations. Carbamoyl phosphate (CP) enters the cytosol where it stimulates de novo synthesis of UMP, increasing the UDP-GNAc pool, while ammonium ions are directly incorporated into glucosamine-6-phosphate by the action of glucosamine-6-phosphate isomerase. Modified from (Grammatikos et al., 1998). Ado = adenosine, Urd = uridine and GlcN = glucosamine.
Pederson et al reported that glucosamine causes a rapid increase in the UDP-hexosamine pool, as a result of its direct phosphorylation and incorporation into the pathway. This is accompanied by UTP limitations thereafter (Pederson et al., 1992). Alternatively, the deamination of glucosamine by glucosamine-6-phosphate deaminase releases an ammonium molecule and may contribute to the accumulation of intracellular ammonium (Comb and Roseman, 1958). However, provision of uridine alongside glucosamine will result in a sustained increase the UDP-GNAc pool, since this can be converted to UDP by uridine kinase. Glucosamine has also been reported to have other important effects in cell culture such as its competitive inhibition of glucose uptake, inhibition of the formation of the lipid-linked oligosaccharide precursor (Spiro, 1958; Plagemann and Erbe, 1973; Schwarz and Klenk, 1974; Datema and Schwarz, 1979).

The discovery of a direct link between this elevation of UDP-GNAc sugars and glycosylation by Rijcken et al prompted the Wagner group to explore this theme further in BHK cells. They established perfusion cultivation in 2.5 L bioreactors, with the aim of testing various conditions in this set-up by rapid washout of medium at relevant time points. They tested the growth of the cell line in glutamine-free medium, in medium with elevated concentrations of ammonium (15 mM) and with glucosamine-supplemented medium (10 mM). Their overall aim was to determine how the glycosylation of recombinantly expressed IL-Mu6 (an IL-2 glycoprotein variant) changes under these conditions. Analysis of the N-linked glycans demonstrated that with increasing ammonium concentrations, a decrease in sialylation and fucosylation was accompanied by an increase in antennarity. O-glycosylation was unaffected, but the UDP-GNAc pool was increased, as expected. These cells were found not to require glutamine for growth.

Glucosamine was supplied to cells after a period of growth without glutamine (i.e. low ammonium concentrations) and antennarity effects similar to those observed for elevated ammonium concentrations were noted, although they were less pronounced. However, there was a larger decrease in sialylation. In retrospect, these findings are the first indications that elevated ammonium concentrations caused changes to antennarity but not sialylation through the UDP-GNAc pool. The authors note that since glucosamine did not have the same effect as ammonium, other physiological factors were involved (Gawlitzek et al., 1998).
The pathways by which these ammonium-dependent glycosylation changes were effected were then examined in more detail. Firstly, uridine and glucosamine were simultaneously supplied to the cells in the absence of ammonium. This caused an artificial increase in the UDP-GNAc pool without decreasing the UTP pool. An increase in antennarity was found, while the sialylation remained constant. The effects of ammonium in the absence of an increase in UDP-GNAc pool were examined by pre-treatment of the cells with adenosine to decrease the UDP-GNAc pool and subsequent addition of ammonium to the culture. The adenosine was added prior to the ammonium in order to prevent any existing UTP from feeding into the pool with the addition of ammonium. As expected, the UDP-GNAc pool remained constant while the UTP was depleted. The decrease in sialylation and lack of impact on antennarity proved that the ammonium-dependent increase in UDP-GNAc pool alters antennarity, but that sialylation effects occur through a different mechanism (Grammatikos et al., 1998).

They suggest that direct incorporation of the excess ammonium occurs at the stage of amination of fructose 6-phosphate to glucosamine-6-phosphate by the action of glucosamine-6-phosphate deaminase (also known as glucosamine-6-phosphate isomerase, or GPI, Figure 1.16). Furthermore, they postulate that the elevated ammonium concentration causes the carbamoyl phosphate (which is synthesised in the mitochondrion by carbamoyl synthetase I) to enter the cytosol. This then stimulates de novo synthesis of UMP, further increasing the effect (Grammatikos et al., 1998).

$^{15}\text{NH}_4\text{Cl}$ was then used to prove that the excess ammonium was incorporated into UDP-GNAc sugars at this point in the network, and that they were subsequently incorporated into glycoproteins. The increase in the intracellular concentration of UDP-GNAc was proportional to the amount of UDP-GNAc containing $^{15}\text{N}$, but a maximum of 80% of the N-linked glycans contained this label. This illustrates that there are other contributors to the UDP-GNAc pool under these conditions. Glucosamine-6-phosphate synthetase (GPS) can also transaminate fructose-6-phosphate to glucosamine-6-phosphate, using glutamine as the amido donor (Valley et al., 1999).

The control of synthesis of glucosamine-6-phosphate is strongly dependent on the cultivation mode. In vivo, GPS is responsible for the production of glucosamine-6-phosphate, while GPI acts as a regulatory enzyme to prevent accumulation. GPI catalyses the reaction in both directions, but the $K_m$ for glucosamine-6-phosphate as substrate is just
0.16 mM, while for ammonium this value is 15.8 mM. Under these conditions the UDP-GNAc pool is tightly regulated by transcriptional control of the enzymes. Under *in vitro* conditions, the elevated ammonium concentration causes GPI to shift towards synthesis of glucosamine-6-phosphate. This deregulation causes an increase in the UDP-GNAc pool (Figure 1.17, (Cayli *et al.*, 1999)).

The characteristics of GPI were investigated by purification of this enzyme from a large-scale cultivation of this BHK cell line. The activity of the purified enzyme was induced by ammonium, and was found to decrease with elevated ammonium concentrations. Glucosamine-6-phosphate, glutamine and glucose-6-phosphate were found to activate enzyme activity, while mannose-6-phosphate was found to be an inhibitor. As a result, addition of mannose (the precursor to mannose-6-phosphate) was found to decrease the UDP-GNAc pool in the presence of ammonium. Antisense RNA against GPI was also used to reduce the accumulation of UDP-GNAc in the presence of ammonium. But, the instability of the construct means it is not suitable for long-term use *in vitro* (Cayli *et al.*, 1999). This group has formulated a complete explanation for the changes in glycosylation in response to elevated ammonium concentrations ((Valley *et al.*, 1999), Figure 1.18).

It is interesting to note that within CHO cells, addition of 30 mM NH₄Cl was found to decrease the antennarity of EPO, while also causing an increase in the UDP-GNAc pool. The authors suggest that these conflicting results may be as a result of cell line or protein-specific differences (Yang and Butler, 2002). However, since an elevation in the UDP-GNAc pool was noted in both cases, it is likely that the mechanisms determined by the Wagner group are accurate, and that differences lie in the availability of the UDP-GNAc sugars for incorporation into glycoproteins.

A decrease in the UDP-GNAc pool has also been found to reduce the *N*-glycan site occupancy. Under glucose-limited conditions, the site occupancy of IFN-γ was found to vary within a small range at various dilution rates in CHO cell continuous culture. This was found to occur not as a direct result of the changed dilution rate, but due to the reduction in availability of NTP sugars and the UDP-GNAc pool. In glucose-limited cultures, the carbon pool is preferentially used for energy synthesis, so there is a reduction in the levels of NTPs since they are used to make DNA and RNA. The reduced NTP pool leads to a decrease in NTP-sugars. As a result, the UDP-GNAc pool decreases and also the occupancy of potential *N*-linked glycosylation sites (Nyberg *et al.*, 1999).
Figure 1.17 Control of the UDP-GNAc pool by GPS and GPI \textit{in vivo} and \textit{in vitro}.

\textit{In vivo}, GPS catalyses the conversion of fructose-6-phosphate to glucosamine-6-phosphate, while GPI acts as a regulatory enzyme. This control breaks down at the elevated concentrations of ammonium found \textit{in vitro}. GPI also forms glucosamine-6-phosphate, thus causing an increase in the UDP-GNAc pool. See Section 1.5.8.3 for further details.
Figure 1.18 – Mechanisms of the deregulation of N-glycan assembly in response to elevated ammonium concentrations.

The normal pH balance of the cell is disturbed with increasing ammonium - an increase in the extracellular pH and of intracellular compartments such as the Golgi, is accompanied by a decrease in cytosolic pH. Mitochondrial pH is increased when ammonium is supplied externally, while a decrease is seen with ammonium derived from cellular metabolism. An increase in the UDP-GNAc pool is caused by two mechanisms - carbamoyl phosphate (CP) exits the mitochondrion and stimulates de novo synthesis of UMP, while ammonium is directly incorporated into GlcN-6-P (glucosamine-6-phosphate) by the action of GPI (glucosamine-6-phosphate isomerase). This increases the concentrations of intermediate metabolites in the synthesis of CMP-NeuAc. However, at high CMP-NeuAc concentrations feedback inhibition of UDP-GlcNac-2-epimerase causes an eventual decrease in its synthesis. Additionally, elevated UDP-GNAc levels inhibit the transport of CMP-NeuAc into the Golgi, and the GPS (glucosamine-6-phosphate synthetase) catalysed reaction of glutamine amido incorporation into GlcN-6-P. The sum of pH changes, elevated UDP-GlcNAc concentrations, impaired CMP-NeuAc synthesis and transport causes deregulated N-glycan synthesis in the presence of high ammonium concentrations. (Valley et al., 1999)
1.5.9 Methods to reduce the influence of ammonium on cell cultures

The inhibitory effects of ammonium on growth, metabolism and glycosylation can be minimised by reducing its accumulation and concentration in culture. A variety of approaches have been used to this end, and they involve overcoming the chemical degradation of glutamine, controlling the addition of nutrients in order to minimise accumulation or replacing glutamine with suitable alternatives (Schneider et al., 1996).

By using a dipeptide, such as L-alanine-L-glutamine (e.g. GlutaMAX-I), as an alternative to glutamine, the chemical degradation is avoided. The dipeptide is hydrolysed by a cellular peptidase and the amino acids are taken up individually, reducing the extracellular accumulation of ammonium. This supplement has been found to increase the final cell yields, and reduce the consumption rates of glucose and amino acids and production of lactate and ammonium (Christie and Butler, 1994). As a result, use of glutamax reduces the negative effects of ammonium while still providing glutamine for cellular consumption.

Under normal cultivation conditions, the metabolism of cells in culture is inefficient. Glucose is consumed at higher rates than is necessary for cell growth and maintenance, and results in a large amount of lactate production. The high metabolic rates also lead to what is termed ‘overflow metabolism’. An example of this is the production of amino acids under these conditions, despite the low energetic yield from such a metabolism. By controlling the addition of glucose and glutamine, so that concentrations remain below 1 mM, a more efficient metabolic state can be attained. This can improve the cell metabolism and glycosylation and has been tested by limiting glucose and glutamine individually and in combination, in a range of cell lines.

Limiting glutamine concentrations in the presence of excess glucose causes a reduction in the concentrations of ammonium and alanine, without causing a dramatic impact on glucose metabolism (Ljunggren and Haggstrom, 1994; Cruz et al., 1999; Wong et al., 2005; Maranga and Goochee, 2006).

When the concentration of glucose is limited either in fed-batch or continuous culture scenario, a reduction in lactate production is seen. While this indicates a more efficient use of glucose, it is also accompanied by an increase in the consumption of glutamine and a reduction in the alanine concentrations. In some cases the rate of glutamine consumption itself does not change, but an increase in the production of ammonium occurs. This
indicates the reciprocal relation between carbon and energy supply by glucose and glutamine – at low glucose concentrations, glutamine can be oxidatively deaminated to glutamate by glutamate dehydrogenase to release ammonium and generate energy. However, the lack of glutamine cannot be compensated by the use of glucose, due to the fact that glutamine carries out other functions. This was found to occur in hybridoma, CHO and PER.C6 cells, indicating the universality of this response (Ljunggren and Haggstrom, 1994; Wong et al., 2005; Maranga and Goochee, 2006).

Limiting both glucose and glutamine has been demonstrated as an effective method to control the production of lactate and ammonium. In both hybridoma and PER.C6 cells, glucose was efficiently metabolised to pyruvate and channelled into the TCA cycle with minimal production of lactate. Alanine concentrations decrease alongside ammonium indicating the efficient metabolism of glutamine to α-ketoglutarate by deamination of glutamate (Ljunggren and Haggstrom, 1994; Maranga and Goochee, 2006).

The glycosylation pattern of IgG secreted by BHK cells was unaffected by low concentrations of glucose and glutamine (Cruz et al., 2000). However, a reduction in sialylation, sugar attachment to bi- and tri-antennary structures and in progression from mannose structures to complex structures was noted in a glutamine limited CHO fed-batch culture (Wong et al., 2005). This effect was intensified with limiting glucose concentrations, and the authors suggest that inhibition of the formation of UDP-GlcNAc may have mediated these effects.

Another method to reduce ammonium accumulation involves replacement of glutamine and glucose. Just as reducing glycolytic flux by controlling the addition of glucose results in more favourable metabolism, replacement with more slowly metabolised hexoses such as fructose, mannose or galactose causes a similar effect. For CHO cells, the use of fructose or galactose in the presence of glutamine caused a decrease in lactate production, but an increase in glutamine consumption and ammonium production was needed in order to maintain the energy requirements of the cell. In a further experiment, use of glutamate and galactose resulted in the lowest concentrations of lactate and ammonium, but reduced the cell growth (Altamirano et al., 2000).

BHK, fibroblasts and Vero cells have been found to adapt well to growth in the presence of glutamate or α-ketoglutarate but without glutamine supplementation. An increase in cell
yield and reduction in ammonium and lactate production was found at these conditions (Hassell and Butler, 1990).

A similar approach has been used for CHO cells, where overexpression of glutamine synthetase has led to comparable growth in glutamine-free, but glutamate-supplemented medium (Zhang et al., 2006).

1.6 Altering the rate of protein transit through the Golgi

Varying the rate at which proteins are transported through the endoplasmic reticulum and Golgi can change the N-linked glycosylation of proteins. This process can be controlled by a variety of methods; for example, partially inhibiting protein synthesis has been shown to increase the glycan processing time of the proteins. Reducing the culture temperature and decreasing ATP levels have also been shown to decrease or even halt the transit rate of glycoproteins through the Golgi. Since reducing temperature brings about a decrease in the cell metabolism in a similar way to ATP reduction, controlling the growth rate of cells has also been explored as a technique to alter the rate of Golgi transit. The impact of each of these approaches on cell metabolism and glycosylation is explored in Sections 1.6.1 through 1.6.4.

1.6.1 Inhibiting protein synthesis

Cycloheximide, which inhibits the elongation step of protein synthesis, has been found to decrease the rate of production of the lipid-linked oligosaccharide precursor (Grant and Lennarz, 1983; Gao and Lehrman, 2002). The site occupancy of prolactin secreted from C127 cells was also found to increase with cycloheximide treatment. The authors suggest that slowing the rate of protein synthesis increases the time for transfer of the lipid-linked oligosaccharide precursor to the protein of interest, explaining the increase in site occupancy (Shelikoff et al., 1994).

1.6.2 Temperature

It has been shown that cultivation of a variety of cell lines at approximately 20°C halts the progression of recombinant proteins through the Golgi (Matlin and Simons, 1983; Griffiths et al., 1985; Wang et al., 1991). However, the effect of reduced temperature on the glycosylation of glycoproteins is variable, with temperatures of approximately 32°C resulting in no change in N-linked glycosylation in some instances, (Matlin and Simons, 1983; Chuppa et al., 1997; Yang and Butler, 2002; Bollati-Fogolin et al., 2005; Ahn et al.,
2008) an increase in the extent of glycosylation and site occupancy in other cases (Wang et al., 1991; Andersen et al., 2000; Gawlitzek et al., 2009) and a decrease in the proportion of acidic isoforms and antennarity in further instances (Yoon et al., 2004b; Ahn et al., 2008).

Lowering the culture temperature to values between 33°C and 25°C also causes a reduction in the growth rate (Jenkins and Hovey, 1993; Chuppa et al., 1997; Kaufmann et al., 1999; Andersen et al., 2000; Yoon et al., 2002; Yoon et al., 2004a; Bollati-Fogolin et al., 2005; Yoon et al., 2005; Trummer et al., 2006; Berrios et al., 2007; Yoon et al., 2007; Ahn et al., 2008). The decreased growth rate is accompanied by an overall decrease in cell metabolism (Chuppa et al., 1997; Moore et al., 1997; Bollati-Fogolin et al., 2005; Berrios et al., 2007). Analysis of the cell cycle distribution at low culture temperature has illustrated that there is an increase in the percentage of cells in the G1 phase, with a proportional decrease of cells in the S phase (Jenkins and Hovey, 1993; Moore et al., 1997; Kaufmann et al., 1999).

Despite the decrease in growth rate and metabolic activity, an increase in the volumetric and specific productivity of recombinant proteins is usually observed (Jenkins and Hovey, 1993; Kaufmann et al., 1999; Yoon et al., 2002; Yoon et al., 2004a; Yoon et al., 2004b; Bollati-Fogolin et al., 2005; Yoon et al., 2005; Galbraith et al., 2006; Trummer et al., 2006; Yoon et al., 2007; Ahn et al., 2008). However, some literature disputes this fact, and observes no change in these parameters at a minimal culture temperature of 33°C (Chuppa et al., 1997; Andersen et al., 2000). This is likely to be a cell clone dependent response to reduced temperature.

1.6.3 Reducing ATP content

Decreasing the ATP content of cultured cells by inhibiting the process of oxidative phosphorylation has also been found to reduce the transit of glycoproteins through the Golgi (Datema and Schwarz, 1981; Persson et al., 1988). Use of CCCP, which uncouples the process of oxidative phosphorylation was also found to inhibit the formation of dolichol-P-mannose, although the N-linked glycosylation was otherwise unaffected (Datema and Schwarz, 1981). Similarly, the use of 2-deoxyglucose, which reduces the rate of glycolysis by inhibition of hexokinase and glucose phosphoisomerase, has been found to inhibit the formation of lipid linked oligosaccharides in tumour cells (Kurtoglu et al., 2007).
1.6.4 Varying the dilution rate of continuous cultures

Sections 1.6.2 and 1.6.3 explored how reducing the culture temperature and ATP content also causes inhibition of Golgi transit. Since a decrease in culture temperature also brought about a reduced growth rate, it is logical to suggest that inducing changes in the growth rate by another method may also cause alterations in Golgi transit rates, and consequently on N-linked glycosylation. The use of chemostat or continuous culture is an ideal method to study the effects of varying growth rate on cultured cells. The dilution rate of medium can be easily changed to achieve the desired change in growth rate, within the limits of cell growth.

Some of the earliest work with chemostat culture explored the effect of varying the rate of perfusion of medium on cell growth, and demonstrated that increasing the feed rate for HeLa cells also resulted in an increased number of viable cells (Cohen and Eagle, 1961). More recent experiments have shown that decreasing the dilution rate of medium also results in a significant decrease in the cell viability, which is caused by an apoptosis-induced increase in the death rate (Miller et al., 1988; Frame and Hu, 1991; Hayter et al., 1993; Martens et al., 1993; Lee et al., 1995; Follstad et al., 2000; Majid et al., 2007). In many cases, at low dilution rates, the growth rate deviates from the specified dilution rate, illustrating that a minimal growth rate is necessary for cell survival (Miller et al., 1988; Hayter et al., 1993; Martens et al., 1993). The cell size has also been found to increase at high dilution rates (Frame and Hu, 1991; Martens et al., 1993).

Decreasing the dilution rate tends to produce a more efficient metabolism, as demonstrated by the decrease in glucose and glutamine consumption and lactate production (Miller et al., 1988; Hayter et al., 1993; Follstad et al., 2000). In one case, the flux from pyruvate to the TCA cycle increased at reduced dilution rates, highlighting this efficiency (Follstad et al., 2000). An increase in the percentage of cells in the G1 phase of the cell cycle with proportional decrease of cells in the S phase has also been noted at decreasing dilution rates (Martens et al., 1993).

The productivity of recombinant proteins is seen to increase at low dilution rates (Miller et al., 1988; Frame and Hu, 1991; Linardos et al., 1991; Martens et al., 1993). It has been suggested that this may be a stress response to the decreased dilution rate, or due to a reduction in the length of mitosis, during which protein production ceases.
Varying the dilution rate has been found to impact on N-linked glycosylation. A decrease in the proportion of glycosylated recombinant interferon-\(\gamma\) is accompanied by a proportional increase in the non-glycosylated fraction at dilution rates lower than 0.3/d for a CHO cell cultivation (Hayter et al., 1993). However, this occurred alongside glucose and glutamine limitation, which suggests that the effect may not be dilution rate specific in this case. A significant decrease in the galactosylation of IgG was also noted at decreased dilution rates (Majid et al., 2007). In this same publication, bcl-2 was overexpressed in the same cell clone and displayed the same impact on glycosylation, despite the high cell viability at low dilution rate. This indicates that the reduction in galactosylation was not likely to have occurred due to the secretion of cellular glycosidases at low culture viability.

1.7 Generation number and cell growth, metabolism and productivity

Cell culture is generally employed as a method to mimic in vivo situations, whether normal or aberrant. With the introduction of techniques to immortalise cell lines, a wider array of experiments can be undertaken. However this process is not without disadvantages, since the ability to cultivate a cell line for an extended period of time may cause a variety of changes that results in alterations to the cell lines themselves.

Both generation number and passage number are used to describe the 'age' of cells in culture. The generation number is defined as the number of times a population has doubled, while a passage number is a measure of how many times a culture has been subcultured into a new vessel. The relationship between these parameters is illustrated in Figure 1.19.

The morphology of Caco-2 cells in culture has been found to alter after an extended period in culture (Yu et al., 1997; Behrens and Kissel, 2003). However, electron microscopy analysis has shown that there were no major morphological differences in this cell line when cultivated for up to 109 passages, and that the varied morphology was related to the growth of the cells under the cultivation conditions used (Briske-Anderson et al., 1997). LNCaP cells, a prostate cancer cell line, have been shown to vary in their response to a variety of different stimuli, such as androgens, at high passage number (Langeler et al., 1993; Esquenet et al., 1997). Additionally, different changes in gene expression pattern were noted when Syrian hamster embryo cells were cultivated for up to 64 passages and subject to UV or gamma irradiation (Chang-Liu and Woloschak, 1997).
In this example, the cell population doubles three times between each subculture, indicating that three generations accumulate during this time. In order to regain the same seeding concentration each time, this culture should be split 1:8. The upper numbers represent the generation number while the lower numbers represent the passage number (Freshney, 2006).
Additionally, cell metabolism was found to alter at extended passage number. After 64 passages, the doubling time of Syrian hamster embryo cells was found to decrease (Chang-Liu and Woloschak, 1997). In cultivating a hybridoma cell line for 88 days in a continuous culture set-up, a cell population with low IgG content appeared at day 55. This was accompanied by an increase in the consumption rate of glucose and lactate, suggesting this metabolism was less efficient. A decrease in the production rate of ammonium was also noted, indicating that glutamine metabolism was also less efficient at this point in the culture. Since these changes occurred in a time-dependent manner, the authors suggest that they may possibly be a result of the culture age (Coco-Martin et al., 1993). A different type of hybridoma cell was cultivated for up to 57 passages, and the metabolism over a 10-day batch incubation was examined at three distinct passage numbers. There was an increase in the maximal number of viable cells reached, but the maximum and cumulative IgG content decreased with increasing passage number. A significant decrease in the yield of lactate from glucose (DL/DG) was observed at high passage number, while there was no change in the yield of ammonium from glutamine (Schmid et al., 1990). This increase in the efficiency of glucose metabolism and conservation of glutamine metabolism is in contrast to the results of Coco-Martin et al., but may be as a result of the different cell lines in use (Coco-Martin et al., 1993).

The expression of cellular proteins in Caco-2 cells and LNCaP cells has also been found to change at extended generation number (Briske-Anderson et al., 1997; Yu et al., 1997; Behrens and Kissel, 2003; Youm et al., 2008). Use of high passage number RAW 264.7 cells has no impact on the rate of recombinant protein transfection, although the subsequent protein expression is significantly reduced (ATCC, 2006). The effect of generation number on the expression of recombinant proteins has been investigated widely due to its importance to the biotechnology industry. Scale-up of culture volumes to production quantities of approximately 20,000 L can take months and so there is the potential for a large number of generations to accumulate during this time. A reduction in productivity during production can be costly and may even impede regulatory approval for a particular process if the effect is severe. A comprehensive review of the effect of culture age on recombinant protein production was published in 2003 (Barnes et al., 2003).

A reduction in the expression of recombinant proteins at high generation number has been widely reported for CHO cells (Kaufmann and Schimke, 1981; Weidle et al., 1988; Pallavicini et al., 1990; Kim et al., 1998b; Fann et al., 2000). The use of amplification
procedures has been found to cause chromosomal rearrangements of CHO cells, suggesting that this cell line becomes unstable in this situation (Flintoff et al., 1984). However a reduction in productivity at high generation number has been noted for a range of other cell lines, including hybridoma and NS0 cells (Frame and Hu, 1990; Ozturk and Palsson, 1990b; Lee et al., 1991; Chuck and Palsson, 1992; Coco-Martin et al., 1992; Coco-Martin et al., 1993; Merritt and Palsson, 1993; Bae et al., 1995; Couture and Heath, 1995).

The loss in productivity has been found to occur reproducibly in a series of hybridoma subclones (Merritt and Palsson, 1993). However, the relative reduction in productivity is dependent on the cell line used (Bae et al., 1995). In some CHO cultures, the production levels stabilise after an initial decrease in the first 30 to 50 days (Kim et al., 1998a). Additionally, serum levels and inoculum state have been found to impact on the decrease in productivity (Chuck and Palsson, 1992; Gaertner and Dhurjati, 1993).

Theoretically, a change at any one of the control points of transcription, translation and post-translational processing could lead to altered productivity levels (Barnes et al., 2003). However, a more distinct set of factors has been found to explain the instability in productivity. Some literature has shown that a simple reduction in the productivity of each cell causes this effect (Kim et al., 1996; Borth et al., 1999). The appearance of non-producing cells within the total cell population has also been found to cause a reduction in the overall production (Frame and Hu, 1990; Lee et al., 1991; Chuck and Palsson, 1992; Bae et al., 1995). It has been suggested that non-producing cells have a growth advantage over producers and that there is the possibility for these cells to overtake the entire cell population. However, in most cases, a balance between the two populations is reached. A loss of the recombinant protein gene from the cellular genome has also been observed (Pallavicini et al., 1990; Merritt and Palsson, 1993; Kromenaker and Srienc, 1994; Kim et al., 1998b; Hammill et al., 2000).

Methods to overcome this decrease in productivity focus on attempting to place the gene of interest into the most suitable place in the genome. Incorporation of the gene into the genome usually occurs at random, but some regions result in better productivity than others. For example, incorporation near the telomeres is more likely to result in stable and high expression rates (Yoshikawa et al., 2000). Similarly, random insertion near an endogenous promoter or enhancer will result in higher productivity than in
heterochromatin which is transcriptionally inactive (Barnes et al., 2003). In addition, site-specific recombination using the Cre/LoxP or Flp/FRT system has been used for targeting integration of recombinant genes into highly expressed regions. A cell line is firstly transfected with a vector containing a reporter gene flanked by two target sequences (either LoxP or FRT). The highly producing clones are transfected with a new vector containing the gene of interest flanked by two targeting sequences. The gene of interest is then inserted with the use of recombinase (Cre or Flp). The technique can be further improved with the use of recombinase-mediated cassette exchange, which replaces the reporter gene with the gene of interest (Barnes et al., 2003). The change in protein expression when genes are inserted at a chromosomal location that is different to the native state is known as “the position effect” (Wilson et al., 1990). Modulation of this position effect or overcoming random insertion may result in improvements in protein productivity in both long- and short-term cultures.

1.8 Aims of thesis

1) Elevated concentrations of ammonium have been shown to reduce the growth rate of cells in culture while increasing the rate of consumption and production of key metabolites. The sialylation and galactosylation of N-linked glycoproteins has also been shown to decrease at high ammonium concentrations, while significant changes to the antennarity have also been observed. Since most ammonium is derived from the degradation and metabolism of glutamine, CHO cells were inoculated in a batch mode of cultivation with varying glutamine concentrations (between 0mM and 20mM) for five days to assess the impact of reduced ammonium concentrations on these parameters. Use of L-alanine-L-glutamine (GlutaMAX-1™) as a glutamine replacement strategy was also explored to this end.

2) A continuous method of culture provides an ideal set up to assess changes in cellular behaviour in response to stimuli, while culture parameters remain stable. This is in contrast to the batch cultivation, where culture conditions vary during the experimental time. Consequently, a continuous culture mode was employed to determine the true effect of varying glutamine concentrations (at 0mM and 8mM) on the cell growth, metabolism, productivity of CHO cells, and the resulting glycosylation of HCG. The intracellular content of nucleotides and sugar nucleotides were assessed to explore the mechanisms of the changes in glycosylation. Additionally, the cell cycle distribution was monitored at each steady state to investigate any glutamine-dependent effects.
3) By altering the dilution rate of culture medium in the continuous culture system, the growth rate of CHO cells can be accurately controlled. Consequently, this method of cultivation is ideally suited to assess the effect of varying growth rate on cellular metabolism and N-linked glycosylation of HCG. In addition, changes in the cell cycle distribution and intracellular content of nucleotides and sugar nucleotides were monitored. A low and high dilution rate of 0.2/d and 0.5/d, respectively, were examined.

4) After a prolonged time in culture, the productivity and metabolism of cells may change. High generation number cells were harvested from each continuous culture, and were subsequently inoculated in a batch culture to assess these parameters.
Chapter 2

Materials and Methods
2.1 Materials

Acetic acid, acetonitrile, ADP, ammonium acetate, AMP, ATP, bovine fetuin, bovine serum albumin, citric acid, CMP-NeuAc, CTP, dipotassiumhydrogenphosphate, DMSO, GDP, GDP-Man, GlycoProfile™ 2-AB labelling kit, GTP, hydrochloric acid, kerosene, KOH, methanol, methionine, phosphate citrate / ureum peroxide tablets, orthophosphoric acid, perchloric acid, propidium iodide, potassium phosphate monobasic, RNase A, sodium carbonate, sodium chloride, sodium citrate, sodiumdihydrogenphosphate monohydrate, sodium phosphate dibasic dihydrate, sulfuric acid, tetrabutylammonium bisulfate hydrogen sulfate, 3,3',5,' tetra methyl benzidine dihydrochloride tablets, trichloroacetic acid, trizma, tween-20, UDP, UDP-Gal, UDP-GalNAc, UDP-Glc, UDP-GlcNAc, urea and UTP all from Sigma Aldrich Corporation, (Missouri, USA).

InstantBlue® protein stain was supplied by Gentuar (Kampenhout, Belgium).

L-glutamine, HT supplement, CD-CHO medium (for cell culture), 12% BisTris gels, LDS sample buffer, MES SDS-PAGE running buffer, NuPAGE antioxidant and reducing reagent for SDS-PAGE were supplied by Invitrogen (California, USA).

Anode fluid 3, cathode fluid 10, Servalyt Precote pl 3-10 gel and Serva Violet 17 were supplied by Serva Electrophoresis GmbH, (Heidelberg, Germany).

PBS was prepared by the service group of P & MSc - CCPD (Process & Manufacturing Sciences Cell Culture Process Development) Merck, Sharp & Dohme, Oss, the Netherlands.

Ammonia (25%), Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA).

BioRad dye was supplied by BIO-RAD Laboratories GmbH, (Munich, Germany).

Ethanol 70%, Merck & Co., Inc., (New Jersey, USA).

Ovitrelle, produced by Merck Serono S.A., (Geneva, Switzerland).

Peptide-N-glycosidase F (PNGaseF), Sialidase A (ABS), Sialidase S (NAN1), bovine kidney fucosidase (BKF), bovine testis galactosidase (BTG), Hexase-I (N-56
acetylglucosaminidase, GUH) and Jack bean mannosidase (JBM) all from Prozyme, San Leandro, USA.

2-AB-glucose homopolymer (2-AB dextran ladder) from Ludger Ltd., Oxford, UK.

2.2 Preparation of solutions
Reagents of less than 5 g were weighed with either a Mettler College analytical balance, or a Sartorius ED224S analytical balance. Reagents above 5 g were weighed with either a Mettler Toledo top-loading balance or a Sartorius CP 3202. MilliQ water was used for preparation of aqueous solutions.

2.3 Pipetting
A set of Gilson pipettes was used to measure volumes in the range of 1 µl to 5 ml.

2.4 pH measurements
Measurement and adjustment of pH was carried out with either a Corning pH meter (model 240) or a Knick pH meter 765 calimatic. Both meters were calibrated before use with standard buffer solutions at pH values of 4.0, 7.0 and 10.01.

2.5 Spectrophotometry
Spectrophotometric measurements were made on a µQuant 96-well plate reader (Bio-Tek Instruments, Inc) or a Unicam Helios spectrophotometer.

2.6 Centrifugation
Volumes of less than 1.5 ml were centrifuged on a bench-top Eppendorf Centrifuge (model 5415R or 5417R). Larger volumes were routinely centrifuged using an Eppendorf centrifuge 5810.

2.7 Growth and maintenance of CHO cell line
2.7.1 Cell line and culture medium
The CHOK1 cell line overexpressing HCG which is used for this work was a kind gift from MSD, Oss, the Netherlands. The cells were cultivated in CD-CHO medium that was routinely stored at 4°C and supplemented with 8 mM L-glutamine and HT supplement (100 µM sodium hypoxanthine, 16 µM thymidine). HT supplement was used to aid cell growth, and no other gene amplification procedures were carried out. With the exception
of the medium used for experiments detailed in Section 3.3, the same lot of medium was used and prepared in 10L batches by the service group of P & MSc - CCPD (Process & Manufacturing Sciences, Cell Culture Process Development) in MSD, Oss, the Netherlands.

2.7.2 Initial inoculation of a culture

49 ml of medium was pre-incubated for 30-45 minutes in a 250 ml Erlenmeyer shake flask. A vial was retrieved from the liquid nitrogen tank and thawed rapidly in a warm bath of ethanol. The contents of the vial were then transferred to the shake flask and incubated.

2.7.3 Routine maintenance

Unless otherwise stated, the cells were routinely cultured in 250 ml and 1 L shake flasks in a Kuhner ClimoShaker ISF1-X incubator at 150 rpm, 5% CO₂ and 80% RH (Adolf Kuhner AG). The cells were maintained between a cell density of 3 x 10⁵ cells/ml and 2 x 10⁶ cells/ml by passaging every two to three days. Twenty to thirty percent of the total volume of the shake flask was used for routine culture.

2.7.4 Cryopreservation

The cells were grown to late log phase and counted to obtain an accurate value for the number of viable cells. Culture medium, culture medium with 20% DMSO, cryogenic vials and the rack to hold the vials were cooled at 2-8°C for 30-45 minutes. Cells were centrifuged at 1000 g for 5 minutes and then resuspended in the cool normal medium before merging with an equal volume of the DMSO-containing medium. A final concentration of 10% DMSO and cell density of 10 x 10⁶ cells/ml was obtained. The vials were kept on ice during all the manipulations, and subsequently placed in a Nalgene Cryo freezing container overnight in a -80°C freezer. The vials were then stored in the liquid phase of a liquid nitrogen tank.

2.7.5 Daily analytical procedures

For all experiments, a number of daily analytical procedures were carried out. The number of viable cells and percentage viability was determined either by manual counting (Section 2.7.6), or by use of the CEDEX AS²⁰, (Roche Innovatis AG). The extracellular concentrations of ammonium, glucose, glutamate, glutamine and lactate were determined with a Nova Bioprofile (model 100 plus or 400, both Nova Biomedical Corporation). BGA parameters (pH, pO₂, and pCO₂) were determined using the Bayer Rapidlab 248 (Siemens 58
Healthcare Diagnostics) or the Nova Bioprofile 400. Excess sample was centrifuged at room temperature for 5 minutes at 1000 g and subsequently filtered through a 0.2 μm filter. These cell-free supernatants were stored at -20°C for further analyses.

2.7.6 Manual cell counting

A sample of cell suspension was mixed with trypan blue dye. The mixture could be left to stand for 5 to 15 minutes, during which time the non-viable cells would take up the dye and stain blue. A small amount was then transferred under the coverslip, into the chamber of the hemacytometer by capillary action (bright light hemacytometer with improved neubauer rulings, Hausser Scientific). The hemacytometer is divided into five main 1mm² chambers (Figure 2.1), and all cells within each chamber and touching the top and left perimeter of the chamber were counted. Both viable and non-viable cells were counted.

Cell numbers and viability were determined according to the formulae detailed below:

\[
\text{Cell number (cells/ml)} = \frac{\text{No. of cells in each chamber}}{\text{No. of chambers}} \cdot 10^4 \cdot \text{dilution factor}
\]

\[
\text{Cell viability (％) = \frac{\text{No. of viable cells}}{\text{No. of cells (total)}} \cdot 100}
\]

2.8 Bioreactor set-up for continuous cultures

The continuous cultures were carried out in 2L bioreactors (Applikon) with a working volume of 1.7 L. Culture pH, as measured by a PT100 electrode (Mettler Toledo), was maintained at 7.20 (± 0.02) by addition of CO₂ via the headspace and 0.5M NaOH via an inlet port. The dissolved oxygen was measured with an O₂ electrode (Mettler Toledo), and was controlled at 50% of air saturation via a macrosparger at 5 L/h. These variables were controlled with the use of an ADI 1030 bioprocessor (Applikon). The temperature was maintained at 37°C by use of a heating blanket and water jacket which was controlled by a Julabo 4 HD water bath. Agitation rate was set at 325 rpm using a P100 stirrer and ADI 1032 stirrer controller (both Applikon). The effluent air was cooled before leaving the reactor by a connection to a Julabo F25 HD waterbath. Airflow over the headspace was maintained at 10 L/h. Control of the level in the reactor was carried out using an APS501 level controller (AppliSens). The harvest pump ran continuously and was therefore rate
Figure 2.1 The bright light hemacytometer.

The hemacytometer is divided into nine regions, and all the cells lying within and touching the top and left perimeter of the five main chambers (labelled 1 through 5) were counted. The number of viable cells and percentage viability were determined according to Section 2.7.6.
setting. The feed pump was switched on when necessary by the level controller. Fresh medium was added and culture harvest was removed using Watson Marlow 101U/R pumps. Medium was stored at 4°C and filtered through a 0.2 µm Sartobran P filter (Sartorius) before addition to the reactor. Harvest culture was filtered through a 0.8 µm Sartoclean-CA filter before collecting in the harvest reservoir. Each day, the feed and harvest reservoirs were weighed with a Sartorius QC65EDE-5 balance to determine the flow rate of medium.

The bioreactors were inoculated at an initial cell density of 3 x 10^5 cells/ml and allowed to proceed for three days in batch mode. At this point, the perfusion of medium was begun.

2.8.1 Steady state analytical procedures

A theoretical steady state is defined 5 volume changes after the last perturbation, and if stable conditions were attained at this point, further analyses were carried out. A new harvest line was attached to the bioreactor to collect cell suspension for purification of HCG (Section 2.15). The new harvest reservoir was maintained at 4°C. In this way, the harvest filters were bypassed to avoid encountering dead and dying cells. An assessment of the glycosylation of HCG was carried out by IEF and analysis of N-linked glycan structures (Sections 2.19 and 2.20, respectively). Extra samples were taken for analysis of extracellular amino acid concentrations (Section 2.11), cell cycle distribution (Section 2.13) and intracellular nucleotide and sugar nucleotide content (Section 2.14). This was carried out for two consecutive days during the steady state period.

2.9 Calculation of cell culture kinetic parameters

Data analysis was based on kinetic and cumulative calculations. Cumulative calculations represent the total amount of metabolite or viable cells that is produced or consumed. Specific rates are calculated by dividing the total amount by the viable cell concentration and time, as shown below for glucose.

\[
\frac{-d[Gluc]}{dt} = q_{Gluc} \cdot VC \quad \text{or} \quad q_{Gluc} = \frac{-d[Gluc]}{dt} \cdot \frac{1}{VC}
\]

The specific glucose consumption rate (qGluc) is defined at the rate of change in glucose concentration per unit time (d[Gluc]/dt) and per viable cell (VC). This value is made negative to result in positive trends. The calculation is the same for glutamate.
Similarly for lactate,

\[
\frac{d[Lac]}{dt} = qLac \cdot VC \quad \text{or} \quad qLac = \frac{d[Lac]}{dt} \cdot \frac{1}{VC}
\]

However, the specific rates of glutamine consumption and ammonium production need to take into account the chemical degradation of glutamine to ammonium and pyrrolidone carboxylic acid. The equations are formed in a similar manner to those for glucose and lactate, though the apparent consumption and production rates are a product of the true cell-specific consumption and production rate and the chemical degradation rate \((k \cdot [Gln])\).

\[
-\frac{d[Gln]}{dt} = qGln \cdot VC + k \cdot [Gln] \quad \text{and} \quad -\frac{d[NH_4^+]}{dt} = qNH_4^+ \cdot VC + k \cdot [Gln]
\]

Which rearranges to

\[
qGln = -\frac{d[Gln]}{dt} \cdot \frac{1}{VC} - k \cdot [Gln] \quad \text{and} \quad qNH_4^+ = \frac{d[NH_4^+]}{dt} \cdot \frac{1}{VC} - k \cdot [Gln]
\]

The value for the first order degradation rate constant, \(k\) was determined for CD-CHO medium in a shake flask and bioreactor system. This was necessary since \(k\) is dependent on medium composition, pH, ionic strength and temperature (Ozturk and Palsson, 1990a). Glutamine supplemented medium was incubated in shake flasks for five days, and the metabolite concentrations were determined daily. A plot of the percentage remaining glutamine as a function of time was constructed, and the data was fitted to the function \(y = e^{-kx}\), (a representative plot is given in Figure 2.2). A mean value of 0.069d\(^{-1}\) was determined for a shake flask system. A value of 0.078d\(^{-1}\) was used for bioreactor experiments, and this value was determined under similar experimental conditions – i.e. controlled pH and temperature in a bioreactor (Lammers, 2008).

A more detailed derivation of specific rate calculations based on material balancing is given for batch and continuous culture systems in Section 2.9.1 and 2.9.2, respectively.
Figure 2.2 Representative plot of percentage glutamine remaining as a function of time.

CD-CHO medium was incubated under shake flask experimental conditions and the concentration of glutamine was determined daily. The glutamine degradation constant, $k$, was determined from 4 separate evaluations.
2.9.1 Batch culture

For a batch culture system, all calculations are based on the following balance equation:

\[ \text{State}_t = \text{state}_{t-1} + \text{in} - \text{out} + \text{production} \]

i.e., the current state is a product of the previous state, plus any in flows and production of metabolites or biomass, minus the flows out of the system. However, by definition, no inflows exist in a batch system, so this term can be neglected.

From this, the following volume balance equation can be made:

\[ V_t = V_{t-1} - V_{\text{sample}} \]

So, the volume at any given time point \( V_t \) is a product of the volume at the previous time point \( V_{t-1} \), minus the volume removed at the time of sampling.

A balance equation for metabolites in culture can be formed, such as for glucose concentration (in mmol) as shown below.

\[ \text{Gluc}_t = \text{Gluc}_{t-1} - \text{Gluc}_{\text{sample}} - \text{Gluc}_{\text{cons}} \]

Or more simply,

\[ G_t = G_{t-1} - G_s - \text{Gluc}_{\text{cons}} \]

This relationship can be rearranged to determine the specific glucose consumption rate.

Firstly, the amount is related to the concentration and volume as follows;

\[ G = [G] \cdot V \]

So, converting the previous relationship to this format gives;

\[ [G]_t \cdot V_t = [G]_{t-1} \cdot V_{t-1} - [G]_s \cdot V_s - \text{Gluc}_{\text{cons}} \]

Which rearranges to;

\[ \text{Gluc}_{\text{cons}} = [G]_{t-1} \cdot V_{t-1} - [G]_t \cdot V_t - [G]_s \cdot V_s \]
But, the rate of glucose consumption at time (t) is based on the change in glucose concentration between time (t-1) and time (t), and the sampling time point occurs at (t-1). As a result, the glucose concentration in the sample equals the glucose concentration in the shake flask at that time point, i.e.

\[ [G]_s \cdot V_s = [G]_{t-1} \cdot V_s \]

Substituting these values into the previous equation gives;

\[
Gluc_{cons} = [G]_{t-1} \cdot V_{t-1} - [G]_t \cdot V_t - [G]_{t-1} \cdot V_s
\]

Which rearranges to;

\[
Gluc_{cons} = [G]_{t-1} (V_{t-1} - V_s) - [G]_t \cdot V_t
\]

And to calculate the glucose consumption rate per viable cell, this term is merely divided by the average viable cell number between times (t-1) and (t);

\[
Gluc_{cons} = \frac{[G]_{t-1} (V_{t-1} - V_s) - [G]_t \cdot V_t}{(VC_{t-1} (V_{t-1} - V_s) + VC_t \cdot V_t) \cdot 0.5}
\]

The expression is converted to pmol per viable cell, per unit time with the following method, assuming all quantities are in L.

\[
Gluc_{cons} = \frac{[G]_{t-1} (V_{t-1} - V_s) - [G]_t \cdot V_t \cdot 1}{(VC_{t-1} (V_{t-1} - V_s) + VC_t \cdot V_t) \cdot 0.5 \Delta t \cdot 10^9}
\]

This expression can also be used to describe the specific consumption rate of glutamate and the apparent consumption rate of glutamine. For production rates, use of this formula will lead to negative values, so the following calculation can be used alternatively. The apparent ammonium production rates can be calculated similarly.

\[
\frac{[G]_t \cdot V_t - [G]_{t-1} (V_{t-1} - V_s)}{(VC_{t-1} (V_{t-1} - V_s) + VC_t \cdot V_t) \cdot 0.5 \Delta t \cdot 10^9}
\]
2.9.2 Continuous culture

In this case, the balance equation is given by:

\[ \text{State}_t = \text{state}_{t-1} + \text{in} - \text{out} + \text{production} \]

i.e., the current state is a product of the previous state, plus flows into and out of the system, plus production of other metabolites and cells.

For volume, the following balance equations can be made:

\[ V_t = V_{t-1} + V_{\text{in}} - V_{\text{out}} \]

where

\[ V_{\text{in}} = V_{\text{feed}} + V_{\text{OH}} \]

and

\[ V_{\text{out}} = V_{\text{harvest}} + V_{\text{sample}} \]

So, the volume on any given day \( V_t \) is made up of the product of the volume the previous day \( V_{t-1} \), combined with any additional volume changes, either through feed or NaOH addition \( V_{\text{feed}} \) and \( V_{\text{OH}} \), respectively) and harvest or sample removal \( V_{\text{harvest}} \) and \( V_{\text{sample}} \), respectively).

A balance equation for metabolites in culture can be formed, such as for glucose concentration (in mmol) as shown below.

\[ \text{Gluc}_t = \text{Gluc}_{t-1} + \text{Gluc}_{\text{feed}} - \text{Gluc}_{\text{sample}} - \text{Gluc}_{\text{harvest}} - \text{Gluc}_{\text{cons}} \]

Or more simply,

\[ G_t = G_{t-1} + G_f - G_s - G_h - G_{\text{cons}} \]

This relationship can be rearranged to determine the specific glucose consumption rate. Firstly, the amount is related to the circulating concentration and volume as follows;

\[ G = \{G\} \cdot V \]

So, converting the previous relationship to this format gives;
\[ [G]_t \cdot V_t = [G]_{t-1} \cdot V_{t-1} + [G]_f \cdot V_f - [G]_h \cdot V_h - \text{Gluc}_{\text{cons}} \]

Or,

\[ \text{Gluc}_{\text{cons}} = [G]_{t-1} \cdot V_{t-1} - [G]_t \cdot V_t + [G]_f \cdot V_f - [G]_h \cdot V_h \]

Again,

\[ [G]_t \cdot V_t = [G]_{t-1} \cdot V_{t-1} \]

Also,

\[ [G]_h = ([G]_{t-1} + [G]_f) \cdot 0.5 \]

i.e. the concentration of glucose in the harvest changes over time, and as a result is more accurately described as the average glucose concentration over that period. So,

\[ \text{Gluc}_{\text{cons}} = [G]_{t-1} \cdot V_{t-1} - [G]_t \cdot V_t + [G]_f \cdot V_f - (([G]_{t-1} + [G]_f) \cdot 0.5 \cdot V_h \]

But, \( V_t = \Phi_f \cdot \Delta t \) (the feed flow per unit time)

And, \( V_h = \Phi_h \cdot \Delta t \) (the harvest flow per unit time)

\[ \text{Gluc}_{\text{cons}} = [G]_{t-1} (V_{t-1} - V_t) - [G]_t \cdot V_t + [G]_f \cdot \Phi_f \cdot \Delta t - ([G]_{t-1} + [G]_f) \cdot 0.5 \cdot \Phi_h \cdot \Delta t \]

Assuming all amounts are in units of L, the expression can be converted to the specific glucose consumption rate in pmol per viable cell per day;

\[ \text{Gluc}_{\text{cons}} = \frac{[G]_{t-1} (V_{t-1} - V_t) - [G]_t \cdot V_t + [G]_f \cdot \Phi_f \cdot \Delta t - ([G]_{t-1} + [G]_f) \cdot 0.5 \cdot \Phi_h \cdot \Delta t}{(VC_{t-1} (V_{t-1} - V_t) + VC_1 \cdot V_t) \cdot 0.5} \cdot \frac{1}{\Delta t \cdot 10^9} \]

For production rates, this use of this formula will lead to negative values, so the following calculation can be used as an alternative;

\[ \text{Gluc}_{\text{cons}} = \frac{[G]_f \cdot \Phi_f \cdot \Delta t - ([G]_{t-1} + [G]_f) \cdot 0.5 \cdot \Phi_h \cdot \Delta t}{(VC_{t-1} (V_{t-1} - V_t) + VC_1 \cdot V_t) \cdot 0.5} \cdot \frac{1}{\Delta t \cdot 10^9} \]

Note that the concentration of glucose, glutamine and glutamate used for the feed term, \([G]_f\) in the calculation of specific consumption rate was 30mM, 8mM and 1.71mM, respectively. This term was neglected for calculation of specific production rates.
2.9.3 *Dilution rate*

The dilution rate (D) of a continuous culture can be described by the following equation

\[ D = \frac{F}{V} \] (d⁻¹)

The volume in the reactor (V) is given in ml, and a value of 1700ml was used since the level sensor was calibrated to this value. F is the flow rate of medium, and it is defined as the change in harvest weight per unit time, or

\[ F = \frac{(\text{harvest}_t - \text{harvest}_{t-1})}{\Delta t} \] (ml/d)

The relationship between the flow rate, calculated dilution rate and the length of time for the total volume of the bioreactor to be replaced by new medium (residence time) is shown in Table 2.1. The residence time can also be described as the inverse of the dilution rate. At least 5 residence times since the last perturbation are required for a steady state to be achieved. After this time, most parameters such as viable cell number, metabolite concentrations and specific rates of consumption and production of these metabolites should be stable, and a steady state can be defined.

<table>
<thead>
<tr>
<th>Dilution rate (d⁻¹)</th>
<th>Flow rate (ml/d)</th>
<th>1 Residence time (days)</th>
<th>5 Residence times (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>340</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>0.4</td>
<td>680</td>
<td>2.5</td>
<td>12.5</td>
</tr>
<tr>
<td>0.5</td>
<td>850</td>
<td>2</td>
<td>10</td>
</tr>
</tbody>
</table>

*Table 2.1 Relationship between dilution rate, flow rate and residence time.*

2.9.4 *Growth rate*

The growth rate of cells is calculated as the change in viable cell number per unit time, quoted in units of d⁻¹ or /d. For batch cultures, the growth rate is calculated as follows;

\[ \frac{\ln(CVC_t - CVC_{t-1})}{\Delta t} \] (d⁻¹)

While for continuous cultures, growth rate is calculated with the following formula, the growth rate is theoretically equal to the dilution rate at steady state.
CVC represents the cumulative viable cells, i.e. the total amount of viable cells in the culture, effluent and in the sample taken at that time. The CVC can be calculated for batch systems by the following equation;

\[
\frac{(\text{CVC}_t - \text{CVC}_{t-1})}{V_t \cdot \text{VC}_{\text{ave}} \cdot \Delta t} \quad \text{(d}^{-1})
\]

For continuous cultures, the flow of medium needs to be taken into account, so the following formula is used as an alternative

\[
\text{CVC}_t = \text{CVC}_{t-1} + (VC_t \cdot V_t) - (VC_{t-1} \cdot V_{t-1} - V_t)
\]

2.9.5 Generation number
The number of generations (GN) can be calculated by use of the following formula.

\[
\text{GN}_t = \text{GN}_{t-1} + \left(\frac{\mu \cdot \Delta t}{\ln(2)}\right)
\]

2.10 Measurement of oxygen uptake rate
The rate of oxygen uptake by cells within a continuous bioreactor was measured by a dynamic method (Singh, 1996). Firstly, all the air on the headspace was replaced with nitrogen to prevent any transport of oxygen back to the culture from the headspace. This was carried out at 10 L/h and took approximately 30 minutes. After this time, the pO₂ was increased to almost 90% of air saturation via pure oxygen macrosparging at 5 L/h. The decreasing pO₂ profile from this setpoint was plotted against time to determine the rate of oxygen uptake in %/hr, i.e. the OUR\text{measure}. This change in pO₂ profile was measured by the O₂ electrode and the data was retrieved via an interface with the ADI1030 bioprocessor and the MFCS (multi fermentor control system) data registration software.

A correction for the desorption of oxygen to the headspace was carried out by repeating the procedure in the same system when the culture was replaced with CD-CHO medium. This value is termed the OUR\text{blank}. (See Figure 2.3)
Figure 2.3 Representative plot of the rate of oxygen uptake in the presence (red) and absence of CHO cells (blue) in CD-CHO medium.

Both sets of data were fit to a first order linear regression, \( y = mx + c \), where \( m \) gives the rate of oxygen uptake. The actual oxygen uptake rate and subsequently the specific oxygen uptake rate by the cells were determined according to Section 2.10. The original timescale was reset to an arbitrary value for clarity.

<table>
<thead>
<tr>
<th>( D ) (( d^{-1} ))</th>
<th>( qO_2 ) (mmol/( 10^9 )cells/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>0.069 ± 0.007</td>
</tr>
<tr>
<td>0.4</td>
<td>0.102 ± 0.010</td>
</tr>
<tr>
<td>0.5</td>
<td>0.106 ± 0.009</td>
</tr>
</tbody>
</table>

Table 2.2 The specific oxygen uptake rate at various dilution rates.

Values were converted to mmol/\( 10^9 \)cells/hr for use in the metabolic flux analysis model (Section 2.12) Values were determined at the end of each continuous cultivation. Joke Borsje (SSCC & D department in MSD, Oss, the Netherlands) contributed additional data points via the establishment of an identical cultivation.
The actual oxygen uptake rate was then calculated by using the assumption that under these circumstances, 1% air saturation corresponds to 0.00224mM dissolved oxygen (using Henry’s law, (Sander, 1999)).

\[
OUR_{\text{act}} = (OUR_{\text{measure}} - OUR_{\text{blank}}) \cdot 0.00224 \\
\text{(mM/h)}
\]

The specific oxygen consumption rate \( (qO_2) \) in pmol/VC/hr was calculated from this using the equation given below. The values determined at each dilution rate is shown in Table 2.2.

\[
qO_2 = \frac{(OUR_{\text{act}})}{VC \cdot 1000} \cdot 10^9 \cdot 24
\]

2.11 Extracellular amino acid assay
Analysis of the extracellular concentrations of amino acids was carried out by SAFC biosciences, a part of Sigma Aldrich biotechnology. Cell-free culture supernatants were diluted with a deproteinising solution (13.5% w/v 5-sulfosalicylic hydrate) and an internal standard solution (glucosaminic acid) before filtering through a 0.2 \( \mu \text{m} \) filter. This prepared sample was kept between 2-8°C before analysis on a Beckman Instruments HPLC.

The following components from these results were incorporated into a metabolic flux analysis model: alanine, arginine, asparagine, aspartic acid, cystine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine and valine (Section 2.10). Although no cysteine was detected in the samples, small quantities of cystine were observed. It was assumed that complete decomposition of cysteine to cystine occurred.

2.12 Metabolic flux analysis
This technique was used to estimate how the changes in the consumption and production rates of metabolites relate to differences in the distribution of intracellular fluxes. By combining material balances of carbon and nitrogen with measurements of specific production rates of metabolites via a reaction network, the metabolic fluxes were estimated.

The analysis can be formulated by the matrix equation:
A \cdot x = r

where,

\[ \begin{align*}
A &= \text{the matrix of stoichiometric coefficients for the biochemical network,} \\
x &= \text{the vector of the metabolic fluxes} \\
r &= \text{the vector of metabolite rates of change.}
\end{align*} \]

The specific production rates of extracellular metabolites were measured (calculated as in Section 2.9.2). The rate of change of intracellular metabolites was assumed to be zero (due to the pseudo steady state assumption). As a result, the matrix can be solved for the flux vector \( x \), using the method of least squares, shown below.

\[ x = (A^T A)^{-1} \cdot A^T r \]

Cell metabolism was simplified in order to consider the main pathways of material flows, that is, the metabolism of carbohydrates and amino acids, and the synthesis of biomass and HCG (Figure 2.4). The 10 reactions of glycolysis were consolidated into one flux from glucose to pyruvate, the flux from pyruvate to lactate was also included. The TCA cycle was simplified from 8 fluxes. The catabolism of 19 amino acids was considered; tryptophan was excluded since it made no significant contribution towards energy generation, and is used sparingly in protein synthesis. In addition to the generation of metabolic intermediates, the use of amino acids in protein and nucleotide synthesis was considered. With the exception of alanine, the synthesis of non-essential amino acids was assumed to be negligible due to the high concentrations present in cell culture medium. The conversion of glutamine to glutamate and \( \alpha \)-ketoglutarate was also included.

The synthesis of cellular building blocks is described by the biomass equation. Lumped into this equation is the use of glucose in the synthesis of lipids and nucleotides, amino acids for use in cellular proteins and HCG, the consumption of glutamine, aspartate and glycine in the synthesis of nucleotides and the production of glutamate in cellular metabolism. The cellular molecular weight was calculated as 25.474 g/mol using an elemental cell composition of \( C_{1.975}H_{1.2605}N_{0.389}O_{0.489} \). The dry cell weight of the cell was assumed to be 240 pg/cell (Europa et al., 2000; Gambhir et al., 2003). The cell composition in literature was used to derive the coefficients for glucose and amino acids in the biomass equation; protein, 75%; RNA, 7.5%; DNA, 3.75%; lipids and macromolecules, 13.75% (Darnell et al., 1986).
Figure 2.4 The reaction network under consideration.

The black arrows represent the fluxes calculated and AA represents the total amino acid pool. Individual amino acids are given by their three letter symbols, while the structures given in the blue circle represent the TCA cycle. AcCoA; acetyl-CoA, CoA; coenzyme A, αKG; α-ketoglutarate, SuccCoA; succinyl-CoA, Fum; fumarate, Mal; malate, OAA; oxaloacetate.
Stoichiometric coefficients for protein synthesis from amino acids were derived from literature data of the amino acid composition of mammalian cells (Okayasu et al., 1997). The value used for the biomass was the specific growth rate.

The equation for HCG production is based on the amino acid composition of a typical IgG molecule (Edeman et al., 1969). A molecular weight of 40610.6 g/mol, and the relation 15 IU/μg was used to convert the specific productivity of HCG from μIU/VC/day to mmol/10⁹cells/hr. For material balancing, an elemental composition of C₁H₁.₅₇₆N₀.₂₄₆O₀.₃₈₁ was determined for HCG with 50% glycosylation.

The evolution of carbon dioxide as a metabolite was not measured. However, the respiratory quotient was assumed to be 1, meaning the oxygen uptake rate equals the carbon evolution rate. The oxygen uptake rate was determined according to Section 2.10. The equation for each of the reactions considered is given in Figure 2.5. From this, the stoichiometric matrix which is composed of 30 columns of reactions and 32 rows of metabolites was constructed (Figure 2.6).

The specific production rate of each metabolite was composed into a vector of 32 rows before the matrix was solved for metabolic fluxes. The carbon and nitrogen material balance was assessed and the final fluxes are quoted as mmol carbon/10⁹cells/hr. For example, the flux from glucose to pyruvate is quoted as Jₘₑₜ-gluc-pyr, and a negative flux indicates that the flux is in the opposite direction to that quoted.

The analysis model in use was developed by Jongchan Lee and Wei-Shou Hu at the University of Minnesota, and is freely distributed with lecture notes for the “Advanced Course in Bioprocessing” organised by the Hu research group. The model has been used as a vital analysis tool in recent publications by the group (Europa et al., 2000; Gambhir et al., 2003). A Microsoft Excel version of the algorithm was used for this work.
A - Glycolysis:
1) GLC → 2 PYR
2) PYR → LAC

B - TCA cycle:
3) PYR → AcCoA + CO₂
4) AcCoA + OAA → αKG + CO₂
5) αKG → SucCoA + CO₂
6) SucCoA → FUM
7) FUM → MAL
8) MAL → OAA

C - Glutaminolysis:
9) GLN → GLU + NH₃
10) GLU → αKG + NH₃
11) MAL → PYR + CO₂

D - Synthesis of Biomass and HCG:
12) 0.0208 GLC + 0.0377 GLN + 0.0133 ALA + 0.0070 ARG + 0.0 ASN + 0.0261 ASP + 0.0004 CYS + 0.0006 GLU + 0.0165 GLY + 0.0033 HIS + 0.0084 ILE + 0.0133 LEU + 0.0101 LYS + 0.0033 MET + 0.0055 PHE + 0.0081 PRO + 0.0099 SER + 0.0080 THR + 0.0040 TYR + 0.0096 VAL → BIOMASS (CH₁₉₇₅N₀₂₆₀₅O₄₈₉)
12a) 0.0104 GLN + 0.0110 ALA + 0.0050 ARG + 0.0072 ASN + 0.0082 ASP + 0.005 CYS + 0.0107 GLU + 0.0145 GLY + 0.0035 HIS + 0.0050 ILE + 0.0142 LEU + 0.0145 LYS + 0.0028 MET + 0.0072 PHE + 0.0148 PRO + 0.0267 SER + 0.0160 THR + 0.0085 TYR + 0.0189 VAL → HCG (CH₁₅₇₅N₀₂₅₆O₄₈₁)

E - Amino Acid Metabolism
13) PYR + GLU → ALA + αKG
14) SER → PYR + NH₃
15) 2GLY → SER + CO₂ + NH₃
16) CYT → PYR + NH₃
17) ASP + αKG → OAA + GLU
18) ASN → ASP + NH₃
19) HIS → GLU + 2NH₃ + CO₂
20) ARG + αKG → 2 GLU + 2NH₃ + CO₂
21) PRO → GLU
22) ILE + αKG → SUCCoA + AcCoA + GLU
23) VAL + αKG → GLU + CO₂ + SUCCoA
24) MET + SER + αKG → CYT + SUCCoA + GLU
25) THR → SUCCoA + NH₃
26) PHE → TYR
27) TYR + αKG → GLU + FUM + 2 AcCoA + CO₂
28) LYS + 2 αKG → 2 GLU + 2 CO₂ + 2 AcCoA
29) LEU + αKG → GLU + 3 AcCoA

Figure 2.5 Equations describing the reaction network.

The three letter symbols for each amino acid are used, while all other abbreviations are detailed at the beginning of the text.
Figure 2.6 The stoichiometric matrix.

The stoichiometry of the reaction network was used to form a matrix of composed of 32 reactions and 30 compounds. The terms shown in blue are those that form a part of the TCA cycle.
2.13 Flow cytometric analysis of cell cycle distribution

2.13.1 Fixation of cells

A sample of 2 x 10⁶ cells was centrifuged for 3 minutes, at 100 g and room temperature. The supernatant was carefully removed, and the cells were resuspended in 100 µl of ice-cold PBS. The cells were fixated by addition of 500 µl of ice-cold 70% ethanol. A 30 minute incubation at 4°C was necessary for completion of the fixation process. Alternatively, the cell pellets could be immediately stored at -20°C for several weeks before analysis.

2.13.2 Propidium iodide staining

On day of analysis the cells were re-pelleted and carefully washed once with PBS. The cells were incubated with RNase A (50 µg/ml) for 20 minutes at 37°C to destroy RNA before analysis. A second washing step with PBS was followed by incubation with propidium iodide (50 µg/ml) for 15 minutes at room temperature. Excess propidium iodide was removed with a final PBS wash. Unstained samples were analysed similarly.

2.13.3 Flow cytometer set up

The analysis was carried out in a 96-well plate format with a BD FACSCanto II flow cytometer (Beckton Dickinson, New Jersey, USA). Excitation was achieved with the argon laser (wavelength of 488nm) and emission was collected on a linear scale in the PE channel (with a 620nm bandpass interference filter). The sample flow rate was kept at 0.5 µl/second to discourage analysis of cell doublets. A dot plot of forward scatter versus side scatter was formed to give an initial indication of the spread of the cell population (Figure 2.7 A). Additionally, a PE-W versus PE-A dot plot was formed and used to gate single cells from aggregates, since the aggregates have larger pulse width values (i.e. pulse width versus pulse area, Figure 2.7 B). This gating was re-plotted in a histogram with PE-A versus count in order to gate the single cell populations of G1, S and G2/M (Figure 2.7 C). The amount of fluorescence is proportional to the DNA content of each cell, and this content varies during the cell cycle phases. Cells in G0/G1 are diploid and have DNA content of 2N; while in the G2/M phase this value is doubled. Cells in the S phase have DNA content between 2N and 4N. Polyploid cells have greater than 4N DNA content, while apoptotic cells have less than 2N DNA content. At least 30,000 cells were analysed per sample. All data were recorded and analysed using the FACSDiva Software (Becton Dickinson).
Figure 2.7 Representative dot plots and histograms for cell cycle distribution analysis.

Part A shows a dot plot illustrating the scatter of size (forward scatter) and granularity (side scatter) in the cell population. The green area shows the single cell population that is gated in B (termed P8). B shows the accurate gating of single cells based on propidium iodide incorporation, with the ungated areas containing cell debris and aggregates. The histogram in part C illustrates the gating of cell populations based on cell cycle phase; cells in G1 are gated in P9, S phase in P10 and G2/M as P11.
2.14 RP-HPLC analysis of intracellular nucleotides and nucleotide sugars

Analyses of intracellular nucleotides and nucleotide sugars were carried out by modification of the method of Kochanowski et al (Kochanowski et al., 2006).

2.14.1 Extraction of intracellular nucleotides and nucleotide sugars

3 x 10^6 cells were removed from the bioreactor and immediately centrifugated for 5 minutes at 100 g and 4°C. The supernatant was discarded and 185 μl of cold 0.5 M perchloric acid was added to the pellet. The sample was mixed vigorously and incubated for 10 min on ice before centrifugation for 5 minutes at 10,000 g and 4°C. The supernatants containing the soluble molecules were neutralised with 42 μl of cold 2.5 M KOH in 1.5 M K₂HPO₄ and incubated on ice for 2 minutes. In order to remove potassium perchlorate precipitate, the neutralised samples were again centrifuged for 5 minutes at 10,000g and 4°C and filtered with 0.2 μm filters (Millex GV units from Millipore.) The samples were stored at -80°C prior to analysis.

2.14.2 Ion-pair RP-HPLC analysis

Two buffers were used for analysis – buffer A was composed of 100 mM potassium phosphate plus 8 mM tetrabutylammonium bisulfate hydrogen sulfate (pH 6.5). Buffer B was composed of 70% buffer A and 30% methanol. The buffers were filtered through 0.22 μm filters before use. Analyses were carried out on an Agilent 1100 Series HPLC system composed of an autosampler, online vacuum degasser, binary pump and UV detector.

Separation was carried out with a Supelcosil LC-18-DB column (15 cm x 4.6 mm x 3 μm, Supelco) at 40°C. The elution gradient was as follows: 0% B for 17 minutes (0.5 ml/min), 0-30% B for 27 minutes (1 ml/min), 30% B for 5 minutes (1 ml/min) and 0% B for 20 minutes (0.5 ml/min). UV detection of substances was carried out at a wavelength of 254nm, and the standard injection volume was 50 μl. Integration was carried out with Chemstation software from Agilent Technologies.

A standard mix of fourteen compounds (250 pmoles of each) was made to identify key compounds within the CHO extracts. The compounds used were as follows - ADP, AMP, ATP, CMP-NeuAc, CTP, GDP, GDP-Man, GTP, UDP, UDP-Gal, UDP-GalNAc, UDP-Glc, UDP-GlcNAc and UTP. Resolution of all 14 compounds was achieved under the separation conditions noted (Figure 2.8). Identification of each peak was achieved by spiking the standard mix with increased amounts of each compound (data not shown, but
results noted in Figure 2.8). In order to calibrate the peak area with amount, peak areas integration was performed using the mixture of standards from a range of 50 pmoles to 2500 pmoles. A linear response was detected in this range, with $r^2$ values exceeding 0.993 for all compounds (Figure 2.9). A standard mix was run between every tenth sample to detect any shifts in retention time. Table 2.3 illustrates the small deviations in retention time noted during the experimental time period.

CHO extracts were separated under the same conditions, and differences between the retention times of peaks were noted. Consequently, the peaks were re-identified via spiking experiments, with the exception of ADP and ATP. These two compounds had similar retention times in both pools, so re-identification was deemed to be unnecessary. A decrease in retention time can be observed in the elution profile (Figure 2.8) and the extent of this shift is explored in Table 2.3. CMP-NeuAc was not readily identified in CHO extracts due to the failure of the spiking experiments in this instance. Co-elution of GDP-Man and UDP-Gluc was observed for CHO extracts under these conditions, so analysis of the quantity of this pool was carried out via merging the linearity response of both compounds determined in the standard mixture. Results for this pool are therefore subject to error, and only changes in relative amounts should be used.

2.15 Immunoaffinity purification of HCG from culture supernatant

A column of 1 cm diameter and 10 cm length (Omnifit) was carefully filled with NHS-sepharose 4 FF resin conjugated to the monoclonal antibody anti β-hCG 119A, which was a gift from MSD (Oss, The Netherlands). A bed height of 6.5 cm was achieved, and the column was attached to a Watson Marlow 503S pump using various adapters.

All purification steps were carried out at 2-8°C. Buffers were filtered through a 0.22 μm filter (Sartorius), degassed and stored at 2-8°C. All steps were carried out at 2 ml/min, with the exception of loading, which proceeded at the reduced flow rate of 1 ml/min. Methionine was included in the buffers due to its anti-oxidant properties.

On the day of harvest, a sufficient volume of culture supernatant was made cell-free by centrifuging for 5 minutes at 1000 g, followed by filtration through a 1.2 μm filter. This supernatant was stored at -80°C. The cell-free culture supernatant was thawed overnight at room temperature. The following morning, it was mixed thoroughly to ensure homogeneity.
Figure 2.8 Representative RP-HPLC elution profiles showing the separation of nucleotides and sugar nucleotides in a standard mix (red) and CHO extract (blue).

Both samples were separated according to the conditions noted in Section 2.14. Part A illustrates the elution profile over 62 minutes and the close elution times of UTP, GTP, ADP and ATP in both pools can be seen. B shows the detailed elution profile from 7 to 16 minutes. All 14 compounds were separated in the standard mix and identified with spiking experiments. Only 12 peaks were observed for the CHO extract, since CMP-NeuAc could not be detected, while UDP-Gluc and GDP-Man co-eluted under these separation conditions. The retention time and identification of all compounds in CHO extracts, with the exception of ADP and ATP, were carried out via spiking experiments. A decrease in retention time was observed for the CHO samples (see Table 2.3 for more detail).
Table 2.9 Calibration of peak area and amount of nucleotides and sugar nucleotides in a standard mix.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Equation</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDP-Gal</td>
<td>( y = 0.8989x + 12.218  )</td>
<td>0.9998</td>
</tr>
<tr>
<td>UDP-Gal / GDP-Man</td>
<td>( y = 1.163x - 1.225 )</td>
<td>1</td>
</tr>
<tr>
<td>UDP-GalNAc</td>
<td>( y = 1.0604x + 5.8268 )</td>
<td>0.9984</td>
</tr>
<tr>
<td>UDP-GlcNAc</td>
<td>( y = 0.882x + 6.9241 )</td>
<td>0.9995</td>
</tr>
<tr>
<td>UDP</td>
<td>( y = 0.775x - 33.738 )</td>
<td>0.9986</td>
</tr>
<tr>
<td>GDP</td>
<td>( y = 1.3393x - 37.794 )</td>
<td>0.9998</td>
</tr>
<tr>
<td>CTP</td>
<td>( y = 0.8697x - 10.829 )</td>
<td>0.9993</td>
</tr>
<tr>
<td>AMP</td>
<td>( y = 1.8529x + 22.415 )</td>
<td>0.9991</td>
</tr>
<tr>
<td>UTP</td>
<td>( y = 0.5655x - 28.346 )</td>
<td>0.9938</td>
</tr>
<tr>
<td>GTP</td>
<td>( y = 0.9533x - 7.9857 )</td>
<td>0.9958</td>
</tr>
<tr>
<td>ADP</td>
<td>( y = 1.2976x - 34.376 )</td>
<td>0.9985</td>
</tr>
<tr>
<td>ATP</td>
<td>( y = 0.8012x - 7.5826 )</td>
<td>0.9999</td>
</tr>
</tbody>
</table>

With a standard mix of known compounds, amounts ranging from 50 pmol to 2500 pmol were injected to determine the corresponding peak area. The linearity of the relationship was determined by plotting (A) and analysing the trend using first order linear regression (B). The mathematical relationship between the two values is given in the equation column, and the fidelity of this relationship for each compound is given in the R² column. The equations were used to determine the quantity of each compound in CHO extracts. Values for CMP-NeuAc are not quoted since it was not detected in CHO extracts. UDP-Gluc and GDP-Man were found to co-elute in CHO extracts, so an approximate value was determined by merging the results obtained for the individual compounds.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Rt (min)</th>
<th>Compound</th>
<th>Rt (min)</th>
<th>Δ Rt (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard mix</td>
<td></td>
<td>CHO extract</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMP-NeuAc</td>
<td>7.85 ± 0.009</td>
<td>UDP-Gal</td>
<td>9.50 ± 0.006</td>
<td>0.25</td>
</tr>
<tr>
<td>UDP-Gal</td>
<td>9.75 ± 0.014</td>
<td>UDP-Gal / GDP-Man</td>
<td>9.97 ± 0.006</td>
<td>n/a</td>
</tr>
<tr>
<td>UDP-Gluc</td>
<td>10.26 ± 0.016</td>
<td>UDP-Gluc / GDP-Man</td>
<td>9.97 ± 0.006</td>
<td>n/a</td>
</tr>
<tr>
<td>GDP-Man</td>
<td>10.49 ± 0.012</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UDP-GalNAc</td>
<td>11.22 ± 0.018</td>
<td>UDP-GalNAc</td>
<td>10.94 ± 0.009</td>
<td>0.28</td>
</tr>
<tr>
<td>UDP-GlcNAc</td>
<td>11.54 ± 0.020</td>
<td>UDP-GlcNAc</td>
<td>11.27 ± 0.008</td>
<td>0.27</td>
</tr>
<tr>
<td>UDP</td>
<td>12.58 ± 0.026</td>
<td>UDP</td>
<td>12.49 ± 0.010</td>
<td>0.09</td>
</tr>
<tr>
<td>GDP</td>
<td>13.71 ± 0.023</td>
<td>GDP</td>
<td>13.35 ± 0.010</td>
<td>0.36</td>
</tr>
<tr>
<td>CTP</td>
<td>14.44 ± 0.035</td>
<td>CTP</td>
<td>13.90 ± 0.014</td>
<td>0.53</td>
</tr>
<tr>
<td>AMP</td>
<td>15.06 ± 0.017</td>
<td>AMP</td>
<td>14.78 ± 0.009</td>
<td>0.28</td>
</tr>
<tr>
<td>UTP</td>
<td>23.05 ± 0.053</td>
<td>UTP</td>
<td>22.38 ± 0.020</td>
<td>0.67</td>
</tr>
<tr>
<td>GTP</td>
<td>23.42 ± 0.046</td>
<td>GTP</td>
<td>22.82 ± 0.017</td>
<td>0.60</td>
</tr>
<tr>
<td>ADP</td>
<td>28.44 ± 0.032</td>
<td>ADP</td>
<td>28.12 ± 0.009</td>
<td>0.32</td>
</tr>
<tr>
<td>ATP</td>
<td>35.68 ± 0.032</td>
<td>ATP</td>
<td>35.30 ± 0.010</td>
<td>0.37</td>
</tr>
</tbody>
</table>

Table 2.3 Comparison of the retention times of compounds in the standard mix and CHO extracts.

The mean retention time (Rt) for the compounds in the standard mix was determined through the use of data from spiking experiments, linearity experiments, and standard mix runs during analyses of CHO extracts. Values shown are the mean of 38 samples ± the SEM. Only minor deviations in retention times were seen for all compounds within the standard mix, with the maximal change in retention time noted for UTP at 0.05 min. Mean retention times for CHO extracts were determined via use of data from spiking experiments and experimental samples (61 values in total, mean ± SEM). CMP-NeuAc was not detected, while UDP-Gluc and GDP-Man co-eluted in CHO extracts. Within CHO extracts, the retention times between samples varies by a small amount, with a maximum shift also seen in UTP at 0.02 min. A decrease in retention time of all compounds and pools of compounds was seen with CHO extracts when compared to the standard mix. This decrease ranged from 0.09 min in the case of UDP, to 0.67 min for UTP. Compounds were re-identified in CHO extracts with the use of spiking experiments.
and diluted 1:1 with PBS-methionine buffer (4.1 mM Na₂HPO₄.2H₂O, 1.76 mM KH₂PO₄, 0.13 M NaCl, 0.1 mg/ml methionine, pH 7.2). The column was equilibrated using at least 50 ml of PBS-methionine before loading of the 1:1 dilution of culture medium onto the column. Two washing steps were then performed; firstly using 50 ml of wash 1 buffer (2 M NaCl, 4.1 mM Na₂HPO₄.2H₂O, 1.76 mM KH₂PO₄, 0.1 mg/ml methionine, pH 7.2), and then with another 50 ml of PBS-methionine. Elution of HCG from the column was achieved using 45 ml of 10 mM ammonium acetate buffer at pH 2.7 (pH adjustment with acetic acid). This elution fraction was immediately neutralised to a pH between 7 and 8, using 25% (v/v) ammonia. The column was regenerated at this stage during every tenth purification run using 10 ml of 1.65 M citric acid buffer (pH to 2.0 with sodium citrate). The column was neutralised using 50 ml of 1 M Tris, pH 8.0 and stored at 2-8°C after flowing 50 ml of PBS (6.5 mM phosphate, 0.13 M NaCl, pH 7.2) in 20% ethanol through the system. A representative plot of pH and absorbance during this process is shown in Figure 2.10, while the absorbance spectrum of purified HCG is shown in Figure 2.11.

The volume of neutralised elution fraction was reduced using Amicon Ultra-15 units (Millipore) with a molecular weight limit of 3 kDa. The elution fraction was split into four parts, and each one was applied to the Amicon unit and centrifuged for 40 minutes at 4000 g, 4°C in a Heraeus cryofuge 8500i centrifuge. After this time, the 25-fold concentrated sample was recovered using a Gilson pipette and made up to a total volume of 2.5ml with water.

The purified HCG protein was then desalted using PD-10 columns (GE Healthcare). One column was equilibrated with 25 ml of water. The protein was loaded onto the column and allowed to be absorbed by the resin, while the flow-through was discarded. The sample was then eluted in 3.5 ml of water, and divided into two 1.75 ml aliquots. These aliquots were then made to a total volume of 2.5 ml. The column was rinsed with 25 ml of water, one of the aliquots was allowed to absorb to the resin and the flow-through was discarded. The first desalted aliquot was then eluted in 3.5 ml of water. The same column was rinsed with another 15 ml of water, and the procedure was repeated for the second 2.5 ml aliquot. The two desalted aliquots were then pooled together, frozen in a dry ice - ethanol bath and lyophilised overnight. The sample was re-hydrated in 1 ml of water and a BioRad protein assay was performed. The HCG was aliquoted into distinct amounts for subsequent analysis and lyophilised once more. The efficiency of the purification process is shown in Table 2.4.
The vertical dashed lines indicate the time points at which the composition of buffer flowing through the system was changed, dividing the purification into the following stages – equilibration, loading, wash 1, wash 2, elution, regeneration, neutralisation and rinsing for storage (left to right). In this case, 50 ml of culture supernatant was diluted 1:1 with PBS - methionine buffer and loaded onto the column. A total of 101 fractions were collected in regular volumes. After the initial equilibration of the column, the supernatant dilution was loaded. The absorbance fluctuates around 2.7 at this time, due to non-specific host cell proteins eluting from the column while HCG was associated via its β subunit to the antibody immobilised on the resin. The subsequent two wash steps rinse away any remaining substances from the supernatant that are still present in the column. The low pH of the elution buffer disrupts the attraction between HCG and antibody causing elution of the protein, which is illustrated by the increase in absorbance at this time. These elution fractions were pooled and neutralised for subsequent analysis. The column was regenerated, before the pH was returned to physiological levels with the neutralisation buffer. To prevent microbial growth, the column was stored in a PBS-ethanol buffer.
HCG was purified and desalted according to instructions in Section 2.11. The protein content was assayed and determined to be 880 µg/ml. The absorbance of this 1 ml pool between wavelengths of 200 to 800 nm was carried out with a Varian Cary Model 500 spectrophotometer. A broad peak in absorbance was observed between 250 and 300 nm, with its maximum value at 277 nm (inset). This indicates that the purification process was monitored at this wavelength in order to specifically detect HCG.
<table>
<thead>
<tr>
<th></th>
<th>Volume (ml)</th>
<th>Content (IU/ml)</th>
<th>Total Content (IU)</th>
<th>Total Content (µg)</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-load</td>
<td>200</td>
<td>165</td>
<td>33000</td>
<td>2178.0</td>
<td>-</td>
</tr>
<tr>
<td>Load</td>
<td>200</td>
<td>&lt; 17</td>
<td>&lt; 3400</td>
<td>224.4</td>
<td>&lt; 10.3</td>
</tr>
<tr>
<td>Wash 1</td>
<td>50</td>
<td>14</td>
<td>700</td>
<td>46.2</td>
<td>2.1</td>
</tr>
<tr>
<td>Wash 2</td>
<td>50</td>
<td>&lt; 2.1</td>
<td>&lt; 105</td>
<td>6.9</td>
<td>&lt; 0.3</td>
</tr>
<tr>
<td>Elution</td>
<td>45</td>
<td>660</td>
<td>29700</td>
<td>1960.2</td>
<td>90.0</td>
</tr>
<tr>
<td>Neutralisation</td>
<td>50</td>
<td>&lt; 2.1</td>
<td>&lt; 105</td>
<td>6.9</td>
<td>&lt; 0.3</td>
</tr>
<tr>
<td>Rinse</td>
<td>50</td>
<td>&lt; 2.1</td>
<td>&lt; 105</td>
<td>6.9</td>
<td>&lt; 0.3</td>
</tr>
</tbody>
</table>

Table 2.4 The efficiency of HCG purification.

100 ml of culture supernatant was diluted with 100 ml of PBS-methionine buffer (pre-load) and purification proceeded with the collection of pools of flow-through at each stage. Extracellular HCG content was assayed by the quality unit in MSD, Oss, the Netherlands. The total content of HCG was converted from IU to µg by use of the relationship 15 IU = 1 µg. This purification resulted in the recovery of 90% of the HCG at the elution stage. However, the ~ 10% loss at loading indicates that the capacity of the resin was exceeded. This suggests that the actual efficiency may be closer to 100% if the entire capacity of the resin was not used. Small losses occur in the washing, neutralisation and rinsing stages. The reducing SDS-PAGE pattern of HCG is shown in Figure 2.16.
2.16 BioRad protein assay
The BioRad protein assay is based on the Bradford assay and is based on the colour change of Coomassie Brilliant Blue G-250 dye in the presence of varying concentrations of protein. A stock of 0.1 mg/ml of bovine serum albumin stock (BSA) was prepared and diluted with MilliQ water and BioRad reagent according to Figure 2.12. This resulted in a range of concentrations from 0 to 20 µg/ml for the microassay procedure. BioRad reagent concentrate was the last component to be added to the mixture. Samples of unknown protein concentration were diluted appropriately to place them within the range of the assay.

The samples were mixed and left at room temperature for 30 minutes. After this time, the absorbance was read at 595 nm. A curve of absorbance at 595 nm and BSA concentration was plotted, and the data was analysed using first order linear regression, seen in Figure 2.12. The protein content of unknown samples was determined by use of the equation associated with the linear regression.

2.17 Determination of HCG content by ELISA
The following 10x stock buffers were prepared and stored at 2-8°C for a maximum of six months before use. Tris buffer was composed of 2 M Tris and 2 M NaCl. The pH was initially adjusted to pH 7.4 with concentrated HCl, and after overnight storage the pH was adjusted once more to 7.1. PBS buffer was composed of 0.36 M K$_2$HPO$_4$ and 1.54 M NaCl. The pH of this solution was adjusted to 7.0 with the use of another buffer – 0.4 M NaH$_2$PO$_4$ and 1.54 M NaCl. All other buffers were prepared freshly. All antibody-specific reagents were kindly provided by MSD, Oss, the Netherlands (including pre-coated plates, standard HCG, reference HCG and HRP-labelled conjugate). The plates were coated with a monoclonal antibody directed against the β subunit, which was termed 293A, at a concentration of 20 µg/ml. The conjugate was a monoclonal anti-αHCG-HRP, termed 116C. Samples of unknown HCG content were diluted appropriately to place them within the range of the assay. The reference HCG series was created in order to monitor the accuracy of the standard series.

All samples were diluted (in 1x PBS buffer supplemented with 2 g/L BSA, termed PBS-BSA buffer) and pipetted firstly into the wells of an uncoated 96 well plate. 100 µl of solution from each well was transferred with a multi-channel pipette to a pre-coated plate.
Figure 2.12 The BioRad protein assay.

Part A shows the quantities of various solutions that are required to form each data point of the standard curve. The BSA stock is at a concentration of 0.1 mg/ml. The resulting absorbance at 595 nm is plotted against the known concentration to give the standard curve, a representative plot of which is shown in B.
This was incubated in the dark for 15 minutes at room temperature, before the contents of the wells were discarded for washing steps. This involved rinsing three times with PBS-BSA buffer and three times with MilliQ water. All remaining liquid drops were removed before the plate was incubated for another 15 minutes at room temperature with 90 µl of conjugate solution per well (diluted as per analysis instructions). After this time, the plate was again washed - thrice with Tris-Tween buffer (1x Tris buffer supplemented with Tween-20 at 0.5 ml/L), and thrice with MilliQ water. The plate was again dried sufficiently before incubation in the dark with 90 µl of TMB substrate solution per well. (This solution was made by dissolving one phosphate citrate / ureum peroxide tablet in 100 ml MilliQ water. Two 3,3',5,5' tetra methyl benzidine dihydrochloride tablets were dissolved in 10 ml of this solution just before use.) After sufficient colour development was achieved, usually within 8 minutes, the reaction was stopped with the addition of 90 µl of sulfuric acid (2M) into each well. The plate was left under agitation for at least five minutes before the absorbance at 450 nm was measured. A standard curve of HCG content versus absorbance was constructed. Logarithmic regression of this trend allowed the determination of the extracellular HCG content of unknown samples (Figure 2.13).

2.18 Desalting Ovitrelle
Recombinantly produced HCG (Ovitrelle) was used as a standard for comparison to CHO derived HCG. Microcon centrifugal filter devices with a molecular weight cut-off of 10 kDa (YM-10) were obtained from Millipore. A sample of Ovitrelle was carefully transferred from the syringe to the sample reservoir of the filter device and centrifuged for 10 minutes at 13,000 g and 4°C. Placing the sample reservoir upside down in another specialised Eppendorf tube and subjecting it to centrifugation for 3 minutes at 1,000 g subsequently recovered the sample. The resulting filtrate was made up to a volume of 500 µl with MilliQ water, and the sample was subject one further round of desalting. The protein content of the final desalted sample was determined by BioRad assay. Aliquots of 40 µg were lyophilised for use in isoelectric focusing.

2.19 Isoelectric focusing
Isoelectric focusing was performed on a Multiphor II Electrophoresis System with an EPS3501XL powerpack and the temperature was controlled using a MultiTemp waterbath (all from GE Healthcare).
A standard curve of HCG content from 0.5 to 3 IU/ml is created and the absorbance at 450 nm is measured. The resulting logarithmic trend is used to determine the HCG content of unknown samples. The inter- and intra-assay coefficient of variation was 6%, and 16%, respectively.
Approximately 3 ml of kerosene was applied to the electrophoresis plate that had been precooled to 5°C. A precast Servalyt Precote pI 3-10 gel was orientated correctly and carefully laid down to ensure no air bubbles were trapped underneath it. One of the electrode wicks was wetted in anode fluid 3 and placed on the red side of the gel. Another was soaked with cathode fluid 10 and carefully placed to the blue/black side of the gel. The sample application strip was placed 1 cm away from the cathode wick. The system was connected to the power supply and prefocused for 30 minutes at 2000 V, 7 mA and 12 W.

After this time, lyophilised samples were reconstituted in 10 μl of freshly made 3 M urea. The electrophoresis system was disconnected and the samples were then applied to the application strip. Markers of pI in the range 3 – 10 (Serva or GE) were also loaded directly onto the gel. The gel was focused at 2000 V, 7 mA and 12 W for 7000 Vh. This took an average of 4 hours.

After focusing, excess kerosene was removed from the back of the gel, and it was placed in fixation buffer (20% trichloroacetic acid, w/v) for 40 minutes, at room temperature with agitation. The gel was washed twice for 1 minute in destain (3% orthophosphoric acid) and then stained for 15 minutes. The stain was made directly before use, and was composed of equal volumes of 20% orthophosphoric acid solution and Serva Violet 17 solution (2 g/L). The gel was destained with three changes of buffer every 8 minutes, and washed with MilliQ water twice for 5 minutes. The gel was removed from aqueous solution and scanned for densitometry analysis of the average isoelectric point (pI) with a GS300 calibrated densitometer (BioRad), or a desktop scanner. The gel could be air-dried overnight in the dark, if necessary.

2.19.1 Densitometry analysis of average pI

The intensity and migration distance (from the cathode side of the gel) of the bands were determined using Quantity One or LabWorks software (BioRad and UVP, respectively). The intensity of the bands was corrected for background, and the relative intensity of each band per lane was determined.

A graph of the migration distance of the marker bands (Rf) and corresponding pI values was constructed, as shown in Figure 2.14. A second order polynomial trendline was used to analyse the marker migration, and the resulting equation was used to calculate the pI.
Figure 2.14 Typical standard curve of pI and migration distance of pI markers.

Two pI markers from different manufacturers were used for this work. A plot of the migration distance (Rf) versus isoelectric point (pI) of each band is shown for both markers (GE in blue and Serva in red). Second order polynomial regression was found to give a good fit, with $r^2$ values above 0.98 for both markers used.
values of the sample bands. The average pi for a particular sample was calculated according to the formula given below.

$$\text{Average } pI = \frac{(r_1 \cdot p_1) + (r_2 \cdot p_2) + \ldots + (r_n \cdot p_n)}{100}$$

$r =$ relative intensity per band
$p =$ pi value of the band

2.20 Analysis of N-linked glycan structures of HCG

This was carried out in NIBRT, with minor technical assistance. Trivial alterations to the method of Royle et al were required (Royle et al., 2008). An overview of the process is given in Figure 2.15.

2.20.1 Denaturing SDS – PAGE

Discontinuous denaturing polyacrylamide gel electrophoresis was performed using the NuPAGE system from Invitrogen in order to denature the glycoprotein.

Purified, lyophilised protein samples were prepared for electrophoresis by resuspending in 6.5 μl MilliQ water, 2.5 μl LDS sample buffer (4x stock) and 1 μl reducing reagent (10x stock) before heating at 70°C for 10 minutes.

One litre of MES running buffer was prepared by diluting the stock 20x solution appropriately. For the inner chamber of the electrophoresis system, 500 μl of NuPAGE antioxidant was added to 200 ml of the 1x running buffer, while the other 800 ml was retained for the outer chamber. Precast BisTris gel cassettes (12%) were removed from the pouches and rinsed gently with MilliQ water. The white tape was removed and the gel was correctly orientated in the gel box and secured. A small volume of buffer was placed in the inner chamber to check for a tight seal before both chambers were filled with the appropriate buffer. The well comb was carefully removed followed by rinsing of the wells with buffer. Samples and prestained protein markers were loaded onto the gel using a p20 Gilson pipette. The gel was run at 150 V until the dye front reached the end of the gel, which took approximately 90 minutes.
The bands of HCG from SDS-PAGE were cut out and incubated with PNGase F overnight in order to release the glycans from the protein (1). The released glycans were eluted and dried down (2) before labelling with fluorescent 2-aminobenzamide (3). The labelled glycans were eluted, cleaned up and dried (4) followed by NP-HPLC profiling of the entire glycan pool (5). Preliminary assignments for glycan structures were made with the use of GlycoBase (6). A series of exoglycosidase digestions (7) was carried out alongside WAX fractionation and collection of the glycan pool into differentially charged species (8), followed by sialidase digestion (ABS, 9) and NP-HPLC profiling (10) in order to complete the process of final structural assignments (11). Modified from (Campbell et al., 2008).
After electrophoresis, the gel was carefully removed from the cassette and stained for one hour in InstantBlue solution. The gel was rinsed three times in MilliQ water before scanning migration patterns with a GS300 calibrated densitometer (Figure 2.16). The protein bands were cut out of the gel with a scalpel and sliced into small pieces of approximately 1 mm³ and stored at -20°C.

2.20.2 N-Glycan release

The gel pieces were transferred to a 96-well filter plate (Whatman protein precipitation plate) and washed with 1 ml acetonitrile with shaking on a plate mixer (Sarstedt, Leicester, UK) for 10 min. After this time, the liquid was removed with the use of a vacuum manifold for 96-well plates. This procedure was repeated with 1 ml of PNGase F buffer (20 mM NaHCO₃, pH 7.0), 1 ml of acetonitrile, 1 ml of PNGase F buffer and 1 ml of acetonitrile. The filter plate was placed over a new collection block (2 ml deep polypropylene 96-well blocks, Porvair, Surrey, UK). The glycans were released by overnight incubation with a 100 mU/ml solution of PNGase F (peptide N-glycosidase F) at 37°C. A sufficient volume of enzyme solution was added to ensure all gel pieces had reswollen (approximately 50 μl). After five minutes, the gel pieces were then covered with an additional 50 μl of PNGase F buffer and sealed with adhesive film (SealPlate, Web Scientific, Crewe, UK) before incubation.

2.20.3 N-Glycan extraction

A volume of 200 μl of MilliQ water was added to the gel pieces, and the plate was placed on the shaker for 10 minutes. After this time, these released glycans were vacuumed to the collection block and retained. The procedure was repeated with 200 μl of MilliQ water, 200 μl of MilliQ water, 200 μl of acetonitrile, 200 μl of MilliQ water and 200 μl of acetonitrile. The total volume was then dried down in a vacuum centrifuge. The released glycans were converted to aldoses by reaction with 20 μl of 1% formic acid at room temperature for 40 minutes. The glycans were dried down to remove the formic acid, before resuspending in a known volume of MilliQ water. At this point, a portion of the unlabelled glycans could also be retained for further analysis at a different time point. Samples to be labelled at this time with 2-AB were dried down once more.
Denaturing SDS-PAGE of HCG was carried out according to Section 2.20.1. M denotes protein marker and numbers represent the molecular weight of each band in kDa. A plot of migration distance versus molecular weight was constructed for the protein marker, and linear regression analysis was carried out (data not shown, $r^2 = 0.98$). The molecular weights of the three HCG bands were calculated using this analysis. The molecular weight of the glycosylated $\alpha$ and $\beta$ subunits of HCG are quoted in literature as 14.9 kDa and 23 kDa, respectively (de Medeiros and Norman, 2009). However, glycosylated proteins appear to have larger molecular weights in SDS-PAGE due to the water molecules that associate with the glycans in this procedure. As a result, the $\alpha$ subunit is found at a molecular weight of 18 kDa and the $\beta$ subunit is seen to separate into two bands at 28 and 33 kDa, most likely due to changes in glycosylation site occupancy. All three bands were cut out and merged for analysis of $N$-linked glycan structures.
2.20.4 2-AB Labelling and cleanup

A volume of 5 μl of 2-AB (2-aminobenzamide) labelling solution was added to the tube containing the dry glycans. The tube was vortexed and spun down before incubation at 65°C for 30 minutes. After this time, the samples were re-vortexed and spun before re-incubating for another 2.5 hours. During this time, 1 cm² pieces of 3MM Whatman chromatography paper were prepared by washing in 3 changes of MilliQ water and drying at 65°C for at least an hour. The paper was then folded in four and placed into a Whatman protein precipitation plate (pre-washed with 200 μl acetonitrile vacuumed to waste, repeated with 200 μl water.) The 2-AB sample was applied to the paper and left to dry for 15 minutes at room temperature. The free 2-AB was washed away with 5 acetonitrile washing steps as follows - addition of 1.5 ml acetonitrile to each well, agitation on a shaker for 15 minutes and vacuum to waste. The glycans were eluted by firstly placing the Whatman plate on top of a new collection block and addition of 900 μl MilliQ water with shaking for 30 minutes. This liquid was vacuumed to the collection block before the step was repeated once more. This volume was dried overnight in a vacuum centrifuge and resuspended in 100 μl MilliQ water the following day.

2.20.5 NP-HPLC Profiling of 2-AB labelled N-glycans

The 2-AB labelled glycans were separated with a Waters 2695 separations module with a 2475 fluorescence detector. The detector was set to maximum sensitivity, with excitation and emission wavelengths of 330 and 420 nm respectively. A TSKgel Amide-80 column was used (3 μm x 250 mm x 4.6 mm, Anachem). Solvent A was composed of 50 mM ammonium formate, pH 4.4 while solvent B was acetonitrile. Samples were made up in 80% acetonitrile. The elution gradient was as follows: a linear gradient of 35 to 47% solvent A over 48 minutes, followed by 1 minute at 47% to 100% A, returning to 35% A over 1 minute and then finishing with 35% A for 6 minutes, all at a flow rate of 0.48 ml/min, and 30°C. All samples were prepared in 80% acetonitrile. In order to condition the column before use, a water blank was used to initiate a run (20% v/v in acetonitrile). A dextran ladder was run every 24 hours in order to calibrate and assign GU values for resulting peaks.
2.20.6 Exoglycosidase digestions

In order to identify the sequence, monosaccharide type and linkage of the sugar residues, exoglycosidase digestions were carried out. The enzymes used were as follows; Sialidase A (ABS), Sialidase S (NAN1), bovine kidney fucosidase (BKF), bovine testis galactosidase (BTG), Hexase-I (N-acetylglucosaminidase, GUH) and Jack bean mannosidase (JBM). The specificity of the enzymes used is detailed in Table 2.5. A pool of representative samples was made and aliquots were taken for digestion analysis (and WAX HPLC, Section 2.20.7). Aliquots of 20 µl were dried down in 200 µl microcentrifuge tubes, before the required enzyme array was added to each tube, as described in Table 2.6. The buffer used was 500 mM sodium acetate, pH 5.5. The samples were mixed well before incubation at 37°C overnight. After the overnight incubation, half of the ABS, BKF, BTG, GUH digest was further treated with JBM to digest to the 2-AB core (this digest was set up initially with double the amount of glycans to accommodate for this). This involved addition of 4 µl of enzyme, 4 µl of MilliQ water and 2 µl of JBM buffer (100 mM sodium acetate, 2 mM Zn^{2+}, pH 5.0). This digest was incubated at 37°C for a further 24 hours.

The enzymes were removed from the mixture with use of a Nanosep centrifugal device (Pall Life Sciences, MI, USA), which had a 10 kDa molecular weight cut-off. The membrane was pre-washed with 200 µl MilliQ water by centrifuging at 14,000 g for 10 minutes. The flow through was discarded, and the digested sample was applied to the membrane and centrifuged for 2 minutes at 14,000 g. The digestion tube was washed out with 20 µl of MilliQ water, which was then applied to the membrane, and the centrifugation process was repeated. The membrane was rinsed with a final 100 µl of water at 14,000g for 2 minutes. The samples were dried down and resuspended in 20 µl water, made to 100 µl with acetonitrile before NP-HPLC analysis. The resulting digest profiles are shown in Figures 2.18, 2.19 and 2.20.

2.20.7 WAX – HPLC fractionation

In order to confirm the structures present in the undigested N-glycan pools, additional weak anion exchange HPLC analysis was carried out. Separation was carried out using a 2695 separations module with a 474-fluorescence detector (Waters) and a Prozyme GlycoSep C polymeric DEAE anion exchange column (10 µm x 75 mm x 7.5 mm). Solvent A was 20% acetonitrile, and solvent B was 0.1 M ammonium acetate buffer pH 7.0 in 20% v/v acetonitrile. The elution gradient was as follows: 100% A for 5 minutes, a linear gradient of 100% to 0% A over 15 minutes, a hold at 0% A for 2.5 minutes, 0 to
Table 2.5 Exoglycosidase enzymes used in this work, their source and specificity of action.

All enzymes were sourced from Prozyme (San Leandro, USA) and were used according to manufacturer’s instructions.

<table>
<thead>
<tr>
<th>Exoglycosidase</th>
<th>Specificity</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sialidase A (ABS)</td>
<td>α2-6, 3 &amp; 8 non-reducing terminal sialic acids</td>
<td>Arthrobacter ureafaciens</td>
</tr>
<tr>
<td>Sialidase S (NAN1)</td>
<td>α2-3 &amp; 8 non-reducing terminal sialic acids</td>
<td>Recombinant from <em>Streptococcus pneumoniae</em>, expressed in <em>E.coli</em></td>
</tr>
<tr>
<td>α-Fucosidase (BKF)</td>
<td>α(1-2,3,4,6) fucose</td>
<td>Bovine kidney</td>
</tr>
<tr>
<td>β-Galactosidase (BTG)</td>
<td>β(1-3,4) galactose</td>
<td>Bovine testes</td>
</tr>
<tr>
<td>Hexosaminidase (GUH)</td>
<td>βGlcNAc (not bisecting GlcNAc linked to Man)</td>
<td>Recombinant from <em>Streptococcus pneumoniae</em>, expressed in <em>E.coli</em></td>
</tr>
<tr>
<td>Mannosidase (JBM)</td>
<td>α(1-2,3,6) mannose</td>
<td>Jack bean</td>
</tr>
</tbody>
</table>

Table 2.6 The composition of the panel of digests used for HCG N-glycan structure assignment.

A total of 6 digests were set up, with the ABS, BKF, BTG, GUH digest carried out with double the amount of glycan carriers. This was done in order to carry out a JBM digest on appropriate structures and under the correct conditions (termed digest 7). The conditions of this particular digest are described in Section 2.20.6.
Figure 2.17 Legends for sugar symbols, linkage type and position.

The nomenclature of the Dublin-Oxford Glycobiology lab is in use and the GU increment per sugar are those that were determined for HCG.
Figure 2.18 Sialidase A (A) and α-fucosidase, sialidase A (B) digest pattern for HCG with accompanying structure assignment.

Digests were carried out as in Table 2.5 (digests 1 and 2 respectively), and structures were assigned with reference to GlycoBase. No differences were seen between the NAN1 and ABS digests (data not shown), indicating that all sialic acid linkages were either α2-3 or α2-8.
Figure 2.19 Sialidase A, galactosidase (A) and sialidase A, galactosidase and α-fucosidase (B) digest pattern for HCG with accompanying structure assignment.

Digests were carried out as in Table 2.5 (digests 3 and 4, respectively), and structures were assigned with reference to GlycoBase.
Figure 2.20 Sialidase A, galactosidase, α-fucosidase and hexosaminidase (A) and Sialidase A, galactosidase, α-fucosidase, hexosaminidase and mannosidase (B) digest pattern for HCG with accompanying structure assignment.

Digests were carried out as in Table 2.5 (digests 5 and 7, respectively), and structures were assigned with the reference to GlycoBase.
100% A over 0.5 minute and a final 7 minutes at 100% A. Throughput the separation, a flow rate of 0.75 ml/min and a temperature of 30°C was used. All samples were prepared in MilliQ water, and a fetuin N-glycan standard was used for calibration. The remainder of the pool of glycans was separated and collected in fractions corresponding to neutral, mono-, di-, tri-, and tetra-sialylated species (Figure 2.21). These fractions were dried down completely, resuspended in 20 µl and split into two pools. One half was digested with ABS (as described in Section 2.20.6), and the remainder was retained without digestion. All samples were then separated on NP-HPLC (Figure 2.22).

2.20.8 Assigning N-glycan structures

HPLC traces were integrated with the use of Empower software (Waters) and the GU value of each peak was determined with reference to the dextran ladder. Assigning glycan structures was carried out by use of all the digest and WAX fractionation results, and GlycoBase; an online database of glycan structures and GU values (http://glycobase.nibrt.ie:8080/database/show_glycobase.action).

For example, the largest peaks in Figure 2.18 A (which eluted at 27 and 29 minutes, with GU values of 7.09 and 7.54, respectively) were found to comprise of Gal₂GlcNAc₂Man₃ ± Fuc (or F ± A₂G₂, the fucosylated structures elute later). Part B of this same figure shows that these two structures merge to form one peak when a fucosidase is included in the digest panel (resulting in Gal₂GlcNAc₂Man₃/A₂G₂ at 7.10 GU, and a proportionally increased area.) However, neglect of the fucosidase and inclusion of a galactosidase in the digest mixture causes the two peaks to re-emerge at a lower GU value that corresponds to a loss of two galactose residues from each structure, i.e. GlcNAc₂Man₃ ± Fuc / F ± A₂ (Figure 2.19 A, structures at 19 and 21 minutes, with GU values of 5.28 and 5.75 respectively). A combination of fucosidase and galactosidase in the digest panel results in elution of one peak of GlcNAc₂Man₃ / A₂G₂ at GU 5.30 (Figure 2.19 B). Inclusion of a hexosaminidase further digests this structure down to Man₃ / M₃ with a GU of 4.32 (at 15 minutes, Figure 2.20 A). The Man₁ core / M₁ structure elutes at 9 minutes with complete digestion of the glycans with the total digest panel including a mannosidase (Figure 2.20 B). All structures were assigned in a similar manner, and the structures assigned to peaks in the undigested pool of HCG glycans are shown in Figure 2.23 and Table 2.7.
2.20.9 *Abbreviations used to identify 2-AB labelled N-glycans*

All complex N–linked glycans have 2 core GlcNAcs and 3 mannose residues, which are assumed in all structures. The number of GlcNAcs as antennae are represented by A1, A2 and A3. Galactose residues β-linked to the antennae are represented by G, and the numbers following indicate the number of galactose residues. Sialic acids are indicated by S, and again the number following the letter indicates the number of residues. Alpha linked fucose is represented by F, and a core-linked fucose is written at the beginning of a structure. For mannose structures, the number of mannose residues is indicated after an M. For example, NeuAc$_2$Gal$_2$GlcNAc$_2$Man$_3$ + Fuc would be represented as FA2G2S2. Legends for sugar symbols, linkage type and position are shown in Figure 2.17.
A fetuin standard (black) and HCG glycan pool (blue) were separated on a WAX HPLC. The fetuin standard is seen to separate into neutral, mono-, di-, tri-, and tetra-sialylated species (N, S1, S2, S3 and S4, respectively). Fractions from 2 – 9 minutes, 9 – 14 minutes, 14 – 19 minutes, 19 – 23 minutes, and 23 - 30 minutes, corresponding to F1, F2, F3, F4 and F5 within the HCG pool were collected and dried down. F3 contained a pool of di- and tri-sialylated structures. The neutral component of HCG also contains free 2-AB which elutes alongside. All fractions were split into two parts and one half was subject to ABS digestion. All undigested and digested fractions were separated on NP-HPLC to aid the process of assigning structures in the total glycan pool. (Figure 2.16 and Section 2.20.7).
The 2-AB labelled N-glycans of a HCG pool were separated by WAX-HPLC, and five fractions corresponding to the neutral, mono-, di- and tri-, tetra-sialylated regions were collected (F1, F2, F3, F4 and F5 respectively, see Figure 2.15). These pools were dried down and resuspended in 80% acetonitrile for NP-HPLC analysis. Overlaying the resulting traces illustrates the contribution of each fraction to relevant peaks. Colour scheme: neutral = black, monosialylated = green, di- and tri-sialylated = blue.
Figure 2.23 NP-HPLC trace of total glycan pool of HCG with assigned structures.
<table>
<thead>
<tr>
<th>Peak No.</th>
<th>GU value</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.89 ± 0.00</td>
<td>A1</td>
</tr>
<tr>
<td>2</td>
<td>5.32 ± 0.00</td>
<td>A2</td>
</tr>
<tr>
<td>3</td>
<td>5.80 ± 0.00</td>
<td>A1G1</td>
</tr>
<tr>
<td>4</td>
<td>6.16 ± 0.00</td>
<td>M5</td>
</tr>
<tr>
<td>5</td>
<td>6.29 ± 0.00</td>
<td>A2G1, A1G1S1</td>
</tr>
<tr>
<td>6</td>
<td>6.63 ± 0.01</td>
<td>A2G1S1</td>
</tr>
<tr>
<td>7</td>
<td>6.72 ± 0.00</td>
<td>FA2G1</td>
</tr>
<tr>
<td>8</td>
<td>7.13 ± 0.00</td>
<td>A2G2, FA2G1S1</td>
</tr>
<tr>
<td>9</td>
<td>7.53 ± 0.00</td>
<td>FA2G2, A2G2S1</td>
</tr>
<tr>
<td>10</td>
<td>8.00 ± 0.00</td>
<td>A2G2S2, FA2G2S1</td>
</tr>
<tr>
<td>11</td>
<td>8.40 ± 0.00</td>
<td>A3G2S1, FA2G2S2</td>
</tr>
<tr>
<td>12</td>
<td>8.80 ± 0.01</td>
<td>A3G2S2, A3G3S1</td>
</tr>
<tr>
<td>13</td>
<td>9.17 ± 0.01</td>
<td>FA3G3S1, A3G3S2</td>
</tr>
<tr>
<td>14</td>
<td>9.62 ± 0.01</td>
<td>A3G3S3, FA3G3S2</td>
</tr>
<tr>
<td>15</td>
<td>10.00 ± 0.01</td>
<td>FA3G3S3</td>
</tr>
</tbody>
</table>

Table 2.7. Composition of each peak of the N-glycan pool of HCG and associated GU value.
Chapter 3

Investigating the Effect of Glutamine on CHO Metabolism and HCG Glycosylation in a Batch Mode of Culturing
3.1 Introduction

Glutamine is an amino acid that is routinely supplemented to cells in culture since it is considered to be essential for growth (Eagle et al., 1956). Some cell lines use glutamine as a major energy source, by converting it to glutamate and α-ketoglutarate in order to fuel the TCA cycle. This occurs via catalysis by glutaminase and glutamate dehydrogenase, respectively and generates 27 ATP. Alternatively, glutamate can be transaminated to α-ketoglutarate by a transaminase, a reaction yielding just 9 ATP (Reitzer et al., 1979; Street et al., 1993; Neermann and Wagner, 1996; Cruz et al., 1999). Glutamine is a necessary component for all growing cells since it donates its amido group for the synthesis of purine nucleotides, pyrimidine nucleotides and nicotinamide coenzymes. However, glutamine results in the formation of ammonium via metabolic pathways and chemical degradation (Ozturk and Palsson, 1990a). Ammonium is a toxic metabolite that is usually metabolised by the enzymes of the urea cycle, located in the liver. However, cells in culture have only a small capacity to detoxify ammonium by transaminating glutamate, which conserves an extra amino group within a new amino acid, most commonly alanine, glycine or aspartate (Street et al., 1993).

Excess ammonium decreases the growth rate of cells and increases the consumption and production of key metabolites (Kurano et al., 1990; Mc Queen and Bailey, 1990; Ozturk et al., 1992; Yang and Butler, 2000a; Yang and Butler, 2000b; Yang and Butler, 2002). A reduction in N-linked sialylation and galactosylation was observed at elevated ammonium concentrations (Thorens and Vassalli, 1986; Andersen and Goochee, 1995; Rijcken et al., 1995a; Gawlitzek et al., 1998; Grammatikos et al., 1998; Gawlitzek et al., 2000; Yang and Butler, 2000b; Yang and Butler, 2000c; Yang and Butler, 2002; Gillmeister et al., 2009). The antennarity of the glycans was also affected; some studies recording a decrease (Yang and Butler, 2000c; Yang and Butler, 2002) while others detected an increase in antennarity at elevated ammonium concentrations (Gawlitzek et al., 1998; Grammatikos et al., 1998).

Since these are undesirable effects, a variety of strategies may be employed to reduce the formation of ammonium. Neglecting glutamine from the culture medium reduces the ammonium formation. However, use of a cell line that does not have an absolute requirement for glutamine and can convert glutamate to glutamine via glutamine synthetase (or by some other mechanism) is necessary so that some amount of cell growth still occurs. CHO cells are known to have a low level of endogenous glutamine synthetase activity which can usually maintain cell growth in the absence of glutamine (Zhang et al., 112)
2006). As a result, lack of glutamine supplementation was explored as a possible method for reduction of the toxic effects of ammonium.

Another possibility that is explored in this chapter involves replacement of glutamine in the cell culture medium with the dipeptide L-alanine-L-glutamine (GlutaMAX-I). A cellular peptidase hydrolyses glutamax extracellularly, before each amino acid is taken up individually (Christie and Butler, 1994). As a result, the chemical degradation of glutamine is avoided, thus reducing extracellular concentrations of ammonium. Final cell yields are increased and a reduction in the consumption rates of glucose and amino acids and production of lactate and ammonium have been observed (Christie and Butler, 1994). Additionally, glutamax has been shown to increase the galactosylation of tPA in CHO cells (Gillmeister et al., 2009). This suggests that glutamax may have a significant impact on glycosylation via reducing the negative effects of ammonium while still providing glutamine for cellular consumption.

A batch mode of cultivation was employed to test these parameters. The cells were supplied with an initial bolus of CD-CHO medium supplemented with a specific concentration of glutamine or glutamax. Cell growth proceeded without any additional feeds, and with small volumes of supernatant withdrawn in order to monitor the culture. Incubation of the shake flasks in a CO\textsubscript{2} incubator means that very limited pH control occurred. A variation in the growth rate, as well as the concentrations and rates of consumption and production of metabolites occurs under these conditions. Despite this inherent instability of culture parameters, batch cultivation still provides a good method for testing ammonium reduction strategies.

Aims

This chapter is split into two parts – the first investigated the effect of varying the concentration of glutamine and glutamax within a large concentration range on the metabolism of CHO cells and subsequent glycosylation of HCG in 250 ml shake flasks (Section 3.3). As such, this aimed to establish whether the effects of glutamine limitation and glutamine replacement in this cell line were comparable to other studies described in the literature. Major changes in glycosylation were assessed at the end of cultivation time using IEF.
The second section deals with the effect of varying glutamine within a smaller concentration range, but in a larger culture volume (0, 4 and 8 mM glutamine in 500 ml shake flasks). This allowed the investigation of minor changes to metabolism at a lower concentration of glutamine. Additionally, any time-dependent changes in the glycosylation of HCG were assessed with more sensitive structural analysis of the N-linked glycans on days 1, 3 and 5 of culture (Section 3.4).

3.2 Methods

3.2.1 Experimental culturing and analysis for small-scale investigation of glutamine and glutamax
Basal CD-CHO medium supplemented with 0, 8 or 20 mM glutamine or glutamax was prepared. The medium also contained HT supplement (100 μM sodium hypoxanthine, 16 μM thymidine) and was routinely stored at 4°C without glutamine or glutamax supplementation. (Glutamax and glx are used interchangeably to refer to the same L-alanyl-L-glutamine supplement termed GlutaMAX-I™, which is produced by GIBCO). Before inoculation, the medium was pre-incubated at experimental conditions. CHO cells were routinely maintained with rotation via a Bellco orbital shaker at 200 rpm in a Thermo Steri-Cycle CO₂ incubator at 5% CO₂ and 37°C. These experiments were carried out in the School of Biochemistry and Immunology, Trinity College Dublin, Ireland.

Cells were precultured in 250 ml and subsequently in 1 L shake flasks while maintaining the cell density between 0.3 x 10⁶ cells/ml and 2 x 10⁶ cells/ml. When a sufficient number of cells were obtained, an appropriate volume of cells was spun down to establish cultures at an initial cell density of 0.5 x 10⁶ cells/ml. The cells were resuspended in 50 ml of fresh medium in 250 ml shake flasks. Each day, 2 ml of culture supernatant was removed for analysis of cell growth and viability (by manual counting, Section 2.7.6) and assaying key cell culture parameters such as pH, pO₂ and pCO₂, as well as extracellular concentrations of glutamine, glutamate, ammonium, glucose and lactate (using the Nova Bioprofile 400). All experiments were carried out in triplicate, where the 8 mM glutamine condition was examined over three separate occasions, on two separate occasions for 0 and 20 mM glutamine and on just one occasion for glutamax experiments.

On day 5, the culture was harvested by centrifugation at 1000 g for five minutes and filtration through a 0.2 μm filter. 1 ml of this cell-free supernatant was frozen separately and reserved for extracellular HCG content analysis by ELISA (Section 2.17).
remaining supernatant was frozen at -80°C for purification of HCG and subsequent glycosylation analysis by IEF (Sections 2.15 and 2.19 respectively).

3.2.2 Experimental culturing and analysis for large-scale investigation of glutamine

This work was composed of experiments carried out at separate locations, using different shakers and with minor variations in analytical equipment. Cultures were harvested after 1, 3 and 5 days incubation with 0 mM, 4 mM and 8 mM glutamine in order to assess the changes in glycosylation of HCG at these time points.

A single incubation with varying glutamine concentrations was carried out in the School of Biochemistry and Immunology, Trinity College Dublin, Ireland (i.e. 3 shake flasks inoculated per glutamine concentration used, and one shake flask of each concentration harvested on days 1, 3 and 5).

A similar duplicate incubation was carried out in the small scale cell culture process development lab, MSD, Oss, the Netherlands. This involved inoculation of six shake flasks per concentration of glutamine used, and two shake flasks at each concentration were harvested carried out on days 1, 3 and 5.

Relevant distinctions between results from both locations are noted in text as the ‘single incubation’ and ‘duplicate incubation’.

Basal CD-CHO medium supplemented with 0, 4 or 8mM glutamine was prepared. The medium also contained HT supplement (100 μM sodium hypoxanthine, 16 μM thymidine) and was routinely stored at 4°C. Before inoculation, the medium was pre-incubated at experimental conditions.

Cells for the duplicate incubation were routinely maintained in a Kuhner ClimoShaker ISF1-X incubator at 150 rpm, 5% CO₂ and 80% RH, while the single incubation was incubated on a Bellco orbital shaker at 200 rpm in a Thermo Steri-Cycle CO₂ incubator at 5% CO₂ and 37°C.

Cells were precultured in 250 ml and subsequently in 1 L shake flasks while maintaining the cell density between 0.3 x 10⁶ cells/ml and 2 x 10⁶ cells/ml. When a sufficient number of cells were obtained, an appropriate volume of cells was spun down to establish the
cultures at an initial cell density of $0.5 \times 10^6$ cells/ml. At time zero, a culture volume of 150 ml was used inoculated in a 500 ml shake flask. Each day, 3 ml of culture supernatant was removed for routine analytical procedures. An analysis of cell growth and viability was carried out by an automated method using the CEDEX$^{\text{AS20}}$ for the duplicate incubation, while manual counting was used for the single incubation (Section 2.7.6) A Nova Bioprofile 400 was used for the single incubation to determine the following parameters - pH, pO$_2$ and pCO$_2$, as well as concentrations of glutamine, glutamate, ammonium, glucose and lactate. In the duplicate incubation, the BGA parameters (pH, pO$_2$ and pCO$_2$) were determined with a Bayer Rapid Lab 248, while concentrations of glutamine, glutamate, ammonium, glucose and lactate were assayed with the Nova Bioprofile 100.

On days 1, 3 and 5 the culture was harvested by centrifugation at 1000g for five minutes and filtration through a 0.2 μm filter. The supernatant was frozen at -80°C for purification of HCG and subsequent glycosylation analysis. The same batch of resin was used for both purification processes. The change in IEF pattern was determined for both incubations, while structural analysis of the N-linked glycans was carried out for the duplicate incubation only (Sections 2.19 and 2.20 respectively).

Additionally, 1 ml of cell-free supernatant from the duplicate incubation was frozen separately and reserved for extracellular HCG content analysis by ELISA (by the quality and regulatory unit in MSD, Oss, the Netherlands).

The metabolic data plotted in Figures 3.19 to 3.31 were composed of results from all data sets.
3.3 Results of the small-scale investigation of glutamine and glutamax

3.3.1 The growth of cells in culture is increased at 8 mM glutamax

Before commencing experiments, the normal growth characteristics of this cell line were explored. GIBCO recommends supplementation of CD-CHO medium with 8 mM glutamine, so this was considered as the control condition. Triplicate shake flasks were inoculated at an initial cell density of 0.4 x 10^6 cells/ml, and the maximal growth phase occurred over the first five days of a 7-day incubation (Figure 3.1). The stationary phase of growth occurred from days 5 to 6, while a decrease in viable cell number, caused by increase in cell death, (as measured by decreasing percentage viability of the cells, data not shown) was noted from days 6 to 7. As a result, a five-day culture period was chosen for experimentation. The remainder of this section explores the effect of 0 mM, 8 mM and 20 mM glutamine or glutamax supplementation on the metabolism, productivity and glycosylation of this CHO cell line.

The type of glutamine supplementation was found to impact on the viable cell density (Figure 3.2). On day 2, the number of viable cells was significantly decreased to 1.1 ± 0.0 x 10^6 cells/ml at 0 mM glutamine (compared to 1.7 ± 0.09 x 10^6 cells/ml at 8 mM glutamine at the same time point, significance was only noted with the other glutamine-supplemented cultures, p<0.01). The maximal cell density of 6.37 ± 0.89 x 10^6 cells/ml was reached with 8 mM glutamax supplementation on day 5. This value was higher than all other conditions, with the exception of 20 mM glutamax (p<0.001). This was the only time a difference in viable cell numbers was noted between the glutamax cultures. In the case of the glutamine-supplemented cultures, no significant difference in the viable cell number was noted at any time-point.

The growth rate was calculated from these values, and significant differences were only noted on days 2 and 5 (Figure 3.3). The decrease in viable cell number at 0 mM glutamine on day 2 was paralleled by a significant reduction in growth rate, although the result was not significant when comparing to 20 mM glutamax (p<0.05). On day 5, the growth rate at 8 mM glutamax was significantly increased at 0.44 ± 0.20/d, but only when compared to the 8 mM glutamine control (p<0.05, 0.07 ± 0.03/d).
Figure 3.1 Maximal CHO cell growth occurs over the first five days of batch cultivation.

Triplicate shake flasks were inoculated at an initial cell density of 0.4 x 10^6 cells/ml and with 8 mM glutamine supplementation under the conditions described in Section 3.2.1. The number of viable cells was determined daily for a period of seven days by manual cell counting (Section 2.7.6). The viability remained high until day 7, when it was seen to decrease to 90% (data not shown). Results are expressed as mean values ± SEM, and at points where no error bar is shown, the SEM was within the size of the symbol. This was carried out in order to determine the time period of growth for this cell line under the experimental conditions used. Consequently, a five-day time course was selected since this comprises the growth period under these conditions.
Figure 3.2 Lack of glutamine supplementation reduces the number of viable cells on day 2 of culture. Glutamax increases the number of viable cells relative to their glutamine-supplemented counterparts on day 5 of cultivation.

Triplicate shake flasks were incubated at an initial cell density of 0.5 x 10^6 cells/ml in 50 ml fresh medium in 250 ml shake flasks with 0 mM glutamine (—), 8 mM glutamine (——), 20 mM glutamine (——), 8 mM glutamax (—–) and 20 mM glutamax (––) supplementation. Each day the viable cell number and viability was determined by manual counting with trypan blue dye (Section 2.7.6). Results are expressed as mean values ± SEM, and at points where no error bar is shown, the SEM was within the size of the symbol. The viability was determined to be 100% throughout the time period. The change in the number of viable cells was assessed each day by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 3.3.1.
Figure 3.3 The growth rate of cells supplemented with 0 mM glutamine is significantly decreased on day 2 of culture, with the exception of the comparison to growth at 20mM glutamax.

Triplicate shake flasks were incubated at an initial cell density of $0.5 \times 10^6$ cells/ml in 50 ml fresh medium in 250 ml shake flasks and with 0 mM glutamine (---), 8 mM glutamine (—), 20 mM glutamine (—), 8 mM glutamax (---) and 20 mM glutamax (---) supplementation. Each day the viable cell number and viability of cells was determined by manual counting with trypan blue dye (Section 2.7.6). Following this, the growth rate was calculated according to Section 2.9.4. Results are expressed as mean values ± SEM. The daily differences in growth rate on were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 3.3.1.
3.3.2 The rate of glucose consumption and lactate production reduces with use of glutamax or 0mM glutamine

No changes were observed in the extracellular concentration of glucose for the first two days of culture, but from days 3 to 5, the glucose concentration at 0 mM glutamine was higher than all the other cultures (p<0.001, 18.8 ± 0.7 mM at 0 mM glutamine on day 5 compared to 14.3 ± 0.2 mM at 20 mM glutamine at this same time point, Figure 3.4). At day 2, this significance was only observed between the glutamine cultures. No differences were noted between the glutamine or glutamax cultures. The specific glucose consumption rate was found to be significantly different on days 2, 3 and 5 (Figure 3.5). On day 2, there was a lower rate of glucose consumption at 0 mM glutamine, when compared to the other glutamine-supplemented conditions (p<0.05). This value was not found to differ from the glutamax cultures. On day 3, the consumption rate of glucose at 0 mM glutamine was lower than 8 mM glutamine and 20 mM glutamax only (p<0.05, at 1.49 ± 0.12 pmol/VC/day for 0 mM glutamine). On day 5, the rate of glucose consumption at 0 mM glutamine was statistically higher than all other cultures (p<0.001, 1.83 ± 0.22 pmol/VC/day).

No differences were noted in the concentration of lactate in all cultures on days 0 and 1 (Figure 3.6). From day 2 onwards, the lactate concentration at 0 mM glutamine was statistically lower than all other cultures (p<0.05, at a concentration of 6.9 ± 1.1 mM at 0mM glutamine on day 5 compared to 13.7 ± 0.1 mM with 8 mM glutamax). Additionally, on days 3 and 4, no difference was noted in the lactate concentrations for either the glutamine or glutamax cultures. On days 4 and 5, similar lactate concentrations were observed for the 8 mM glutamine and 20 mM glutamax supplemented cultures. No significant differences were seen in the specific lactate production rate of each of the cultures on day 1 (Figure 3.7). The lactate production rate was statistically lower at 0 mM glutamine on day 2, but only compared to the other glutamine conditions (p<0.01, 2.93 ± 0.29 pmol/VC/day at 0mM glutamine and 2.59 ± 0.54 pmol/VC/day at 8 mM glutamine). The rate of lactate production at 8 mM glutamine was significantly lower than the 20 mM glutamine culture on day 2 (p<0.05). On day 3, lactate production at 0 mM glutamine was statistically lower than all the other cultures (p<0.001, 0.82 ± 0.06 pmol/VC/day). No statistical differences were noted between any cultures on day 4. On day 5, there was a small but significant increase in the lactate production rate in the 0 mM glutamine culture when compared to the other glutamine supplemented and the 8 mM glutamax cultures (p<0.01).
Figure 3.4 The extracellular concentration of glucose at 0 mM glutamine is significantly higher than all other cultures from days 3 to 5.

Triplicate shake flasks were incubated at an initial cell density of $0.5 \times 10^6$ cells/ml in 50 ml fresh medium in 250 ml shake flasks and with 0 mM glutamine (—), 8 mM glutamine (—), 20 mM glutamine (—), 8 mM glutamax (—) and 20 mM glutamax (—) supplementation. Each day, 1 ml of supernatant was taken from the culture to analyse the extracellular concentration of glucose with the Nova Bioprofile 400. Results are expressed as mean values ± SEM, and at points where no error bar is shown, the SEM was within the size of the symbol. The daily differences in glucose concentration were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 3.3.2.
Figure 3.5 The consumption rate of glucose at 0 mM glutamine is significantly lower than the glutamine-supplemented cultures on day 2 of cultivation, but increases above all cultures on day 5.

Triplicate shake flasks were incubated at an initial cell density of $0.5 \times 10^6$ cells/ml in 50 ml fresh medium in 250 ml shake flasks and with 0 mM glutamine (—), 8 mM glutamine (—), 20 mM glutamine (—), 8 mM glutamax (—) and 20 mM glutamax (—) supplementation. Each day, 1 ml of supernatant was taken from the culture to analyse the extracellular concentration of glucose with the Nova Bioprofile 400. The viable cell number was also determined each day, as described in Section 2.7.6. The specific consumption rate of glucose in pmol per viable cell per day was calculated according to Section 2.9.1, and plotted against the time in culture. Results are expressed as mean values ± SEM. The daily differences in $q_{\text{Gluc}}$ were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 3.3.2.
Figure 3.6 A significantly reduced concentration of lactate is present from days 2 through 5 of cultivation within cultures that were provided with 0 mM glutamine.

Triplicate shake flasks were incubated at an initial cell density of $0.5 \times 10^6$ cells/ml in 50 ml fresh medium in 250 ml shake flasks and with 0 mM glutamine (---), 8 mM glutamine (---), 20 mM glutamine (---), 8 mM glutamax (---) and 20 mM glutamax (---) supplementation. Each day, 1 ml of supernatant was taken from the culture to analyse the extracellular concentration of lactate with the Nova Bioprofile 400. Results are expressed as mean values ± SEM, and at points where no error bar is shown, the SEM was within the size of the symbol. The daily differences in lactate concentration were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 3.3.2.
Figure 3.7 On day 3 of culture, lactate is produced at a significantly lower rate with 0 mM glutamine supplementation.

Triplicate shake flasks were incubated at an initial cell density of $0.5 \times 10^6$ cells/ml in 50 ml fresh medium in 250 ml shake flasks and with 0 mM glutamine (---), 8 mM glutamine (—), 20 mM glutamine (—), 8 mM glutamax (---) and 20 mM glutamax (---) supplementation. Each day, 1 ml of supernatant was taken from the culture to analyse the extracellular concentration of lactate with the Nova Bioprofile 400. The specific production rate of lactate in pmol per viable cell per day was calculated according to Section 2.9.1, and plotted against the time in culture. Results are expressed as mean values ± SEM, and at points where no error bar is shown, the SEM was within the size of the symbol. The daily differences in qLac were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 3.3.2.
The reduced lactate concentration at 0 mM glutamine led to a higher pH value at all time points, with the exception of day 0 (p<0.05, Figure 3.8). The pH of the glutamax cultures began at a lower pH than the glutamine cultures, but the pattern of decreasing pH was similar. On day 1, the pH at 8 mM glutamax was considered to be the same as the glutamine-supplemented cultures. However, from days 2 to 4, the pH was higher than the remaining glutamine and glutamax cultures (p<0.01). On day 5, there was no significant difference in pH between 8 mM glutamine and the glutamax conditions, or between the 20 mM glutamine and glutamax cultures.

3.3.3 Use of glutamax results in the production of glutamine and ammonium

The concentrations of glutamine were found to be significantly different at all time points for the glutamine-supplemented cultures (p<0.05, Figure 3.9). The glutamax cultures were found to produce glutamine from the start of cultivation, although concentrations produced were similar to those determined at 0 mM glutamine on days 0 and 1. The concentrations increased on day 2, but no difference was found in the amount of glutamine produced by either glutamax cultures at this point. From day 3 onwards, a significantly higher concentration of glutamine was found in the 20 mM glutamax culture (p<0.05). On the last day of culture, there was no significant difference in the concentration of glutamine found at either at 8 mM glutamine or glutamax, or at 20 mM glutamine or glutamax. On day 5, the concentration of glutamine in the glutamax cultures were 3.85 ± 0.04 mM at 8 mM glutamax and 6.23 ± 0.23 mM at 20 mM glutamax.

The glutamine consumption rates were corrected for chemical degradation for the glutamine cultures, but not for the glutamax cultures (See Section 2.9, Figure 3.10). The production of glutamine by the glutamax cultures led to a negative production rate as a result of the calculations used. On day 1, the highest consumption rate was found at 8 mM glutamine, and this value was deemed to be higher than all the others at 2.39 ± 0.48 pmol/VC/day (p<0.001). This value decreased on day 2, and was only found to be significantly higher than the 20 mM glutamax culture (p<0.05). No difference was found between the glutamine and glutamax cultures on day 3, though the glutamine consumption rate was higher than the glutamax rate (0.39 ± 0.05 pmol/VC/day for 8 mM glutamine and -0.42 ± 0.00 pmol/VC/day at 8 mM glutamax). On day 4, a significant difference was noted between all cultures (p<0.001). On the final day of cultivation, the consumption rate of glutamine with 20 mM glutamine supplementation was deemed to be higher than the others at 1.06 ± 0.44 pmol/VC/day (p<0.05).
Figure 3.8 The culture pH is significantly higher in cultures supplemented with 0 mM glutamine from days 1 through 5 of cultivation.

Triplicate shake flasks were incubated at an initial cell density of $0.5 \times 10^6$ cells/ml in 50 ml fresh medium in 250 ml shake flasks and with 0 mM glutamine (—), 8 mM glutamine (—), 20 mM glutamine (—), 8 mM glutamax (---) and 20 mM glutamax (---) supplementation. Each day, 1 ml of supernatant was taken from the culture to analyse the culture pH with the Nova Bioprofile 400. Results are expressed as mean values ± SEM, and at points where no error bar is shown, the SEM was within the size of the symbol. The pCO$_2$ remained between 30 and 40 mmHg, while the pO$_2$ remained between 120 and 160 mmHg during the culture time. As such, these values are considered to be stable. The daily differences in pH were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 3.3.2.
Figure 3.9 The extracellular concentration of glutamine increases for the cultures supplemented with glutamax.

Triplicate shake flasks were incubated at an initial cell density of 0.5 x 10^6 cells/ml in 50 ml fresh medium in 250 ml shake flasks and with 0 mM glutamine (—), 8 mM glutamine (—), 20 mM glutamine (—), 8 mM glutamax (—) and 20 mM glutamax (—) supplementation. Each day, 1 ml of supernatant was taken from the culture to analyse the extracellular concentration of glutamine with the Nova Bioprofile 400. Results are expressed as mean values ± SEM, and at points where no error bar is shown, the SEM was within the size of the symbol. The daily differences in glutamine concentration were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 3.3.3.
TriPLICATE shake flasks were incubated at an initial cell density of 0.5 x 10^6 cells/ml in 50 ml fresh medium in 250 ml shake flasks and with 0 mM glutamine (—), 8 mM glutamine (—), 20 mM glutamine (—), 8 mM glutamax (—) and 20 mM glutamax (—) supplementation. Each day, 1 ml of supernatant was taken from the culture to analyse the extracellular concentration of glutamine with the Nova Bioprofile 400. The viable cell number was also determined each day, as described in Section 2.7.6. The specific consumption rate of glutamine in pmol per viable cell per day was calculated according to Section 2.9.1, and plotted against the time in culture. Results are expressed as mean values ± SEM, and at points where no error bar is shown, the SEM was within the size of the symbol. The daily differences in qGln were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 3.3.3.
The extracellular concentration of glutamate was higher at 20 mM glutamine than at 0 mM glutamine on day 2 (p<0.05, 2.38 ± 0.09 mM at 20mM glutamine and 1.99 ± 0.07 mM at 0 mM glutamine, Figure 3.11). From day 2 to 5, the cultures with 8 mM and 20 mM glutamine had significantly higher glutamate concentrations than all other cultures (p<0.05). The mean values were also significantly different from each other at these time points. There was no difference in the concentration of glutamate between the glutamax cultures and 0 mM glutamine on days 3 and 4, but these values deviated on day 5. The concentration of glutamate increased significantly at 8 mM glutamax to 2.10 ± 0.01 mM (p<0.01).

However, these changes in glutamate concentration did not result in significant changes in the glutamate consumption rate for any culture at any time-point (Figure 3.12).

Significantly different concentrations of ammonium were reached for each glutamine-supplemented culture during the cultivation time (p<0.05, Figure 3.13). The concentration was significantly decreased at 0 mM glutamine, and a mean value of 2.9 ± 0.0 mM was reached on day 5 of culture (p<0.001). On days 0 and 1, the ammonium concentrations from glutamax cultures were similar to those at 0 mM glutamine. However, the values were increased statistically from day 2 (p<0.001). Only at day 5 did the concentrations between glutamax conditions change, with a significant increase in concentration found at 20 mM glutamax (p<0.01).

The rate of ammonium production in the glutamine-supplemented cultures was found to be significantly higher than all other cultures on day 1 (p<0.001, Figure 3.14). These rates decreased on day 2, but were still statistically higher than the glutamax cultures (p<0.05, 1.25 ± 0.06 pmol/VC/day at 8 mM glutamine and 0.86 ± 0.05 pmol/VC/day at 8 mM glutamax). From days 3 to 5, the specific ammonium production rate at 0 mM glutamine was lower than all other cultures (p<0.05).
Figure 3.11 The extracellular concentration of glutamate increases significantly on days 3 to 5 of cultivation with 8 mM or 20 mM glutamine.

Triplicate shake flasks were incubated at an initial cell density of $0.5 \times 10^6$ cells/ml in 50 ml fresh medium in 250 ml shake flasks and with 0 mM glutamine (---), 8 mM glutamine (---), 20 mM glutamine (---), 8 mM glutamax (---) and 20 mM glutamax (---) supplementation. Each day, 1 ml of supernatant was taken from the culture to analyse the extracellular concentration of glutamate with the Nova Bioprofile 400. Results are expressed as mean values ± SEM, and at points where no error bar is shown, the SEM was within the size of the symbol. The daily differences in glutamate concentration were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 3.3.3.
Triplicate shake flasks were incubated at an initial cell density of $0.5 \times 10^6$ cells/ml in 50 ml fresh medium in 250 ml shake flasks and with 0 mM glutamine (---), 8 mM glutamine (---), 20 mM glutamine (---), 8 mM glutamax (---) and 20 mM glutamax (---) supplementation. Each day, 1 ml of supernatant was taken from the culture to analyse the extracellular concentration of glutamate with the Nova Bioprofile 400. The viable cell number was also determined each day, as described in Section 2.7.6. The specific consumption rate of glutamate in pmol per viable cell per day was calculated according to Section 2.9.1, and plotted against the time in culture. Results are expressed as mean values ± SEM, and at points where no error bar is shown; the SEM was within the size of the symbol. The daily differences in qGlu were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 3.3.3.

Figure 3.12 The rate of glutamate consumption does not change significantly with altering glutamine and glutamax supplementation.
Figure 3.13 The extracellular concentration of ammonium is significantly reduced with 0 mM glutamine supplementation.

Triplicate shake flasks were incubated at an initial cell density of $0.5 \times 10^6$ cells/ml in 50 ml fresh medium in 250 ml shake flasks and with 0 mM glutamine (---), 8 mM glutamine (—), 20 mM glutamine (—), 8 mM glutamax (---) and 20 mM glutamax (---) supplementation. Each day, 1 ml of supernatant was taken from the culture to analyse the extracellular concentration of ammonium with the Nova Bioprofile 400. Results are expressed as mean values ± SEM, and at points where no error bar is shown, the SEM was within the size of the symbol. The daily differences in ammonium concentration were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 3.3.3.
Figure 3.14 The rate of ammonium production decreases with 0 mM glutamine supplementation.

Triplicate shake flasks were incubated at an initial cell density of $0.5 \times 10^6$ cells/ml in 50 ml fresh medium in 250 ml shake flasks and with 0 mM glutamine (—), 8 mM glutamine (—), 20 mM glutamine (—), 8 mM glutamax (—) and 20 mM glutamax (—) supplementation. Each day, 1 ml of supernatant was taken from the culture to analyse the extracellular concentration of ammonium with the Nova Bioprofile 400. The viable cell number was also determined each day, as described in Section 2.7.6. The specific production rate of ammonium in pmol per viable cell per day was calculated according to Section 2.9.1, and plotted against the time in culture. Results are expressed as mean values ± SEM, and at points where no error bar is shown, the SEM was within the size of the symbol. The daily differences in $\text{qNH}_4^+$ were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 3.3.3.
3.3.4 A significant increase in the total HCG content and productivity is found with 0mM glutamine supplementation

The total extracellular HCG content was determined on day 5 of culture only. A statistically higher amount of HCG was produced at 0 mM glutamine on day 5, when compared to the other glutamine controls (433.7 ± 49.1 IU/ml at 0 mM glutamine and 241.0 ± 18.1 IU/ml at 8 mM glutamine, Figure 3.15). No significant difference was noted when comparing to the glutamax cultures.

This translated into a higher specific rate of production of HCG at 0 mM glutamine versus all other conditions (37.9 ± 4.0 µIU/VC/day at 0 mM glutamine and 20.5 µIU/VC/day at 8 mM glutamine, Figure 3.16).

3.3.5 Lack of glutamine supplementation increases average pl of HCG

HCG, which was purified from culture supernatant that was harvested on day 5, was subject to IEF (Figure 3.17). At 0 mM glutamine, an increase in the intensity of three bands at pl values of 6.72 ± 0.01, 7.04 ± 0.01 and 7.27 ± 0.01 was noted. Meanwhile, the same band pattern at low pl was detected for all conditions.

Densitometry analysis was used to calculate the average pl from these IEF banding patterns. A significant increase in the average pl was seen 0 mM glutamine, when compared to all other cultures (5.21 ± 0.07, compared to 4.80 ± 0.03 at 8 mM glutamine, Figure 3.18).
Figure 3.15 The total amount of HCG increases significantly on day 5 of cultivation with 0 mM glutamine supplementation when compared to glutamine-supplemented cultures.

Triplicate shake flasks were incubated at an initial cell density of $0.5 \times 10^6$ cells/ml in 50 ml fresh medium in 250 ml shake flasks and with 0 mM glutamine (---), 8 mM glutamine (---), 20 mM glutamine (---), 8 mM glutamax (---) and 20 mM glutamax (---) supplementation. On the final day of cultivation, 1 ml of supernatant was taken from the culture and was made cell-free by centrifugation at 1000 g for five minutes and filtering through a 0.2 μm filter. The samples were stored at -20°C before analysis of extracellular HCG content by ELISA (Section 2.17). Results are expressed as mean values ± SEM. Statistical analysis was performed by one-way ANOVA with post-hoc Bonferroni analysis using Prism (** = p<0.01).
Triplicate shake flasks were incubated according to the conditions described in Section 3.2.1. On the final day of cultivation, one ml of supernatant was taken from the culture and was made cell-free by centrifugation at 1000 g for five minutes and filtering through a 0.2 μm filter. The samples were stored at -20°C before analysis of extracellular HCG content by ELISA (Section 2.17). The viable cell number was also determined each day, as described in Section 2.7.6. The specific productivity of HCG at each condition was determined on day 5 (Section 2.9.1, 0 mM glutamine, 8 mM glutamine, 20 mM glutamine, 8 mM glutamax and 20 mM glutamax). Results are expressed as mean values ± SEM. Statistical analysis was performed by one-way ANOVA with post-hoc Bonferroni analysis using Prism (** = p<0.01).
Figure 3.17 Cultivation of CHO cells with 0 mM glutamine for five days results in an increase in the intensity of three high pI bands of HCG in IEF.

Cells were incubated for five days in 250 ml shake flasks with varying glutamine or glutamax supplementation to CD-CHO medium on a Bellco orbital shaker. After this time, the culture supernatants were harvested, and isoelectric focusing was carried out on HCG purified from each sample. This figure shows a representative IEF profile of HCG at each condition, where M denotes the migration pattern of the pI marker (from Serva) while Gln and Glx represents the concentration of glutamine and glutamax used in the cultivation (respectively).
Figure 3.18 The average pi of HCG increases significantly at 0 mM glutamine.

Culture supernatants were harvested after five days incubation with various supplementations and isoelectric focusing was carried out on HCG purified from each sample. The average pi of HCG for each condition (0 mM glutamine, 8 mM glutamine, 20 mM glutamine, 8 mM glutamax and 20 mM glutamax) was determined with reference to a pi marker by densitometry analysis of the IEF pattern shown in Figure 3.17. Results are expressed as the mean ± SEM (error bars). Statistical analysis was performed by one-way ANOVA with post-hoc Bonferroni analysis using Prism. Supplementation of the medium with 8 or 20mM glutamine or glutamax was shown to significantly decrease the average pi (*** = p<0.001).
3.4 Results of large-scale investigation of glutamine.

3.4.1 The number of viable cells decreases significantly on days 2 and 3 of cultivation with 0 mM glutamine

The growth response of CHO cells to 0, 4 and 8 mM glutamine in a different shaker and larger shake flasks is shown in Figure 3.19. On days 2 and 3, there were a significantly lower number of viable cells at 0 mM glutamine (p<0.05, 2.0 ± 0.0 x 10^6 cells/ml at 0 mM glutamine on day 3, 4.0 ± 0.3 x 10^6 cells/ml at 8 mM glutamine on the same day). However, by the end of cultivation, there was no difference between any of the cultivations, with the mean viable cell number at 8 mM glutamine reaching 5.4 ± 0.6 x 10^6 cells/ml. The reasons for the increase in the viable cell number during this cultivation when compared to the small-scale experiments are addressed in Section 3.5.1.

At 4 mM and 8 mM glutamine, the growth rate was maintained around 0.6 ± 0.0/d until day 3, after which point the rate decreased to 0.1 ± 0.0/d on days 4 and 5 (Figure 3.20). No difference in growth was found between these two cultures at any time in the culture period. On day 3, the growth rate at 0 mM glutamine was statistically lower than the glutamine-supplemented cultures at 0.5 ± 0.0/d (p<0.05). Although this was the only point of significance difference, it is of note that the mean growth rate at 0 mM glutamine was lower than at 4 and 8 mM glutamine on days 1 and 2, but increased on days 4 and 5.

3.4.2 Higher glucose and lower lactate concentrations are found when cultivating at 0mM glutamine

There was a higher concentration of glucose remaining in the culture medium at 0 mM glutamine at all time points, with the exception of day 1 (p<0.01, a mean concentration of 20.3 ± 1.3 mM was determined for the culture with at 0mM glutamine on day 5, compared to 14.3 ± 0.1 mM for the 4 mM glutamine culture at the same time point, Figure 3.21). On day 1, the concentration of glucose was significantly different at all conditions (p<0.05).

This implies that there was a lower rate of glucose consumption at 0 mM glutamine. However, there was no statistical difference in the rate of glucose consumption at any time point, regardless of the concentration of glutamine used (Figure 3.22). There was an interesting trend of changes in this rate between the 0 mM glutamine and the glutamine-supplemented cultures. From days 1 to 3, the mean rate of glucose consumption was lower at 0 mM glutamine, but this value was increased relative to the glutamine-supplemented cultures on days 4 and 5.
Figure 3.19 The number of viable cells decreases significantly on days 2 and 3 of cultivation with 0 mM glutamine.

Triplicate shake flasks were incubated at an initial cell density of $0.5 \times 10^6$ cells/ml in 150 ml fresh medium in 500 ml shake flasks with 0 mM glutamine (—), 4 mM glutamine ( ) and 8 mM glutamine ( ) supplementation. The viable cell number and viability was determined daily. Results are expressed as mean values ± SEM, and at points where no error bar is shown, the SEM was within the size of the symbol. The viability was above 98% throughout the time period. The daily differences in viable cell number were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 3.4.1.
Figure 3.20 The growth rate of CHO cells decreases significantly on day 3 of cultivation with 0 mM glutamine.

Triplicate shake flasks were incubated at an initial cell density of $0.5 \times 10^6$ cells/ml in 150 ml fresh medium in 500 ml shake flasks with 0 mM glutamine (—), 4 mM glutamine ( ) and 8 mM glutamine (—) supplementation. The viable cell number and viability was determined each day. The growth rate was calculated according to Section 2.9.4. Results are expressed as mean values ± SEM, and at points where no error bar is shown, the SEM was within the size of the symbol. The daily differences in growth rate were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 3.4.1.
Figure 3.21 The extracellular concentration of glucose increases significantly with 0 mM glutamine supplementation.

Triplicate shake flasks were incubated at an initial cell density of $0.5 \times 10^6$ cells/ml in 150 ml fresh medium in 500 ml shake flasks with 0 mM glutamine (−), 4 mM glutamine (−) and 8 mM glutamine (−) supplementation. The viable cell number and viability was determined each day. Each day, 1 ml of supernatant was removed from culture and the glucose concentration was determined using the Nova Bioprofile. Results are expressed as mean values ± SEM, and at points where no error bar is shown, the SEM was within the size of the symbol. The daily differences in glucose concentration were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 3.4.2.
Figure 3.22 The rate of glucose consumption is not significantly altered by different levels of glutamine supplementation to the culture medium.

Triplicate shake flasks were incubated at an initial cell density of $0.5 \times 10^6$ cells/ml in 150 ml fresh medium in 500 ml shake flasks with 0 mM glutamine (—), 4 mM glutamine (—) and 8 mM glutamine (—) supplementation. Each day, 1 ml of supernatant was taken from the culture to analyse the extracellular concentration of glucose with the Nova Bioprofile. The viable cell number was also determined each day, as described in Section 2.7.6. The specific consumption rate of glucose in pmol per viable cell per day was calculated according to Section 2.9.1, and plotted against the time in culture. Results are expressed as mean values ± SEM, and at points where no error bar is shown. The daily differences in specific glucose consumption were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 3.4.2.
There was a lower concentration of lactate in the culture at 0 mM glutamine than the glutamine-supplemented cultures on days 2 through 5 (p<0.001, Figure 3.23). A mean concentration of 13.2 ± 0.3 mM was determined at 0 mM glutamine on day 5, while a value of 19.2 ± 0.2 mM was found at 4 mM glutamine. On day 1, only the 8 mM glutamine condition was deemed to have a significantly higher concentration of lactate than at 0 mM glutamine (p<0.05). No significant differences were noted in the extracellular concentration of lactate at either 4 or 8 mM glutamine.

A statistically lower lactate production rate of 2.29 ± 0.69 pmol/VC/day was found at 0 mM glutamine on day 2 (compared to a value of 5.58 ± 0.98 pmol/VC/day at 8 mM glutamine at the same time point, p<0.05, Figure 3.24). Additionally, there was a higher lactate production rate on day 5 of 1.11 ± 0.11 pmol/VC/day at 0 mM glutamine (p<0.001, a value of -0.26 ± 0.14 pmol/VC/day was determined for 8 mM glutamine on day 5). This indicates that the trend of changes to lactate production at 0 mM glutamine was similar to those seen with glucose consumption. On days 1 to 3, there was a lower lactate production rate at 0 mM glutamine, but on days 4 and 5, the rate was higher than the glutamine-supplemented cultures.

From days 3 to 5, there was a significantly higher culture pH at 0 mM glutamine (p<0.01, Figure 3.25). On day 5 a pH of 7.00 ± 0.00 was determined at 0 mM glutamine, while this value was 6.86 ± 0.03 at 8 mM glutamine. On day 2, the pH at 0 mM glutamine was found to be higher than the 8 mM glutamine culture only (p<0.05).

3.4.3 The rate of glutamine consumption and ammonium production is largely unchanged by initial concentrations of 4 mM and 8 mM glutamine

The concentration of glutamine was different at all time points examined, with the exception of days 4 and 5 (p<0.001, Figure 3.26). The differences between the concentrations of glutamine were deemed insignificant for the cultures initially supplemented with 0 mM and 4 mM glutamine. However, 0.8 mM is the lowest concentration of glutamine that the Nova Bioprofile can accurately measure. This means the concentrations determined for the non-glutamine-supplemented culture at all time points is likely to be inaccurate. This means the significance noted on days 4 and 5 is also likely to be an error.
Figure 3.23 The concentration of lactate is significantly reduced on days 3 through 5 of cultivation with 0 mM glutamine.

Triplicate shake flasks were incubated at an initial cell density of $0.5 \times 10^6$ cells/ml in 150 ml fresh medium in 500 ml shake flasks with 0 mM glutamine (—), 4 mM glutamine (—) and 8 mM glutamine (—) supplementation. Each day, 1 ml of supernatant was removed from culture and the lactate concentration was determined using the Nova Bioprofile. Results are expressed as mean values ± SEM, and at points where no error bar is shown, the SEM was within the size of the symbol. The daily differences in lactate concentration were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 3.4.2.
Figure 3.24 The rate of lactate production with 0 mM glutamine supplementation is significantly different to the glutamine supplemented cultures on days 2 and 5 of cultivation.

Triplicate shake flasks were incubated at an initial cell density of $0.5 \times 10^6$ cells/ml in 150 ml fresh medium in 500 ml shake flasks with 0 mM glutamine (−), 4 mM glutamine (−) and 8 mM glutamine (−) supplementation. Each day, 1 ml of supernatant was taken from the culture to analyse the extracellular concentration of lactate with the Nova Bioprofile. The viable cell number was also determined each day, as described in Section 2.7.6. The specific production rate of lactate in pmol per viable cell per day was calculated according to Section 2.9.1, and plotted against the time in culture. Results are expressed as mean values ± SEM, and at points where no error bar is shown. The daily differences in specific lactate production were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 3.4.2.
Figure 3.25 The culture pH is significantly higher on days 2 through 5 of culture with 0 mM glutamine supplementation.

Triplicate shake flasks were incubated at an initial cell density of $0.5 \times 10^6$ cells/ml in 150 ml fresh medium in 500 ml shake flasks with 0 mM glutamine (−), 4 mM glutamine (−) and 8 mM glutamine (−) supplementation. Each day, 1 ml of supernatant was taken from the culture to analyse the extracellular pH. Results are expressed as mean values ± SEM. The daily differences in pH were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 3.4.2. The pCO$_2$ remained between 34 and 44 mmHg, while the pO$_2$ remained between 120 and 146 mmHg, and as such, these values are considered to be stable.
Figure 3.26 The extracellular concentration of glutamine is significantly different from day 1 through 3 of all cultures.

Triplicate shake flasks were incubated at an initial cell density of $0.5 \times 10^6$ cells/ml in 150 ml fresh medium in 500 ml shake flasks with 0 mM glutamine (−), 4 mM glutamine (−) and 8 mM glutamine (−) supplementation. Each day, 1 ml of supernatant was taken from the culture to analyse the extracellular concentration of glutamine with the Nova Bioprofile. Results are expressed as mean values ± SEM. The daily differences in glutamine concentration were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 3.4.3.
Statistical analysis showed that there was no difference in the rate of consumption of glutamine between 4 mM and 8 mM glutamine (Figure 3.27). On day 1, there was a trend of higher consumption at 8 mM glutamine, but from day 2 onwards, the values were similar and decreased through the remaining culture time. At 8 mM glutamine the consumption rate was $0.39 \pm 0.09 \text{ pmol/VC/day}$ on day 2 and decreased to $0.09 \pm 0.01 \text{ pmol/VC/day}$ on day 5. The consumption rate at 0 mM glutamine was significantly lower than that determined for 8 mM glutamine at all time-points, but only lower than 4 mM glutamine on days 2 through 4 ($p<0.05$).

A relatively large error occurred in the measurement of glutamate concentrations, although a trend of decreasing concentration was observed for all cultures from approximately day 3 onwards (Figure 3.28). However, this trend was only observed to be statistical at 0 mM glutamine, where a concentration of $1.79 \pm 0.09 \text{ mM}$ on day 5 was deemed lower than those on days 0 through 2 ($p<0.01$, $2.19 \pm 0.04 \text{ mM}$ on day 0). No significant differences were noted for the specific consumption rate of glutamate between any cultures at any time-point (Figure 3.29). In addition, no time-dependent changes in the rate were observed.

Ammonium was seen to accumulate during all cultures, and the concentrations produced were significantly different at all time-points ($p<0.05$, Figure 3.30). The concentration of ammonium with 8 mM glutamine supplementation was approximately three-fold higher than that determined for the 0 mM glutamine culture at the same time point (concentrations reached $7.51 \pm 0.08 \text{ mM}$ on day 5 with 8 mM glutamine, while $2.64 \pm 0.32 \text{ mM}$ of ammonium was produced for the 0 mM glutamine culture at the same time point, $p<0.001$).

The rates of ammonium production decreased throughout the time course (Figure 3.21). There was a lower rate of production of ammonium on days 1 through 3 at 0 mM glutamine, when compared to 8 mM glutamine ($p<0.01$, $0.40 \pm 0.02 \text{ pmol/VC/day}$ at 0mM, $0.50 \pm 0.10 \text{ pmol/VC/day}$ at 8 mM glutamine, both on day 3). Additionally, the rate of ammonium production on day 1 was significantly different for each of the cultures examined ($p<0.05$). There was no significant difference in the rate of ammonium production for any of the cultures on days 4 and 5.
Figure 3.27 The rate of glutamine consumption does not alter when concentrations of 4 mM and 8 mM are supplied to the culture medium.

Triplicate shake flasks were incubated at an initial cell density of $0.5 \times 10^6$ cells/ml in 150 ml fresh medium in 500 ml shake flasks with 0 mM glutamine (−), 4 mM glutamine (−) and 8 mM glutamine (−) supplementation. Each day, 1 ml of supernatant was taken from the culture to analyse the extracellular concentration of glutamine with the Nova Bioprofile. The viable cell number was also determined each day, as described in Section 2.9.4. The specific consumption rate of glutamine in pmol per viable cell per day was calculated according to Section 2.9.1, and plotted against the time in culture. Results are expressed as mean values ± SEM, and at points where no error bar is shown, the SEM was within the size of the symbol. The daily differences in the specific glutamine consumption rates were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 3.4.3.
Figure 3.28 The extracellular concentration of glutamate does not alter significantly when the concentration of glutamine in the culture medium is varied between concentrations of 0, 4 and 8 mM.

Triplicate shake flasks were incubated at an initial cell density of 0.5 x 10^6 cells/ml in 150 ml fresh medium in 500 ml shake flasks with 0 mM glutamine (−), 4 mM glutamine (−) and 8 mM glutamine (−) supplementation. Each day, 1 ml of supernatant was taken from the culture to analyse the extracellular concentration of glutamate with the Nova Bioprofile. Results are expressed as mean values ± SEM. The daily differences in glutamate concentration were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 3.4.3.
Figure 3.29 The rate of glutamate consumption does not alter when the concentration of glutamine supplied in the culture medium is varied between values of 0, 4 and 8 mM.

Triplicate shake flasks were incubated at an initial cell density of $0.5 \times 10^6$ cells/ml in 150 ml fresh medium in 500 ml shake flasks with 0 mM glutamine (——), 4 mM glutamine (---) and 8 mM glutamine (----) supplementation. Each day, 1 ml of supernatant was taken from the culture to analyse the extracellular concentration of glutamate with the Nova Bioprofile. The viable cell number was also determined each day, as described in Section 2.7.6. The specific consumption rate of glutamate in pmol per viable cell per day was calculated according to Section 2.9.1, and plotted against the time in culture. Results are expressed as mean values ± SEM. The daily differences in the specific glutamate consumption rates were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 3.4.3.
Figure 3.30 The concentration of ammonium is significantly different at all time points measured when the glutamine concentration is varied in culture medium between values of 0, 4 and 8 mM glutamine.

Triplicate shake flasks were incubated at an initial cell density of 0.5 x 10^6 cells/ml in 150 ml fresh medium in 500 ml shake flasks with 0 mM glutamine (—), 4 mM glutamine (—) and 8 mM glutamine (—) supplementation. Each day, 1 ml of supernatant was taken from the culture to analyse the extracellular concentration of ammonium with the Nova Bioprofile. Results are expressed as mean values ± SEM. The daily differences in ammonium concentration were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 3.4.3.
Figure 3.31 The rate of ammonium production is significantly decreased on days 1 and 2 of cultivation with 0 mM glutamine supplementation.

Triplicate shake flasks were incubated at an initial cell density of $0.5 \times 10^6$ cells/ml in 150 ml fresh medium in 500 ml shake flasks with 0 mM glutamine (−), 4 mM glutamine (−) and 8 mM glutamine (−) supplementation. Each day, 1 ml of supernatant was taken from the culture to analyse the extracellular concentration of ammonium with the Nova Bioprofile. The viable cell number was also determined each day, as described in Section 2.7.6. The specific production rate of ammonium in pmol per viable cell per day was calculated according to Section 2.9.1, and plotted against the time in culture. Results are expressed as mean values ± SEM. The daily differences in the specific ammonium production rates were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 3.4.3.
3.4.4 The specific productivity of HCG increases when incubated with 0 mM glutamine
The effect of glutamine on the production of HCG on days 1, 3 and 5 of cultivation was
determined for the duplicate incubation only (Figure 3.32). As expected, the amount of
HCG increased during the cultivation period. On day 1, no significant difference was noted
between conditions as regards the total amount produced (24.3 ± 0.8 IU/ml at 0 mM
glutamine and 37.5 ± 0.5 IU/ml at 8 mM glutamine). On day 3 however, 109.0 ± 2.0 IU/ml
of HCG was produced at 0 mM glutamine and this was determined to be significantly
lower than all other conditions (with 186.0 ± 0.0 at 8 mM glutamine). On the final day of
cultivation, the amount of HCG produced at 0 mM glutamine was only significantly lower
than the amount produced at 4 mM glutamine (372.5 ± 12.5 IU/ml at 0mM glutamine
versus 455.0 ± 5.0 IU/ml at 4 mM glutamine, Figure 3.32). On days 1 and 3, there was no
significant difference in the specific productivity of HCG at 0, 4 or 8 mM glutamine
(Figure 3.33). However, on day 5, there was a significantly higher specific productivity at
0 mM glutamine when compared to 8 mM only (39.6 ± 1.4 μIU/VC/day at 0 mM
glutamine and 22.2 ± 0.8 μIU/VC/day at 8 mM glutamine). The reasons for the difference
in HCG productivity with this set-up, when compared to the small scale experiments is
explored in Section 3.5.4.

3.4.5 The proportion of sialylated, triantennary and fucosylated N-glycan structures
decreases while neutral structures increase at 0 mM glutamine
The glycosylation of HCG at 0, 4 and 8 mM glutamine was assessed on days 1, 3 and 5 by
IEF and structural analysis of the N-linked glycans. For the single incubation, IEF was
carried out on one sample per condition (Figure 3.34 A). The IEF pattern demonstrates that
at 0 mM glutamine, there was an increase in the intensity of bands at pI 6.73, 7.10 and 7.26
(± 0.00 for all) while the remaining pI bands were a similar intensity at all glutamine
concentrations. This pattern existed from day 1 of culture. The average pI was calculated
for each of these single data points, but no statistical analysis could be carried out (Figure
3.34 B). However, a trend of increased pI was observed at 0 mM glutamine at all time-
points examined. The IEF pattern of the duplicate set up was determined for HCG purified
on days 3 and 5 only (Figure 3.35 A). The same pattern of changes that was seen for the
single incubation (described above) was also found here. Bands of higher intensity were
found at 6.68 ± 0.01, 7.06 ± 0.02 and 7.31 ± 0.05 pI. The average pI did not differ
significantly between conditions or days for either set up (Figure 3.35 B). The reasons for
the difference in glycosylation with this set-up, when compared to the small scale
experiments is explored in Section 3.5.5.
Figure 3.32 Lack of glutamine supplementation results in a significantly lower amount of HCG on day 3 of culture. However, on day 5, the HCG content is only statistically lower than the culture with 4 mM glutamine supplementation.

1 ml of culture supernatant was made cell-free by centrifugation at 1000 g for 5 minutes before filtering through a 0.2 μm filter and storing at -20°C before analysis. Extracellular HCG content was determined by ELISA and carried out by the quality unit MSD, Oss, the Netherlands. Mean values ± the SEM of duplicate cultures are shown, and at points where no error bar is shown, the SEM was within the area of the border. Statistical analysis was performed by one-way ANOVA with post-hoc Bonferroni analysis using Prism (*=p<0.05, ** = p<0.01).
Figure 3.33 The productivity of HCG was significantly increased on day 5 of cultivation with 0 mM glutamine.

Extracellular HCG content was determined by ELISA and carried out by the quality unit in MSD, Oss, the Netherlands, for days 1, 3 and 5 of culture. The number of viable cells was determined daily using the CEDEX®[^20], and the specific productivity of the cells was calculated according to Section 2.9.1. Mean values ± the SEM of duplicate cultures are shown. Statistical analysis was performed by one-way ANOVA with post-hoc Bonferroni analysis using Prism software (**=p<0.01).
Figure 3.34 No significant changes to the IEF pattern or average pi were found for the single incubation with varying glutamine concentrations.

Cells were incubated for five days with varying glutamine supplementation (0 mM, 4 mM and 8 mM) within the single incubation. On days 1, 3 and 5, HCG was purified from the culture supernatant at each time point. Part A shows a representative IEF profile under these conditions, where M and O denotes the migration pattern of the pi marker and Ovitrelle, respectively (an external recombinant HCG control). Gln represents the concentration of glutamine supplied to the culture, while the numbers denote the day of culture harvest with subsequent protein purification. Part B shows the average pi of HCG for each condition. Results are expressed as the mean ± SEM (error bars). Due to lack of adequate data numbers, no statistical analysis could be carried out.
Figure 3.35 No significant changes to the IEF pattern or average pI were found for the duplicate incubation with varying glutamine concentrations.

Cells were incubated in duplicate for five days with varying glutamine supplementation (0 mM, 4 mM and 8 mM). On days 1, 3 and 5, HCG was purified from the culture supernatant at each time point. A sufficient yield of HCG was obtained on days 3 and 5 for isoelectric focusing. Part A shows a representative IEF profile under these conditions, where M and O denotes the migration pattern of the pI marker and Ovitrelle, respectively (an external recombinant HCG control). Gln denotes the concentration of glutamine supplied to culture. Part B shows the average pI of HCG for each condition. Results are expressed as the mean ± SEM (error bars). Statistical analysis was performed by one-way ANOVA with post-hoc Bonferroni analysis using Prism. No statistical changes were noted.
The nature of these trends of changes in glycosylation was explored further by structural analysis of the N-linked glycans for the duplicate set up only. (See Table 2.7 and Section 2.20.9 for details of N-linked glycan abbreviations). On day 1, a four-fold increase in the proportion of M5 (peak 4) at 0 mM glutamine was noted (8.0 ± 0.5% of total area at 0 mM glutamine and 1.9 ± 0.0% of total area at 8 mM glutamine, Figure 3.36). There was also an increase in the proportion of peak 8, A2G2/FA2G1S1 at 0 mM glutamine (13.5 ± 1.3% of total area at 0 mM glutamine and 7.8 ± 0.2% of total area at 8 mM glutamine).

On day 3, the increase in the proportion of M5 at 0 mM glutamine remained, but there was only a two-fold difference between the glutamine-supplemented and non glutamine-supplemented cultures (8.0 ± 0.5% of total area at 0 mM glutamine, and 3.3 ± 0.1% of total area at 8 mM glutamine, Figure 3.37). There was also an increase in the proportion of peaks 2 and 3 that contain A2 and A1G1, respectively. A two fold-increase was seen in the proportion of A1G1 at 0 mM glutamine (3.7 ± 0.4% of total area at 0 mM glutamine and 3.3 ± 0.1% of total area at 8 mM glutamine). A1 comprised a much smaller proportion of the total N-glycan pool, but the value reached 0.7 ± 0.0% of total area at 0 mM glutamine, compared to 0.5 ± 0.00% of total area at 8 mM glutamine. A significant decrease in the proportion of peaks 10 and 13 were also noted at 0 mM glutamine. Peak 10, which was comprised of A2G2S2 and FA2G2S1, was decreased by approximately 40% at 0 mM glutamine (to 17.1 ± 1.1% of total area compared to 27.1 ± 1.3% of total area at 8 mM glutamine) indicating the first confirmed changes in sialylation. Peak 13 contained the triantennary structures FA3G3S1 and A3G3S2, which were also decreased by approximately 45% at 0 mM glutamine (1.0 ± 0.1% of total area at 0 mM glutamine and 1.8 ± 0.0% of total area at 8 mM glutamine).

On day 5, the largest number of differences between conditions was noted (Figure 3.38). At 0 mM glutamine peaks 3, 4, 6, and 8 were increased, while peaks 7, 10, 13 and 14 were decreased. The two-fold increase in M5 at 0 mM glutamine is maintained, with similar proportions to those determined on day 3. The percentage area for A1G1 was also found at similar proportions to those at day 3. The first statistical increase in A2G1S1 at 0 mM glutamine was seen on day 5 (peak 6, 2.8 ± 0.1% of total area at 0 mM glutamine and 1.4 ± 0.1% of total area at 8 mM glutamine). The increase in peak 8 (comprised of A2G2 and FA2G1S1) at 0 mM glutamine was likely to be connected with the decrease in peak 10 (containing A2G2S2 and FA2G2S1), corresponding to a decrease in the sialylation of
A2G2S2 to form A2G2. A2G2 increased from 7.9 ± 0.4% of total area at 8 mM glutamine to 16.4 ± 0.1% of total area at 0 mM glutamine, while A2G2S2 decreased from 25.6 ± 0.4% of total area at 8 mM glutamine to 17.6 ± 0.4% of total area at 0 mM glutamine. Peak 7, which contained FA2G1, was found to decrease to 0.9 ± 0.2% of total area at 0 mM glutamine from 2.5 ± 0.0% of total area at 8 mM glutamine. Peaks 13 and 14 were composed of the triantennary structures FA3G3S1, A3G3S2 and A3G3S3, FA3G3S2, respectively. The peaks were found to halve at 0 mM glutamine, with percentage areas seen to fall to 1.3 ± 0.1% of total area and 1.1 ± 0.1% of total area, respectively (from 2.5 ± 0.1% of total area and 2.3 ± 0.0% of total area, respectively at 8 mM glutamine). This indicated a decrease in antennarity at 0 mM glutamine.

An estimate of the changes in key glycan structures at each time point was made (Table 3.1). This illustrates that from day 1 of culture, the proportion of sialylated, triantennary and fucosylated structures increases while the neutral structures increases at 0 mM glutamine.

An assessment of the time-dependent changes in glycosylation was also carried out. The proportion of A2 increased significantly from days 1 to 5 in all cultures. The percentage of M5 increased for the glutamine–supplemented cultures only. The proportion of M5 was significantly higher at all time points for the 0mM glutamine culture, and no statistical time-dependent differences were noted for this culture (Figures 3.36 through 3.38)
Figure 3.36 An increase in the proportion of the N-linked glycans termed M5 and A2G2 / FA2G1S1 attached to HCG occurs after one day cultivation with 0 mM glutamine.

Duplicate shake flasks were harvested after one day cultivation with 0 mM, 4 mM and 8 mM glutamine, and the structures of the N-glycans attached to purified HCG were determined (Section 2.20). Results are expressed as the mean ± SEM (error bars). At points where no error bar is shown, the SEM was within the size of the symbol. Statistical analysis was performed by one-way ANOVA followed by post-hoc Bonferroni analysis using Prism, *=p<0.05, **=p<0.01. All triantennary structures were found to be a mixture where branch 1 was either in the β1-6 or β1-2 position. See Table 2.7 and Section 2.20.9 for details of N-linked glycan abbreviations.
Figure 3.37 Three days cultivation with 0 mM glutamine leads to an increase in the neutral
N-linked glycan species attached to HCG, along with a reduction in the sialylation and
proportion of triantennary structures.

Duplicate shake flasks were harvested after three days cultivation with 0 mM, 4 mM and 8 mM
glutamine, and the structures of the N-glycans attached to purified HCG were determined (Section
2.20). Results are expressed as the mean ± SEM (error bars). At points where no error bar is
shown, the SEM was within the size of the symbol. Statistical analysis was performed by one-way
ANOVA followed by post-hoc Bonferroni analysis using Prism, * =p<0.05, **=p<0.01. All
triantennary structures were found to be a mixture where branch 1 was either in the β1-6 or β1-2
position. See Table 2.7 and Section 2.20.9 for details of N-linked glycan abbreviations.
Duplicate shake flasks were harvested after five days cultivation with 0 mM, 4 mM and 8 mM glutamine, and the structures of the N-glycans attached to purified HCG were determined (Section 2.20). Results are expressed as the mean ± SEM (error bars). At points where no error bar is shown, the SEM was within the size of the symbol. Statistical analysis was performed by one-way ANOVA followed by post-hoc Bonferroni analysis using Prism, * =p<0.05, **=p<0.01. All triantennary structures were found to be a mixture where branch 1 was either in the β1-6 or β1-2 position. See Table 2.7 and Section 2.20.9 for details of N-linked glycan abbreviations.
Table 3.1 Estimates of N-linked glycosylation structural changes at each condition.

These values were determined by summing the percentage area of every peak that contained any given structure (for example, the proportion of neutral structures is estimated by addition of the mean percentage area of peaks 1 through 5 and 7 through 9, since each of these peaks has a neutral component). Millimolar concentrations of glutamine are represented herein.
3.5 Discussion

3.5.1 Glutamine and CHO cell growth

Varying the degree of glutamine supplementation had a minimal effect on CHO cell growth for both experimental set-ups. In the small-scale experiments, the only significant difference in growth was noted on day 2, where a reduction in viable cell number and growth rate at 0 mM glutamine was noted. This can also be described as a prolonged lag phase of cell growth (Figure 3.2 and 3.3). In a similar manner, the number of viable cells was significantly lower on days 2 and 3 at 0 mM glutamine in the large scale set up, (Figure 3.19). However, this translated into a lower growth rate on day 3 only (Figure 3.20).

These results are in agreement with the observation that CHO cells have a sufficient level of glutamine synthetase activity to sustain cell growth in the absence of glutamine supplementation (Zhang et al., 2006). This occurs by the conversion of glutamate to glutamine. Increasing ammonium concentrations, whether caused by excessive ammonium or glutamine supplementation is known to decrease the growth rate of cells (Kurano et al., 1990; Mc Queen and Bailey, 1990; Ozturk et al., 1992; Yang and Butler, 2000a). A non-significant trend of decreased growth rate was observed at high ammonium concentrations for these experiments, although the reasons for this were not investigated. Changes on a genomic level or alterations in transmembrane gradients have been postulated to lead to reduced growth in the presence of high ammonium concentrations (Schneider et al., 1996; Chen and Harcum, 2007).

Literature suggests varied responses to decreased glutamine concentrations in CHO cells. Concentrations of less than 1 mM glutamine have been shown to decrease cell growth in both batch and fed-batch situations (Sun and Zhang, 2004; Wong et al., 2005). However, in one case, a lack of glutamine supplementation had no effect on the growth rate in a batch bioreactor (Gawlitzek et al., 1999). These results are most comparable with those of Sun et al, where a 2.6 fold decrease in the number of viable cells was found with 0.3mM glutamine supplementation in a batch cultivation (Sun and Zhang, 2004). However, there is a much lower decrease in growth rate and viable cell number in these experiments, suggesting that the response may be more similar to that described in Wong et al (Wong et al., 2005).
There was a trend of increased growth rate at 0 mM glutamine in the last days of culture for both experimental set-ups, although the difference was not statistically relevant. A molar replacement of glutamine with glutamate caused a similar increase in viable cell number on the last days of CHO batch culture (Altamirano et al., 2000).

It is of note that a higher final viable cell density was reached at all conditions in the large-scale set-up (compare Figure 3.2 and 3.19). At 8 mM glutamine, a mean value of $3.8 \pm 0.2 \times 10^6$ cells/ml was reached on day 5 in the small scale set up, while $5.4 \pm 0.6 \times 10^6$ cells/ml were present at the same time point the large scale set up. This was achieved by an increased growth rate on day 3 ($0.4 \pm 0.0$ in the small-scale set up compared to $0.6 \pm 0.0$ in the large scale set-up, both 8 mM glutamine, day 3). This could be an effect of scaling up from 250 ml shake flasks to 500 ml shake flasks, but is most likely due to the use of different shakers.

Despite the scale-up differences, one can conclude that varying glutamine concentrations does have an impact on CHO cell growth.

3.5.2 Glutamine and the metabolism of glucose and lactate

There was a similar pattern of glucose consumption at varying glutamine concentrations for both the small and large-scale experiments. From day 2 of culture, there was a higher concentration of glucose and a lower concentration of lactate at 0 mM glutamine (Figure 3.4, 3.6, 3.21 and 3.23). The reduction in lactate concentration in these cultures resulted in a higher pH from this time point (Figure 3.8 and 3.25). This suggested that there was a reduction in both glucose consumption and lactate production at all stages of the 0 mM glutamine culture. However, these rates were only reduced significantly on days 2 and 3, and they were increased above the rates of the glutamine-supplemented cultures on day 5 (Figure 3.5, 3.7, 3.23 and 3.24). The lowered glucose consumption and lactate production rates occurred during the extended lag phase period of cell growth, so the occurrences were presumably linked. These rates were maintained at this level for the rest of the culture time, but there was a corresponding decrease in glucose consumption and lactate production for each of the glutamine supplemented cultures at the same time. As a result, the rates at 0 mM glutamine were increased relative to the glutamine-supplemented cultures.
Literature suggests a variable relation between the consumption of glucose and glutamine when the concentration of glutamine is varied. There is no change in the glucose metabolism of BHK and PER.C6 cells when cultivated at concentrations of 0.25 to 4 mM glutamine (Cruz et al., 1999; Maranga and Goochee, 2006). In another experiment with CHO cells, reducing the glutamine concentration to 2 mM in a batch set-up increased the consumption of glucose and production of lactate (Yang and Butler, 2000a). This suggests a reciprocal relationship between use of glucose and glutamine for energy metabolism in this cell strain. Under normal non-limited culture conditions, both glucose and glutamine are used for the generation of energy intermediates, however, when either of these metabolites is limiting, the consumption of the other increases to compensate for this. However, this was not the case here, and the results seemed to mirror those determined for the batch cultivation of CHO cells with molar replacement of glutamine with glutamate. Under these conditions, the rate of glucose consumption and lactate production was determined to be lower than those with glutamine supplementation. Although these rates were calculated over the entire growth period, an increase in the number of viable cells corresponds with decreases in glucose concentrations at the late stages of culture. This suggests that a similar increase in the specific glucose consumption rate and lactate production rate occurs at the late states of culture here also (Altamirano et al., 2000).

3.5.3 The metabolism of varied initial concentrations of glutamine

Since all cells require glutamine to grow, the changes in the concentrations and production rates of glutamine, glutamate and ammonium at 0 mM glutamine can give some insights into how the cell metabolism alters to cope with this lack of glutamine.

Within the small scale set up, a concentration of 20 mM glutamine led to an unusual pattern of glutamine consumption, when the correction for chemical degradation was taken into consideration. Glutamine degradation occurs by first order kinetics, indicating that the reaction rate is dependent on the initial concentration of glutamine (Ozturk and Palsson, 1990a). As a result, when inoculating cells at this initial concentration of glutamine, the reduction in glutamine concentration in the first two days of culture was mostly due to chemical degradation, which resulted in a low true consumption rate (Figure 3.10). This effect was only observed in this culture, most probably due to the extreme concentration used. In the latter days of cultivation, there was a higher rate of glutamine consumption at 20 mM glutamine (Figure 3.10). Within the large-scale experiments, there was no significant difference between the consumption rate of glutamine at either 4 mM or 8 mM,
although this rate decreases at days 4 and 5, most likely due to the reduction in growth rate at this time (Figure 3.27).

The concentration and production rate of ammonium was dependent on the initial glutamine concentration. The production of ammonium by metabolic reactions other than the chemical degradation of glutamine and via the action of glutaminase resulted in a lower concentration and production rate at 0 mM glutamine (Figure 3.13, 3.14, 3.30 and 3.31). Ammonium was produced in a concentration dependent manner at 4 mM, 8 mM and 20 mM glutamine for the first few days of culture. After this time, ammonium was produced at similar rates within each culture, and reduced during the remaining culture time in line with the reducing growth rate. These results are in line with literature (Vriezen et al., 1997; Cruz et al., 1999; Gawlitzek et al., 1999; Altamirano et al., 2000; Yang and Butler, 2000a; Wong et al., 2005; Maranga and Goochee, 2006).

There were no significant differences in the consumption rate of glutamate for either set of experiments which prevents the accurate determination of the mechanisms by which these cells overcome the loss of glutamine supplementation (Figure 3.12 and 3.29). However, trends in changing glutamate concentrations present some angles for speculation.

Within the small-scale experiment, there was a significant increase in the glutamate concentration on days 3 to 5 when glutamine was supplemented to the culture medium (Figure 3.11). This was caused by a trend of increasing production rate on days 1 to 3, suggesting the presence of glutamine resulted in the production of glutamate in the early days of culture (Figure 3.12). However, no change in glutamate concentration or consumption rate was seen at 0 mM glutamine.

The concentration of glutamate decreased from day 3 onwards in the large-scale cultivation, but this trend was only significant at 0 mM glutamine (Figure 3.28). As mentioned previously, this did not lead to a significant difference in the consumption rate at any concentration of glutamate, although there was a clear trend of consumption from day 3 to 5 (Figure 3.29). This suggests that all cultures consumed glutamate in the latter stages of cultivation to offset the other changes at this time point, including a decrease in growth rate.
CHO cells would be expected to synthesise glutamine from glutamate using glutamine synthetase to ensure sufficient intracellular glutamine concentrations that are required to maintain the synthesis of nucleotides, activated sugars and nicotinamide coenzymes. As a result of the lack of distinct changes in glutamate metabolism at 0 mM glutamine, no conclusions can be made about the cellular adaptations to overcome this lack of glutamine. Chapter 4 aims to investigate this further with the use of metabolic flux analysis in a continuous culture of this cell line at 0 mM and 8 mM glutamine.

### 3.5.4 Glutamine and productivity of HCG

Varying glutamine concentrations impacted the production of HCG in both experimental set-ups. The small scale set up demonstrated an increase in the total amount of HCG produced at 0 mM glutamine, resulting in a higher specific productivity (Figure 3.15 and 3.16). These values were determined on the last day of culture. This is in agreement with results determined for batch cultivation of CHO cells where glutamine was replaced with the same molarity of glutamate. An increase in the viable cell number in the late stages of culture resulted in a higher production of tPA and thus higher productivity (Altamirano et al., 2000).

In the large scale set up, the extracellular HCG content and specific productivity was determined on days 1, 3 and 5 (Figure 3.32 and 3.33). The amount of HCG increased at every time point for each culture, although the reduced growth rate at 0 mM glutamine caused a significant decrease in the extracellular HCG content on day 3. On day 5, there was an increase in the HCG content at 4 mM glutamine. In this case, the mean HCG content on day 5 in the culture supplemented with 8 mM glutamine was higher than that determined for the 0 mM glutamine culture. This may be a result of the increased number of viable cells within this set up, likely caused by the use of a different shaker. Despite these changes, and in a similar manner to the small-scale experiments, an increase in the specific productivity of HCG was found at 0mM glutamine on day 5 only. This resulted from a combination of elevated growth rate and similar HCG content to 8 mM glutamine at this time point. The productivity at 8 mM glutamine was similar in both cases. These results are also in agreement with those of Altamirano et al (Altamirano et al., 2000). The alterations in the total and specific productivity of tPA observed by Altamirano et al were suggested to have occurred due to the increased metabolic efficiency under these culture conditions. This may have also occurred in these experiments.
3.5.5 Glutamine and the glycosylation of HCG

The glycosylation of HCG was assessed on the final day of the small-scale experiment only. There were three additional high pi bands on IEF at 0 mM glutamine, although all remaining bands were similar (Figure 3.17). The average pi of HCG was calculated, and the increase in intensity of high pi bands at 0 mM glutamine resulted in a significantly higher average pi (Figure 3.18). This suggests that there was an increase in the proportion of structures with a lower degree of sialylation.

The experiments were scaled up in order to assess the changes in glycosylation throughout the experimental time course. HCG was purified from culture supernatant harvested on days 1, 3 and 5, but at a smaller concentration range of 0 mM, 4 mM and 8 mM glutamine. Incubation of the cells with 0mM glutamine resulted in a similar increase in the intensity of three bands at high pi at each time point examined (Figure 3.34 A and 3.35 A). However, the intensity of these bands in the cultures with glutamine supplementation was increased in comparison to the small-scale experiments. As a result, despite the trend of increased average pi at 0 mM glutamine, there was no significant difference in the average pi between cultures (Figure 3.34 B and 3.35 B).

A more detailed analysis of the N-linked glycan structures attached to HCG was carried out for these cultures at each time point (Figure 3.36, 3.37 and 3.38). These results indicate that notwithstanding the lack of significant changes in average pi, halting glutamine supplementation results in a different pattern of changes in N-linked glycosylation. It is likely that the greater sensitivity of the structural N-linked glycan analysis illustrated changes that were not apparent on IEF.

A significant increase in the proportion of the high mannose structure, M5, was seen from day 1 at 0 mM glutamine. With the exception of the decrease in FA2G1, which occurs on day 5 only, all other changes in glycosylation began as trends and became statistically significant either on day 3 or day 5. This indicated that the change in glycosylation was present from day 1 of culture. Consequently, cultivation of CHO cells at 0 mM glutamine in CD-CHO medium caused an increase in the proportion of neutral glycans and a decrease in the sialylation and antennarity and fucosylation of N-linked glycans attached to recombinant HCG from day 1 of batch culture.
Increased ammonium concentrations are known to cause an increase in sialylation, galactosylation and changes to antennarity (Thorens and Vassalli, 1986; Rijcken et al., 1995a; Gawlitzek et al., 1998; Grammatikos et al., 1998; Gawlitzek et al., 2000; Yang and Butler, 2000b; Yang and Butler, 2000c; Yang and Butler, 2002; Gillmeister et al., 2009). However, the results presented here did not indicate an ammonium-dependent change or improvement in glycosylation, and seem to be in more agreement with the work of Wong et al. This group demonstrated that culture of CHO cells at concentrations of 0.1 to 0.5 mM glutamine in a fed-batch set-up impeded the conversion of high mannose structures to complex structures, reduced the efficiency of sugar transfer to bi- and tri-antennary structures and reduced the sialylation of IFNγ below concentrations of 0.5 mM glutamine (Wong et al., 2005). The authors suggest that this may have occurred due to impaired sialyltransferase activity or a reduction in UDP-GlcNAc, a precursor for CMP-NeuAc synthesis, which would also explain the other glycosylation changes. As a result, glutamine limitation may result in glycosylation changes that are in contrast to those suggested by reducing ammonium accumulation, as observed in these experiments.

However, a multitude of other changes in metabolism and culture conditions occurred during the cultivation time. There was a significant difference in growth rate, glucose metabolism, and pH at 0 mM glutamine. Every one of these parameters is known to alter glycosylation, and as a result, the changes cannot be confidently assigned as glutamine-dependent effects (Hayter et al., 1992; Borys et al., 1993; Wong et al., 2005). As a result, a different approach was implemented to investigate the effect of glutamine on the glycosylation of HCG. Chapter 4 illustrates how coupling analyses that examine the mechanisms behind glycosylation changes to a controlled continuous culture system yielded insights into the effects of glutamine on the metabolism and glycosylation of this CHO cell line.

3.5.6 The effectiveness of glutamax as a glutamine replacement strategy

The use of the dipeptide L-alanine-L-glutamine (glutamax) has been reported to result in improvements in cell growth and more efficient glucose metabolism (Christie and Butler, 1994). Recent investigations have also shown that substitution of glutamine with glutamax increased the galactosylation of tPA produced by CHO cells (Gillmeister et al., 2009). Glutamax also resulted in minimal disruption to lysosomal pH, suggesting that the effect extends to other intracellular organelles, such as the Golgi (Gillmeister et al., 2009). As a result, glutamax may improve N-linked glycosylation, in addition to the metabolic benefits.
In agreement with published literature, glutamax increased the number of viable cells at the end of cultivation ((Christie and Butler, 1994), Figure 3.2). Glucose was consumed at a similar rate to the glutamine controls, with the exception of day 2, where glutamax cultures had significantly reduced consumption rates (Figure 3.4 and 3.5). However, although the lactate concentration was lower at all time points for the glutamax cultures, the production rate of lactate was only significantly lower on day 2 (when compared to their glutamine counterparts, Figure 3.6 and 3.7). This does not correlate with findings for hybridoma cells, where the glucose consumption rate was found to decrease with 6 mM glutamax supplementation (Christie and Butler, 1994). This suggests that the reported effect could be cell-line dependent.

The extracellular concentration of glutamine increased each day during the five-day experimental period, indicating the slow hydrolysis of the dipeptide during culture (Figure 3.9). This indicates a variation in the proposed mechanism of action of the dipeptides. Despite allowing 24 generations for hybridoma cells to adapt to dipeptide supplementation, the cell viability was found to decrease by approximately 50% on the first day of cultivation with ala-gln. This was presumably due to the inability of this cell line to grow in the absence of glutamine. As a result of the decrease in viability, cellular peptidases are released from dying cells, hydrolysing the dipeptide and allowing the individual amino acids to be taken up by the cells. The concentration of glutamine increases in the culture medium on day 1, and decreases thereafter. Consequently, the cells recover, although with a lag in growth (Christie and Butler, 1994). CHO cells have a low level of endogenous glutamine synthetase, meaning they continue to grow in the absence of glutamine, most likely by the synthesis of glutamine from glutamate supplied in culture medium. The viability of this cell line was calculated at 100% for the duration of the experiment, but this value was likely to be associated with some degree of human error due to the manual method of counting. As a result, it is likely that in this case, a minor amount of cell death released small amounts of peptidase each day, which would cause the observed effect.

The production of glutamine by the glutamax cultures was accompanied by an increase in the ammonium concentration (Figure 3.13), although levels were lower than those determined in the corresponding glutamine cultures. This was in agreement with the results for hybridoma cultures (Christie and Butler, 1994). Extracellular glutamate concentrations were also measured, and no significant changes were noted indicating that glutamate was neither consumed nor produced in the presence of glutamax (Figure 3.11, 3.12).
The substitution of glutamine for glutamax had no impact on the total extracellular HCG content or on the specific productivity of HCG (Figure 3.15 and 3.16). This is in agreement with published work (Christie and Butler, 1994), and suggests that glutamax does not affect the expression of recombinant proteins.

The glycosylation of HCG was assessed by IEF after five days of cultivation. The distribution of bands and the calculated average pi was found to be similar to the glutamine-supplemented control (Figure 3.17 and 3.18). However, recent investigations have shown that substitution of glutamine with glutamax caused an increase in the galactosylation of tPA produced by CHO cells (Gillmeister et al., 2009). Glutamax also caused less disruption to lysosomal pH than glutamine, suggesting that the effect extends to other intracellular organelles, such as the Golgi (Gillmeister et al., 2009). This implies that glutamax may impact on other aspects of N-linked glycosylation. However, this was not the case here, suggesting that the observed change in tPA glycosylation may be a protein-specific effect.

In conclusion, use of glutamax increased the viable cell number, and decreased the extracellular concentration of ammonium in this cell line. Despite suggestions in literature, cellular metabolism was not greatly altered, and no detectable changes in glycosylation were observed. Since many of these differences may be cell-line specific, further investigations into the responses of a range of cell lines may be required to ascertain the usefulness of glutamax as a replacement strategy to minimise ammonium and effect positive changes in glycosylation.

3.5.7 Conclusions

Glutamine limitation reduced the growth rate of this cell line on days 2 and 3 of culture, but this lower rate was maintained for a longer period than the glutamine supplemented cultures. As a result, growth was increased relative to these conditions at 0 mM glutamine on day 5. There was a similar trend of glucose consumption and lactate production at this condition. The decrease in lactate production at 0 mM glutamine caused a slower decrease in pH. Increased glutamine concentrations resulted in increased ammonium concentrations, however the minor changes in glutamate consumption yielded no insight on the metabolic adaptations to lack of glutamine supply. There was an increase in the proportion of neutral glycans and a decrease in the sialylation and antennarity of N-linked glycans attached to recombinant HCG from day 1 of culture at 0 mM glutamine. However, these changes
could be due to the variation in growth, glucose metabolism or pH at 0 mM glutamine. Consequently, Chapter 4 addresses this issue in a continuous culture set-up. Use of glutamax as an alternative to glutamine supplementation reduces the ammonium concentration, and increased the viable cell number, but caused no significant change in glycosylation.
Chapter 4

Investigating the Effect of Glutamine on CHO Metabolism and HCG Glycosylation in a Continuous Mode of Culturing
4.1 Introduction

Glutamine is routinely supplemented to cells in culture since many cell lines use glutamine as a major energy source. This occurs by conversion to glutamate by glutaminase and subsequently to α-ketoglutarate in order to fuel the TCA cycle. Glutamate can be deaminated or transaminated to α-ketoglutarate, although transamination produces less energy (Reitzer et al., 1979; Street et al., 1993; Neermann and Wagner, 1996; Cruz et al., 1999). Glutamine is necessary for the synthesis of purine nucleotides, pyrimidine nucleotides and nicotinamide coenzymes via donation of its amido group. Ammonium is a by-product of glutamine metabolism, and is also formed by the chemical degradation of glutamine (Ozturk and Palsson, 1990a). However, ammonium is toxic to cells in culture due to an inactive urea cycle. Transaminating glutamate conserves an extra amino group within a new amino acid, (e.g. alanine, glycine or aspartate) and comprises the cellular detoxification response to high ammonium concentrations (Street et al., 1993).

High ammonium concentrations decrease cell growth rates and increase the consumption and production of key metabolites (Kurano et al., 1990; Mc Queen and Bailey, 1990; Ozturk et al., 1992; Yang and Butler, 2000a; Yang and Butler, 2000b; Yang and Butler, 2002).

The sialylation and galactosylation of recombinant proteins was also found to decrease at high concentrations of ammonium (Thorens and Vassalli, 1986; Andersen and Goochee, 1995; Rijcken et al., 1995a; Gawlitzek et al., 1998; Grammatikos et al., 1998; Gawlitzek et al., 2000; Yang and Butler, 2000b; Yang and Butler, 2000c; Yang and Butler, 2002; Gillmeister et al., 2009). Additionally, high ammonium concentrations have been found to decrease the antennarity of the N-linked glycans in some cases (Yang and Butler, 2000c; Yang and Butler, 2002) and increase in others (Gawlitzek et al., 1998; Grammatikos et al., 1998).

The mechanisms by which increased ammonium concentrations may change N-linked glycosylation have been elucidated. Ammonium disrupts the normal pH distribution of the cells (Schneider et al., 1996). Chloroquine mimics this effect and has been shown to cause the same glycosylation effects as NH₄Cl addition (Thorens and Vassalli, 1986; Andersen and Goochee, 1995; Gawlitzek et al., 2000). This indicates that some of changes are mediated by variations in pH.Irrespective of the route of ammonium formation, an increase in the pH of the Golgi is known to occur. This has been shown to redistribute and
decrease the activity of glycosylation enzymes (Gawlitzek et al., 2000; Axelsson et al., 2001; Rivinoja et al., 2009). Additionally, ammonium causes an increase in the UDP-GNAc pool due to its direct incorporation into glucosamine-6-phosphate by GPI, and the stimulation of de novo synthesis of UMP by carbamoyl phosphate. This result in an initial increase in CMP-NeuAc levels, but due to feedback inhibition of its own synthesis, concentrations decrease. The increased UDP-GNAc pool also inhibits the transport of CMP-NeuAc into the Golgi. As a result, sialylation decreases, and, antennarity may be increased or decreased (Valley et al., 1999).

Since ammonium accumulation causes undesirable effects, some methods to reduce its formation were employed. The previous chapter has illustrated that growth is maintained in this cell line if glutamine was neglected from the culture medium, albeit at a lower rate (Chapter 3). A reduction in consumption and production rates of metabolites was observed. The average pH of HCG was seen to increase at 0 mM glutamine, which corresponded to an increase in the proportion of less sialylated N-linked glycans, while maintaining the most highly sialylated glycoforms, as indicated by IEF. Structural analysis of the N-linked glycans was carried out on HCG purified on days 1, 3 and 5 and it shows that most of these glycosylation changes were present from day 1. The results indicate that there was a decrease in antennarity and sialylation and an increase in the proportion of under processed, neutral glycans at 0 mM glutamine. These glycosylation results merited further investigation since they could not be confidently attributed to a change in glutamine concentration.

The topic was studied further by use of a continuous culture system. With the perfusion of medium, cells reach a steady state that is dependent on the concentration of glutamine supplied to the culture. This allows the growth rate, pH and temperature to be controlled. At each steady state, the concentrations and rates of production and consumption of metabolites are stable. As a result, this system should indicate whether the changes that were observed in batch culture are truly caused by differences in glutamine supplementation.

**Aim**

To understand the mechanisms by which varying glutamine concentrations alters the glycosylation of HCG. At each steady state, metabolic flux analysis was carried out to determine how the cell metabolism is altered in the presence and absence of glutamine.
The N-linked glycan structures were examined, and the concentrations of intracellular nucleotide and sugar nucleotides were determined in order to ascertain whether there was any link between changes in the two parameters, most notably the UDP-GNAc pool. Finally, any glutamine or time-dependent influences on the cell cycle distribution were assessed.

4.2 Methods

4.2.1 Medium preparation and preculture

Basal CD-CHO medium supplemented with either 0 mM or 8 mM glutamine was prepared in 10 L batches by the service group of P & MSc-CCPD (Process & Manufacturing Sciences - Cell Culture Process Development) in MSD, Oss, the Netherlands. The medium also contained HT supplement (100 μM sodium hypoxanthine, 16 μM thymidine). Cells were precultured firstly in 250 ml and subsequently in 1 L shake flasks while maintaining the cell density between 0.3 and 2 x 10^6 cells/ml. After 11 days of preculture, sufficient cells were generated to inoculate duplicate bioreactors according to the conditions detailed in Section 2.8. These experiments were carried out in the small scale cell culture process development lab, MSD, Oss, the Netherlands.

4.2.2 Experimental culturing and analyses

Three days after the inoculation of cells in bioreactors, the perfusion of medium began and was maintained at a rate of 0.4/d throughout the culturing time. The cultures were established with glutamine-supplemented medium (8 mM) and once a steady state had been attained (state 1, as defined in Section 2.8.1) the feed medium was changed to a different batch without glutamine addition. By alternating glutamine supplementation in the feed medium between concentration of 8 mM and 0 mM, four steady states were examined. It was intended that returning the cultures to 8 mM and 0 mM glutamine in the second instance would provide duplicate results for analysis. The time points of these changes in feed medium, and deviations of any culture parameters from setpoint, are described in Table 4.1.

Each day, 10 ml of culture was withdrawn from the bioreactor and a number of different analyses were carried out. The percentage viability and number of viable cells was established with use of the CEDEX AS, the concentrations of ammonium, glucose, glutamate, glutamine and lactate were assayed with use of the Nova Bioprofile 100 plus and BGA parameters (pH, pO_2, and pCO_2) were determined with the Bayer Rapidlab 248.
Excess sample was centrifuged at room temperature for 5 minutes at 1000 g, filtered through a 0.2 μm filter and stored at -20°C for further analysis.

Once a steady state had been reached, extra samples were taken for analysis of extracellular amino acid concentrations (Section 2.11), cell cycle distribution (Section 2.13) and intracellular nucleotide and sugar nucleotide content (Section 2.14). Additionally, a new harvest line was attached to the bioreactor to collect cell suspension for purification of HCG and subsequent assessment of the glycosylation of HCG by IEF and analysis of N-linked glycan structures (Sections 2.15, 2.19 and 2.20, respectively). This was carried out for two consecutive days during the steady state period.

Steady states were defined for the following time periods; days 30 – 33 (state 1), days 45 – 48 (state 2), days 72 – 75 (state 3) and days 88 – 91 (state 4). The metabolism of the cells was assessed over these periods using metabolic flux analysis, where fluxes were predicted using the previously described metabolic flux model and the calculated rates of changes in the consumption and production of key metabolites. Additional analysis of cell cycle distribution, intracellular nucleotide and sugar nucleotide content and supernatant harvest for glycosylation analysis was carried out on the final two days only.

<table>
<thead>
<tr>
<th>Day</th>
<th>Remark</th>
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<tbody>
<tr>
<td>3</td>
<td>Perfusion begins at 8 mM Gln feed</td>
<td>3</td>
<td>Perfusion begins at 8 mM Gln feed</td>
</tr>
<tr>
<td>7</td>
<td>Dilution rate was 0 due to blockage in feed and harvest line</td>
<td>17</td>
<td>Temperature was 38°C for 1 hour</td>
</tr>
<tr>
<td>33</td>
<td>Switch to 0 mM Gln feed</td>
<td>33</td>
<td>Culture level was 1.4 L due to blockage in feed line, Switch to 0 mM Gln feed</td>
</tr>
<tr>
<td>48</td>
<td>Switch to 8 mM Gln feed</td>
<td>48</td>
<td>Switch to 8 mM Gln feed</td>
</tr>
<tr>
<td>61</td>
<td>Stirrer was erroneously 30 rpm over setpoint</td>
<td>75</td>
<td>Switch to 0 mM Gln feed</td>
</tr>
<tr>
<td>75</td>
<td>Switch to 0 mM Gln feed</td>
<td>91</td>
<td>Culture stopped</td>
</tr>
</tbody>
</table>

Table 4.1 – Timeframe for continuous experiments with varying glutamine concentrations. Purposely induced changes in the duplicates cultures A and B are given in bold, while unintended deviations from set point are shown with unformatted text. The impact of the deviations is discussed in the Section 4.3.9.
4.3 Results

4.3.1 The number of viable cells increases at states 3 and 4

In the first few days of culture, the cells grew rapidly to reach an average maximum density of \(5.3 \times 10^6\) cells/ml at day 8 (Figure 4.1). The viable cell number decreased thereafter to reach the first steady state at a viable cell concentration of \(3.0 \pm 0.2 \times 10^6\) cells/ml. There was no significant difference in the number of viable cells at state 2, where a mean value of \(3.4 \pm 0.12 \times 10^6\) cells/ml was reached. However, the viable cell density at states 3 and 4 differed from that at states 1 and 2. The cell number doubled from state 2 to 3 (from \(3.4 \pm 0.1 \times 10^6\) cells/ml to \(6.5 \pm 0.5 \times 10^6\) cells/ml, \(p<0.001\)), while a decrease in cell number occurred from state 3 to 4 (to \(5.1 \pm 0.2 \times 10^6\) cells/ml, \(p<0.001\)). The viability of the cells remained above 98% for the B culture and above 99% for the A culture (data not shown).

The increase in viable cell number up to day 8 was caused by the growth rate exceeding a value of 0.6/d during this time (Figure 4.2). A stable viable cell number was attained due to an eventual stabilisation of the growth rate. The growth rate remained stable during the next state, where no change in viable cell number was observed. At state 3, a significant increase in the viable cell number was achieved by a change in the specific growth rate over several days before the steady state was reached. The A culture showed a gradual pattern of increasing viable cell numbers from day 54 to 62, and the growth rate is increased to 0.5/d for this time period. However, the B culture only showed an increase in viable cell number from days 54 to 55 and 62 to 64. At these times, the growth rate is closer to 0.6/d and 0.5/d, respectively. The decrease in viable cell number at state 4 was achieved through a similar manner as the increase in state 3. The number of viable cells decreased from days 77 through 79 for the B culture, and this was accompanied by a decrease in the growth rate. A similar decrease in viable cell number and specific growth rate was seen from day 78 to 79 in the A culture.

The specific growth rate was controlled by the harvest dilution rate, and no significant differences were noted at each steady state (Figure 4.3). The mean value was close to the set point of 0.4/d at all steady states. The deviation noted on day 7 for the B culture can be seen in Figure 4.3. A blockage in the feed and harvest lines caused the perfusion of medium to stop. As a result, the dilution rate was zero, causing a similar decrease in the specific growth rate (Figure 4.2).
Each day, a sample of culture supernatant was withdrawn from the bioreactor and 500 μl was diluted 1:1 with trypsin for automated determination of viable cell number and percentage viability using the CEDEX AS\textsuperscript{20}. The viable cell numbers of duplicate cultures (A and B) are shown. The vertical dashed lines indicate the time points at which the glutamine concentration in the medium was changed from an initial concentration of 8 mM to 0 mM, 8 mM and 0 mM, as described in Table 4.1. The green boxes indicate the steady states examined. The cell viability was above 97 % for the duration of the culture (data not shown). The change in the number of viable cells at each steady state was assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism (significance not indicated, but $p < 0.001$).
The specific growth rate does not alter significantly at steady state.

Following the daily determination of viable cell numbers (Figure 4.1), the specific growth rate was calculated for duplicate cultures (A and B) according to Section 2.9.2. The vertical dashed lines indicate the time points at which the glutamine concentration in the medium was changed from an initial concentration of 8 mM to 0 mM, 8 mM and 0 mM, as described in Table 4.1. The dilution rate was set at 0.4/d throughout (Figure 4.3). The green boxes indicate the steady states examined. The change in the growth rate at each steady state was assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. However, no significant changes were noted.
Duplicate cultures were established and were allowed to proceed in batch mode for three days. After this time, the perfusion of medium was begun at a rate of 0.3/d for one day, before increasing and maintaining at 0.4/d. This was achieved with the use of Watson Marlow 101U/R pumps for pumping new medium into the reactor (the feed), as well as culture supernatant out of the bioreactor (the harvest). The harvest pump ran continuously (and is therefore considered leading), while the feed pump was turned on when the culture level fell below a set point achieved with the use of level sensors. The harvest dilution rate was calculated daily for both cultures (A and B) by weighing the harvest reservoir. The vertical dashed lines indicate the time points at which the glutamine concentration in the medium was changed from an initial concentration of 8 mM to 0 mM, 8 mM and 0 mM, as described in Table 4.1. Major deviations from the set point are discussed Section 4.3.1. The green boxes indicate the steady states examined. The change in the dilution rate at each steady state was assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. However, no significant changes were noted.
4.3.2 The rate of glucose consumption and lactate production decreases from state 1 to 3

Glucose was provided in CD-CHO medium at a concentration of 35 mM (Figure 4.4). The concentration decreased in both cultures in the first 8 days before stabilising at approx 4 mM. The circulating concentration was $2.2 \pm 0.3$ mM and $2.1 \pm 0.2$ mM at steady states 1 and 2, respectively. No significant difference was noted between the values observed at these steady states. In states 3 and 4, the concentration of glucose was seen to decrease significantly when compared to states 1 and 2 ($p<0.001$). Glucose concentrations were considered limiting at these states since the extracellular concentration was $0.1 \pm 0.1$ mM and $0.2 \pm 0.1$ mM at states 3 and 4, respectively.

Lactate production commenced at the start of culture and concentrations rose until day 8; thereafter values stabilised (Figure 4.4). At states 1 and 2, mean concentrations of $42.1 \pm 0.8$ mM and $42.7 \pm 1.5$ mM, respectively were determined. No significant difference was observed between these two states. At states 3 and 4, lactate concentrations decreased significantly to $32.2 \pm 1.0$ mM and $37.4 \pm 0.9$ mM, respectively ($p<0.05$). Similarly, no significant differences were noted between the concentrations determined at these steady states.

The specific consumption rate of glucose was calculated each day from the extracellular glucose concentration and the number of viable cells. The glucose consumption rate increased from day 8 of culture, and stabilised at steady state 1 with a mean value of $4.27 \pm 0.23$ pmoL/VC/day (Figure 4.5). This value decreased significantly in states 2 and 3 to $3.32 \pm 0.15$ pmoL/VC/day and $2.03 \pm 0.11$ pmoL/VC/day, respectively ($p<0.001$). However, no significant difference was noted between the rate of glucose consumption at states 3 and 4.

The specific lactate production rate was calculated in a similar manner to the glucose consumption rate. The rate of lactate production increased from day 8 and attained a mean value of $6.45 \pm 0.28$ pmoL/VC/day at steady state 1 (Figure 4.6). A significant decrease was seen in states 2 and 3 when this value reached $5.07 \pm 0.31$ pmoL/VC/day and $2.29 \pm 0.18$ pmoL/VC/day, respectively ($p<0.001$). However, no significant difference was noted between the rate of lactate production at states 3 and 4.
Duplicate continuous cultures were established and were allowed to proceed in batch mode for the first three days. The perfusion of medium began at this point. A sample of culture supernatant was removed from the bioreactor each day, and analysis of extracellular concentrations of glucose and lactate were carried out with the use of a Nova Bioprofile 100 plus. The vertical dashed lines indicate the time points at which the glutamine concentration in the medium was changed from an initial concentration of 8mM to 0mM, 8mM and 0mM, as described in Table 4.1. The green boxes indicate the steady states examined. The glucose and lactate concentrations determined daily for duplicates A and B are shown with dashed and full lines, respectively. Meanwhile, glucose concentrations are shown in green and lactate concentrations shown in purple. The change in the glucose and lactate concentration at each steady state was assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism (significance not indicated, but \( p < 0.05 \)).
Duplicate cultures were established and the extracellular concentration of glucose was assayed daily with the Nova Bioprofile 100plus. The viable cell number was also determined each day, as described in Section 4.2.2. The specific consumption rate of glucose in pmol per viable cell per day was calculated according to Section 2.9.2, and plotted against the time in culture (A and B). The vertical dashed lines indicate the time points at which the glutamine concentration in the medium was changed from an initial concentration of 8 mM to 0 mM, 8 mM and 0 mM, as described in Table 4.1. The green boxes indicate the steady states examined. The change in the glucose consumption rate at each steady state was assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. No difference was noted between states 3 and 4, however all other states were found to differ significantly. (Significance not indicated, but p < 0.05)
Duplicate cultures were established and the extracellular concentration of lactate was assayed daily with the Nova Bioprofile 100 plus. The viable cell number was also determined daily as described in Section 4.2.2. The specific production rate of lactate in pmol per viable cell per day was calculated according to Section 2.9.2, and plotted against the time in culture (A and B). The vertical dashed lines indicate the time points at which the glutamine concentration in the medium was changed from an initial concentration of 8 mM to 0 mM, 8 mM and 0 mM, as described in Table 4.1. The green boxes indicate the steady states examined. The change in the lactate production rate at each steady state was assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. No difference was noted between states 3 and 4, however all other states were found to differ significantly. (Significance not indicated, but p < 0.05)
4.3.3 Ammonium production increases with glutamine supplementation

Analysis of the extracellular metabolites was carried out by use of the Nova Bioprofile 100 and using HPLC analysis (by SAFC Biosciences, Section 2.11), and resulted in an overlap of data points for glutamine and glutamate. Table 4.2 shows that there was a broad agreement of the concentrations determined by both methods, although minor variations were detected at states 3 and 4. Since the HPLC method of quantification was more accurate, these values were used for steady state metabolic flux analysis.

A mean circulating concentration of 4.70 ± 0.09 mM glutamine was found at steady state 1, where 8 mM glutamine was supplemented to the culture medium (Figure 4.7). As expected, the glutamine concentration was close to zero at states 2 and 4 (0.18 ± 0.07 mM at state 1 and 0.20 ± 0.02 mM at state 4). There was a significant decrease in the concentration of glutamine at state 3 to 1.99 ± 0.15 mM (p<0.001, when compared to state 1). The extracellular concentration of ammonium was found to differ significantly at all steady states examined (p<0.05, Figure 4.7). In the absence of glutamine supplementation, decreased concentrations were observed. The concentration of ammonium was significantly increased at state 3 to a mean value of 3.67 ± 0.09 mM (compared to 3.15 ± 0.05 mM at state 1, p<0.001). The concentration of glutamate in the culture medium was seen to increase significantly at state 3 to 3.86 ± 0.22 mM (p<0.001, Figure 4.8).

The specific glutamine consumption rate increased at states 1 and 3 to 0.38 ± 0.05 pmol/VC/day and 0.37 ± 0.02 pmol/VC/day, respectively (p<0.001, Figure 4.9). As expected, the consumption rate was close to zero at states 2 and 4 due to lack of glutamine supplementation. While this value reached approximately 0 pmol/VC/day within 3 days of the removal of glutamine from culture medium, the concentration in the bioreactor reached a minimal values 5 days after this point due to the time required for excess glutamine to flush out of the bioreactor (Figure 4.9 and 4.7). There was a trend of decreasing ammonium production from steady states 1 through 4 (Figure 4.10). This difference was considered to be significant when comparing the value at state 1 to those determined at states 3 and 4 (p<0.05 from 0.26 ± 0.05 pmol/VC/day at state 1 to 0.09 ± 0.00 pmol/VC/day at state 4). The glutamate consumption rate was negative at all steady states, indicating that glutamate was produced during these times (Figure 4.11). However, no significant differences were noted in the rate of glutamate production at any steady state.
<table>
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<th>2 (0mM Gln)</th>
<th>3 (8mM Gln)</th>
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<td>-0.02 ± 0.01</td>
<td>-0.14 ± 0.01</td>
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</tr>
</tbody>
</table>

Table 4.2 Concentrations and consumption rates of glutamine and glutamate as determined by HPLC.

These values show an agreement with values determined using the Nova Bioprofile 100 plus (Figure 4.7 and 4.8). Concentrations were determined by SAFC Biosciences and are given in mM, while specific rates are given in pmol/V-day. Mean values of the four days previous to the switch in condition for both cultures ± the SEM of the value are quoted. States 1 and 3 had 8 mM glutamine supplementation, while states 2 and 4 had none.
Duplicate cultures were established and were allowed to proceed in batch mode for the first three days. The perfusion of medium began at this point. A sample of culture supernatant was removed from the bioreactor each day, and analysis of extracellular concentrations of glutamine and ammonium were carried out with the use of a Nova Bioprofile 100 plus. The vertical dashed lines indicate the time points at which the glutamine concentration in the medium was changed from an initial concentration of 8 mM to 0 mM, 8 mM and 0 mM, as described in Table 4.1. The glutamine and ammonium concentrations determined daily for duplicates A and B are shown with dashed and full lines, respectively. Meanwhile, glutamine concentrations are shown in green and ammonium concentrations shown in purple. The green boxes indicate the steady states examined. The change in the both concentrations at each steady state was assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. The concentration of ammonium was found to differ at all steady states, while the concentration of glutamine was similar at states 2 and 4, but differed elsewhere. (Significance not indicated, but p < 0.05)
Duplicate cultures were established and were allowed to proceed in batch mode for the first three days. The perfusion of medium began at this point. A sample of culture supernatant was removed from the bioreactor each day, and analysis of the extracellular concentration of glutamate was carried out with the use of a Nova Bioprofile 100 plus (A and B). The vertical dashed lines indicate the time points at which the glutamine concentration in the medium was changed from an initial concentration of 8 mM to 0 mM, 8 mM and 0 mM, as described in Table 4.1. The green boxes indicate the steady states examined. The change in the glutamate concentration at each steady state was assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism (significance not indicated, but p < 0.001).
Figure 4.9 The consumption rate of glutamine increases significantly at steady states 1 and 3.

Duplicate cultures were established and the extracellular concentration of glutamine was assayed daily with the Nova Bioprofile 100 plus. The viable cell number was also determined daily as described in section 4.2.2. The specific consumption rate of glutamine in pmol per viable cell per day was calculated according to Section 2.9.2, and plotted against the time in culture (A and B). The vertical dashed lines indicate the time points at which the glutamine concentration in the medium was changed from an initial concentration of 8 mM to 0 mM, 8 mM and 0 mM, as described in Table 4.1. The green boxes indicate the steady states examined. The change in the glutamine consumption rate at each steady state was assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism (significance not indicated, but p < 0.001).
Figure 4.10 The production rate of ammonium decreases significantly during culture.

Duplicate cultures were established and the extracellular concentration of ammonium was assayed daily with the Nova Bioprofile 100 plus. The viable cell number was also determined daily as described in Section 4.2.2. The specific production rate of ammonium in pmol per viable cell per day was calculated according to Section 2.9.2, and plotted against the time in culture (A and B). The vertical dashed lines indicate the time points at which the glutamine concentration in the medium was changed from an initial concentration of 8 mM to 0 mM, 8 mM and 0 mM, as described in Table 4.1. The green boxes indicate the steady states examined. The change in the ammonium production rate at each steady state was assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism (significance not indicated, but \( p < 0.05 \)).
Duplicate cultures were established and the extracellular concentration of glutamate was assayed daily with the Nova Bioprofile 100 plus. The viable cell number was also determined daily as described in Section 4.2.2. The specific consumption rate of glutamate in pmol per viable cell per day was calculated according to Section 2.9.2, and plotted against the time in culture (A and B). The vertical dashed lines indicate the time points at which the glutamine concentration in the medium was changed from an initial concentration of 8 mM to 0 mM, 8 mM and 0 mM, as described in Table 4.1. The green boxes indicate the steady states examined. The change in the glutamate consumption rate at each steady state was assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. However, no significant differences were found.
4.3.4 Glycolytic flux and lactate production decreases at states 3 and 4, while TCA cycle changes are noted at state 3

Metabolic flux analysis showed that the metabolism of glucose to pyruvate and lactate via glycolysis and pyruvate dehydrogenase accounted for the greatest proportion of flux within the given reaction network (Figure 4.12). These fluxes were, on average, 8 times higher than the remaining fluxes. (The lowest of these calculated fluxes was $J_{\text{pyr-lac}}$ that had a mean flux of $0.290 \pm 0.032$ at steady state 3. The maximal flux was observed at steady state 1 where $J_{\text{glc-pyr}}$ had a mean flux of $0.997 \pm 0.084$.) The range of the remaining fluxes was from $-0.032 \pm 0.023$ for $J_{\text{mal-pyr}}$ at state 1 to $0.209 \pm 0.009$ for $J_{\text{oaa-akg}}$ at state 4).

The pattern of decreasing consumption and production rates for glucose and lactate from states 1 to 3 was mirrored by similar changes in the fluxes $J_{\text{glc-pyr}}$ and $J_{\text{pyr-lac}}$ (Figure 4.12). In this case, no significant differences were noted in the mean fluxes between states 1 and 2, and 3 and 4. However, a statistical decrease was seen when comparing states 1 and 2 to states 3 and 4. The $\Delta L/\Delta G$ ratio of lactate moles produced per glucose mole consumed also showed this trend of significance.

However, these decreasing fluxes through pyruvate did not result in a significant change in flux to the TCA cycle via acetyl-CoA at any state (Figure 4.12, $J_{\text{pyr-acoa}}$).

The flux from pyruvate to alanine was seen to vary in a glutamine-dependent manner (Figure 4.12). The flux at state 2 was significantly decreased when compared to state 1 and 3 (a mean value of $0.032 \pm 0.001$ was determined at state 2 and $0.046 \pm 0.001$ at state 3). While state 4 was not statistically decreased, it appeared to follow a similar trend.

Changing glutamine concentrations caused significant changes in some of the fluxes within the TCA cycle (Figure 4.13). At steady state 3 there was a decrease in flux from oxaloacetate to $\alpha$-ketoglutarate when compared to state 4 only ($J_{\text{oaa-akg}}$, 0.173 ± 0.002 at state 3 compared to 0.209 ± 0.009 at state 4). The subsequent two fluxes in the TCA cycle were significantly increased at this steady state – $J_{\text{akg-succ}}$ and $J_{\text{succ-fum}}$. $J_{\text{akg-succ}}$ increased from 0.115 ± 0.002 at steady state 2 to 0.141 ± 0.002 at steady state 3, while $J_{\text{succ-fum}}$ changed from 0.100 ± 0.009 at state 2 to 0.126 ± 0.001 at state 3.
Duplicate continuous cultures proceeded through four steady states with varying glutamine concentrations in the CD-CHO medium, where state 1 = 8 mM glutamine, state 2 = 0 mM glutamine, state 3 = 8 mM glutamine and state 4 = 0 mM glutamine (blue, green, white and pink respectively). A sample of culture supernatant was made cell-free and frozen at -20°C before assay of the extracellular concentrations of amino acids (Section 2.11). The results were combined with other metabolic parameters in a metabolic flux analysis model and key fluxes were predicted (Section 2.12). The change in the fluxes at each steady state was assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. (*=p<0.05, **=p<0.01, ***=p<0.001). DL/DG is a measure of the moles of lactate produced per mole of glucose consumed. J represents the flux and abbreviations are noted elsewhere. $J_{\text{pyr-accoa}}$, $J_{\text{pyr-ala}}$, $J_{\text{gly-ser}}$, $J_{\text{ser-pyr}}$, and $J_{\text{cys-pyr}}$ are plotted on the secondary y-axis for clarity. Results are expressed as the mean ± SEM (error bars). At points where no error bar is shown, the SEM was within the size of the symbol.
Figure 4.13 The flux from oxaloacetate to α-ketoglutarate is significantly decreased at state 3, while an increase in the fluxes from α-ketoglutarate to succinyl-CoA and fumarate is also observed at this same state.

Duplicate continuous cultures proceeded through four steady states with varying glutamine concentrations, where state 1 = 8 mM glutamine, state 2 = 0 mM glutamine, state 3 = 8 mM glutamine and state 4 = 0 mM glutamine (blue, green, white and pink respectively). A sample of culture supernatant was made cell-free and frozen at -20°C before assay of amino acids (Section 2.11). The results were combined with other metabolic parameters in a metabolic flux analysis model and key fluxes were predicted (Section 2.12). The change in the fluxes at each steady state was assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism (*=p<0.05, **=p<0.01, ***=p<0.001). J represents the flux and abbreviations are noted elsewhere. J\_aa-rcA is the sum of the fluxes into the TCA cycle, i.e. those from glutamate to α-ketoglutarate, aspartate to oxaloacetate, tyrosine to fumarate and the sum of the amino acid fluxes to acetyl-CoA and succinyl-CoA. Results are expressed as the mean ± SEM (error bars). At points where no error bar is shown, the SEM was within the size of the symbol.
The fluxes around glutamine and fuelling into the TCA cycle showed some significant variations between steady states. Figure 4.14 illustrates that significant differences in fluxes between glutamine, glutamate and α-ketoglutarate occurred from state 1 to 4. At state 1, where glutamine is supplemented at 8mM, there was an average null flux from glutamine to glutamate. However, flux towards α-ketoglutarate from glutamate occurred. At state 2, the first culturing period without glutamine, α-ketoglutarate fluxed towards glutamate and subsequently to glutamine. The return of glutamine supplementation at state 3 reversed this trend, but in contrast to the fluxes observed in state 1, glutamine fluxed towards glutamate and fuelled into the TCA cycle through α-ketoglutarate. At the fourth steady state, α-ketoglutarate was consumed in order to produce glutamate and subsequently glutamine.

No significant differences were noted in the flux from the amino acids histidine, arginine and proline towards the synthesis of glutamate ($J_{\text{as}}-\text{glu}$).

There was a significant decrease in the flux from asparagine to aspartate to oxaloacetate at steady state 3 ($J_{\text{asn-asp}}$ and $J_{\text{asp-oxa}}$, Figure 4.14).

Amino acids formed the greatest flux towards biomass, but no significant differences were observed in any biomass flux during culture (Figure 4.15).

Carbon dioxide production also failed to change significantly (Figure 4.15).

The flux from amino acids towards production of HCG followed a decreasing trend from states 1 to 4, and the values were statistically lower at states 3 and 4 (Figure 4.15). However, this flux was one of the smaller fluxes.

An overall schematic of the change in metabolic fluxes was constructed from this data (Figure 4.16). The schematic was based on textbook metabolic pathway drawings, as illustrated in Figure 2.4. Results of statistical analysis and scale were omitted for clarity; however, related flux pathways were shown at similar scales.
Figure 4.14 The flux from asparagine to aspartate and oxaloacetate decreases significantly at state 3, while a positive flux from glutamine to glutamate and α-ketoglutarate only occurs at state 3.

Duplicate continuous cultures proceeded through four steady states with varying glutamine concentrations, where state 1 = 8 mM glutamine, state 2 = 0 mM glutamine, state 3 = 8 mM glutamine and state 4 = 0 mM glutamine (blue, green, white and pink respectively). A sample of culture supernatant was made cell-free and frozen at -20°C before assay of amino acids (Section 2.11). The results were combined with other metabolic parameters in a metabolic flux analysis model and key fluxes were predicted (Section 2.12). The change in the fluxes at each steady state was assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism (*=p<0.05, **=p<0.01, ***=p<0.001). J represents the flux and abbreviations are noted elsewhere. J_{aa-glu} is the sum of the fluxes from histidine, arginine and proline to glutamate, J_{aa-succ} is the sum of the fluxes from methionine, isoleucine, threonine and valine to succinyl-CoA, J_{aa-acoa} is the sum of the fluxes from lysine, isoleucine, leucine and tyrosine to acetyl-CoA. Results are expressed as the mean ± SEM (error bars). At points where no error bar is shown, the SEM was within the size of the symbol.
Duplicate continuous cultures proceeded through four steady states with varying glutamine concentrations in the CD-CHO medium, where state 1 = 8 mM glutamine, state 2 = 0 mM glutamine, state 3 = 8 mM glutamine and state 4 = 0 mM glutamine (blue, green, white and pink respectively). A sample of culture supernatant was made cell-free and frozen at -20°C before assay of amino acids (Section 4.2.2). The results were combined with other metabolic parameters in a metabolic flux analysis model and key fluxes were predicted (Section 2.12). The change in the fluxes at each steady state was assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism (*=p<0.05, **=p<0.01, ***=p<0.001). For clarity, the flux from amino acids to HCG is plotted on the secondary y-axis. J represents the flux and abbreviations are noted elsewhere. The determination of the relative contributions towards the biomass flux is detailed in Section 2.11, as is the flux towards HCG. The amino acid fluxes were calculated by summing the specific rates of all amino acids used, with the exception of cysteine and proline, while the flux towards CO₂ was assumed to have derived from amino acid metabolism only. Results are expressed as the mean ± SEM (error bars). At points where no error bar is shown, the SEM was within the size of the symbol.
Figure 4.16 Glycolytic flux and lactate production decreases at states 3 and 4. At state 3 the influx to the TCA cycle at oxaloacetate decreases, as do the subsequent 3 fluxes within the TCA cycle. The flux from pyruvate to alanine increases in a glutamine-dependent manner, while the flux to HCG decreases as the time in culture increases.

Duplicate continuous cultures proceeded through four steady states with varying glutamine concentrations in the CD-CHO medium, where state 1 = 8 mM glutamine, state 2 = 0 mM glutamine, state 3 = 8 mM glutamine and state 4 = 0 mM glutamine (blue, green, white and pink respectively) A sample of culture supernatant was made cell-free and frozen at -20°C before assay of amino acids (Section 2.11). The results were combined with other metabolic parameters in a metabolic flux analysis model and key fluxes were predicted (Section 2.12). Results of statistical analysis and scale are omitted for clarity; however, related flux pathways are shown at similar scales. Relative values are shown in Figures 4.11 through 4.15. The flux between pyruvate and acetyl-CoA and alanine are also shown in blue boxes at a different scale in order to illustrate the relative changes at each steady state. AA denotes amino acids, and abbreviations are noted elsewhere. Results are expressed as the mean ± SEM (error bars). At points where no error bar is shown, the SEM was within the size of the symbol.
4.3.5 Total HCG content increases at state 3, but productivity remains stable during culture

Figure 4.17 shows that the amount of extracellular HCG produced during the culture time increased at steady state 3 to a value of 443 ± 3 IU/ml. This value was found to be significantly higher than that at steady state 1 only (p<0.01).

However, this did not translate into a higher specific productivity of HCG at state 3 since an increase in viable cell number was also present during this state in culture. No significant change in productivity was noted during culture, despite the decreasing trend (Figure 4.18; from 44 ± 4 µIU/VC/day at state 1 to 30 ± 5 µIU/VC/day at state 4).

4.3.6 The increase in the proportion of cells in the G1 phase of the cell cycle from state 1 to 3 is accompanied by a corresponding decrease in G2/M

The proportion of the cell population in the G1 phase of the cell cycle was found to increase significantly from state 1 to 2 and 3, although there was no significant change between states 3 and 4 (Figure 4.19, from 54.5 ± 1.3 % in state 1 to 62.0 ± 1.0 % in state 4). No changes were seen in the percentage of the population in the S phase, however the G2/M phase distribution decreased significantly in line with the G1 increase. This indicated a direct shift of cells from the G1 phase to G2/M phase.

4.3.7 Intracellular UTP, CTP and UDP-Gal increases at state 4 while UDP-GlcNAc decreases

Both UDP-Gal and CTP steadily increased in quantity during the four states to become significantly increased at state 4 (from 0.028 fmol/VC to 0.039 fmol/VC for UDP-Gal from state 1 to 4, while CTP increased from 0.068 fmol/VC at state 1 to 0.108 ± 0.005 fmol/VC at state 4, Figure 4.20). UTP was also found to increase steadily through the 4 states to reach a maximum value of 0.433 fmol/VC at the final state (Figure 4.21). UDP-GlcNAc followed a similar increasing trend from states 1 through 3, but the value decreased significantly at state 4 to 0.083 ± 0.007 fmol/VC (from 0.155 ± 0.009 fmol/VC at state 3, Figure 4.21).
Duplicate cultures were established, and a volume of culture supernatant was taken daily from the bioreactor. This supernatant was made cell-free by centrifuging at 1000 g for five minutes and filtering through a 0.2 μm filter. Samples were stored at -20°C before analysis of various samples for extracellular HCG content by ELISA. This was carried out by the quality unit at MSD, Oss, the Netherlands. The vertical dashed lines indicate the time points at which the glutamine concentration in the medium was changed from an initial concentration of 8 mM to 0 mM, 8 mM and 0 mM, as described in Table 4.1. The green boxes indicate the steady states examined. The change in the extracellular HCG content at each steady state was assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism (significance not indicated, but p < 0.01).
Figure 4.18 The specific productivity of HCG is stable during the culture time.

A sample was taken from duplicate cultures each day was treated and stored according to Section 4.2.2. At the time points shown, the extracellular HCG content was assayed by ELISA (Figure 4.17). The specific productivity of HCG by these cells in μIU per viable cell per day was calculated according to Section 2.9.2 (A and B). The vertical dashed lines indicate the time points at which the glutamine concentration in the medium was changed from an initial concentration of 8 mM to 0 mM, 8 mM and 0 mM, as described in Table 4.1. The green boxes indicate the steady states examined. The change in the extracellular HCG content at each steady state was assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. There were no significant changes in productivity at any steady state.
Figure 4.19 As the cell population proceeds from state 1 to 3, a significant increase in the proportion of cells in the G1 phase of the cell cycle is accompanied by a decrease in the percentage of cells in G2/M.

Duplicate continuous cultures proceeded through four steady states with varying glutamine concentrations in the CD-CHO medium, where state 1 = 8 mM glutamine, state 2 = 0 mM glutamine, state 3 = 8 mM glutamine and state 4 = 0 mM glutamine. 2 x 10⁶ cells were harvested in triplicate for both cultures on two consecutive days at each steady state. The percentage of cells in each phase of the cell cycle was determined by flow cytometry analysis (Section 2.12) and the mean value ± SEM was calculated. These values were compared across steady states 1, 2, 3 and 4 (blue, green, white and pink, respectively). Statistical analysis was performed by one-way ANOVA followed by post-hoc Bonferroni analysis using Prism (* = p < 0.05, **=p<0.01, ***=p<0.001).
Duplicate continuous cultures proceeded through four steady states with varying glutamine concentrations in the CD-CHO medium, where state 1 = 8 mM glutamine, state 2 = 0 mM glutamine, state 3 = 8 mM glutamine and state 4 = 0 mM glutamine. 3 x 10^6 cells were harvested in duplicate for both cultures on two consecutive days at each steady state. The amount of ADP, UDP-Gal, UDP-GalNAc, UDP, GDP, AMP and CTP was determined via RP-HPLC (Section 2.14) and the mean value was expressed in fmol per viable cell ± SEM. These values were compared across steady states 1, 2, 3 and 4 (blue, green, white and pink, respectively). Statistical analysis was performed by one-way ANOVA followed by post-hoc Bonferroni analysis using Prism (* = p < 0.05, **=p<0.01, ***=p<0.001).
Figure 4.21 The intracellular content of UTP increases at state 4, while UDP-GlcNAc increases at state 3 before decreasing significantly at state 4.

Duplicate continuous cultures proceeded through four steady states with varying glutamine concentrations in the CD-CHO medium, where state 1 = 8 mM glutamine, state 2 = 0 mM glutamine, state 3 = 8 mM glutamine and state 4 = 0 mM glutamine. 3 x 10^6 cells were harvested in duplicate for both cultures on two consecutive days at each steady state. The amount of ATP, GTP, UTP, UDP-GlcNAc and UDP-Gluc/GDP-Man was determined via RP-HPLC (Section 2.14) and the mean value was expressed in fmol per viable cell ± SEM. These values were compared across steady states 1, 2, 3 and 4 (blue, green, white and pink, respectively). Statistical analysis was performed by one-way ANOVA followed by post-hoc Bonferroni analysis using Prism (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).
There was no distinctive shift in the IEF band pattern of HCG at any steady state (Figure 4.22). Additionally, there were no significant changes in the average pI of HCG at any state (Figure 4.23).

Detailed structural analysis of the N-linked glycans attached to HCG showed significant changes at state 4 (See Table 2.7 and Section 2.20.9 for details of N-linked glycan abbreviations). The percentage area of peaks 3, 4 and 8 increased during culture time, and the largest increase occurred at state 4 (Figure 4.24). A1G1 was seen to increase by 223% from 2.64 ± 0.60% in state 3 to 5.89 ± 0.97% in state 4 (peak 3). The amount of M5 was also seen to increase by a similar amount (217%) from state 3 to 4 (from 6.77 ± 1.01% to 14.70 ± 3.37%, peak 4). A significant increase in peak 8 (composed of A2G2 and FA2G1S1) occurred also (from 9.29 ± 1.31% in state 3 to 14.33 ± 2.08% in state 4).

Peaks 10 and 11 had a decreasing trend through states 1 to 4, with significance only noted between states 1, 2 and 4 (Figure 4.24). These peaks were composed of A2G2S2 / FA2G2S1 and A3G2S1 / FA2G2S2 and showed a 69% and 55% decrease in percentage area respectively (from state 3). An increase of note occurred in state 3 for peaks 12 and 13, with this value being significantly increased versus state 4. Peak 12 contained A3G2S2 and A3G3S1 and saw a shift from 5.54 ± 1.28% in state 3 to 2.23 ± 0.18% in state 4, while states 1 and 2 had a percentage area closer to 2.8%. Peak 13 (FA3G3S1 and A3G3S2) had an area of approximately 2.5% in states 1 and 2, rose to 3.77 ± 0.62% in state 3, and then significantly decreased to 1.23 ± 0.17% in state 4.

Peaks 14 and 15 showed a gradual decrease in percentage area from states 1 through 3, and a significant decrease is seen at state 4 (Figure 4.24). Peak 14, which was made of A3G3S3 and FA3G3S2 decreased from 1.50 ± 0.29% in state 3 to 0.57 ± 0.06% in state 4, which was a 38% drop. The percentage area of peak 15, which was composed of FA3G3S3, decreased by 33% from state 3 (0.90 ± 0.14%) to state 4 (0.30 ± 0.04%).

The overall changes in key structural features were estimated at each steady state. Table 4.3 illustrates that at state 4 a decrease in the sialylation, antennarity and fucosylation of the N-linked glycans attached to HCG was accompanied by an increase in proportion of neutral glycans. The proportion of diantennary structures was unchanged.
Figure 4.22 Varying glutamine concentration has no impact on the IEF pattern of HCG.

Duplicate continuous cultures proceeded through four steady states at varying glutamine concentration, where state 1 = 8 mM glutamine, state 2 = 0 mM glutamine, state 3 = 8 mM glutamine, and state 4 = 0 mM glutamine. Culture supernatant was harvested on two consecutive days at each steady state, and isoelectric focusing was carried out on HCG purified at each of these points. This figure shows a representative IEF profile of HCG at each steady state, where M denotes the migration pattern of the pI marker (from GE healthcare) while numerals 1 through 4 denote the steady state examined (corresponding to the states described above).
Figure 4.23 Varying glutamine concentration has no impact on the average pI of HCG.

Duplicate continuous cultures proceeded through four steady states at varying glutamine concentration, where state 1 = 8 mM glutamine, state 2 = 0 mM glutamine, state 3 = 8 mM glutamine, and state 4 = 0 mM glutamine. Culture supernatant was harvested on two consecutive days at each steady state, and isoelectric focusing was carried out on HCG purified at each of these points. The average pI of HCG for each steady state (numerals 1 through 4) was determined with reference to a pI marker by densitometry analysis of the IEF pattern; a representative profile is shown in Figure 4.22. Results are expressed as the mean ± SEM (error bars). Statistical analysis was performed by one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. However, no significant differences between samples were found.
Duplicate continuous cultures proceeded through four steady states at varying glutamine concentration, where state 1 = 8 mM glutamine, state 2 = 0 mM glutamine, state 3 = 8 mM glutamine, and state 4 = 0 mM glutamine. Results are expressed as the mean ± SEM (error bars). At points where no error bar is shown, the SEM was within the size of the symbol. Statistical analysis was performed by one-way ANOVA followed by post-hoc Bonferroni analysis using Prism (* = p < 0.05, **=p<0.01, ***=p<0.001). All triantennary structures were found to be a mixture where branch 1 was either in the β1-6 or β1-2 position. See Table 2.7 and Section 2.20.9 for details of N-linked glycan abbreviations.
Table 4.2 Estimates of N-linked glycosylation structural changes at various steady states.

<table>
<thead>
<tr>
<th></th>
<th>1 (8mM Gln)</th>
<th>2 (0mM Gln)</th>
<th>3 (8mM Gln)</th>
<th>4 (0mM Gln)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutral</td>
<td>42</td>
<td>46</td>
<td>50</td>
<td>70</td>
</tr>
<tr>
<td>Sialylated</td>
<td>87</td>
<td>84</td>
<td>80</td>
<td>71</td>
</tr>
<tr>
<td>Triantennary</td>
<td>30</td>
<td>28</td>
<td>28</td>
<td>13</td>
</tr>
<tr>
<td>Diantennary</td>
<td>91</td>
<td>92</td>
<td>88</td>
<td>96</td>
</tr>
<tr>
<td>Fucosylated</td>
<td>83</td>
<td>80</td>
<td>73</td>
<td>64</td>
</tr>
</tbody>
</table>

These values were determined by summing the percentage area of every peak that contained any given structure (for example, the proportion of neutral structures was estimated by addition of the mean percentage area of peaks 1 through 5 and 7 through 9, since each of these peaks has a neutral component). States 1 and 3 were supplemented with 8 mM glutamine, while states 2 and 4 had none (Gln = glutamine).
4.3.9 Impact of deviations from setpoint

Table 4.1 lists the deviations that occurred during the culture time period. An assessment of any differences in the cultures around these times indicated whether any significant impact occurred.

One deviation occurred for the A culture on day 61, where the agitation rate of the stirrer was found to be 30 rpm over the set point. This may lead to changes in the cultures due to increased oxygen availability or shear stress. However, from a metabolic and glycosylation perspective, both cultures reached the same steady state. This indicated that this deviation was not significant.

The B culture encountered a total of 3 deviations from set point. Two of these occurred before the first steady state was reached. On day 7, the feed and harvest lines were mistakenly blocked overnight which meant the culture experienced batch-like conditions for this time. This was likely to impact the cells, but more than five residence times of the medium within the bioreactor occurred before a steady state was attained – indicating that sufficient time was allowed for any impact to become apparent. However, the same steady state was achieved for the duplicates indicating that the deviation had no impact.

A temperature increase of one degree occurred for one hour on day 17 for the B culture due to a malfunctioning air conditioning unit in the room where the bioreactors were housed. This was considered to have no impact due to the short length of time and the small shift from set point.

On day 33, a blockage in the feed line caused the culture level to drop to 1.4 L since the harvest pump continued to remove cell suspension during this time. This is likely to alter the cells, but the shift to the next condition was carried out the same day, which gives the cells sufficient time to display any differences from the accompanying replicate before the next steady state. Both cultures achieved identical steady states, indicating that the deviations in the B culture were of no consequence.
4.4 Discussion

4.4.1 Glutamine and the growth of CHO cells

It is evident that glutamine was not required for the growth of this cell line in CD-CHO medium. Cell growth occurred irrespective of whether the culture was initiated at 8 mM glutamine or 0 mM glutamine for the experimental time period (Chapter 3). The growth rate was reduced in the batch experiments at 0 mM glutamine, suggesting that this may have been responsible for the changes in glycosylation (Figure 3.3 and 3.20). The specific growth rate was controlled at 0.4/d by the continuous culture system used here, and no glycosylation changes were seen between states 1 and 2. Consequently, these experiments prove that in the presence of varying glutamine concentrations, the glycosylation effects were not due to changes in growth. Additionally, reports suggest that in some cell lines, glutamine plays an important role in the generation of energy by flux through the TCA at α-ketoglutarate (Reitzer et al., 1979; Neermann and Wagner, 1996). These results indicate that this flux was minimal in this cell line under normal cultivation conditions, where glucose is non-limiting (at steady state 1).

4.4.2 Metabolic fluxes at steady states 1 and 2

The first two steady states examined the effect of varying glutamine at excess glucose concentrations. Circulating concentrations of glucose were above 2 mM at both these steady states, and the specific glucose consumption rate was above 3 pmol/VC/day (Figures 4.4 and 4.5). No changes in the glycosylation of HCG or in the intracellular concentrations of nucleotide or sugar nucleotides were detected (Figures 4.20 through 4.24). Metabolic flux analysis illustrated how the cells adapt under these conditions to the lack of glutamine. A schematic of the changing direction of fluxes around glutamine, glutamate and α-ketoglutarate at each steady state is shown in Figure 4.25.

At the initial steady state where glutamine was supplied, the mean flux from glutamine towards glutamate and the TCA cycle was zero, while the a positive flux to biomass was determined (Figure 4.14 and 4.15). This suggests that glutamine was used purely for the synthesis of nucleotides and nicotinamide coenzymes at this time. With the removal of glutamine at the second steady state, the flux from α-ketoglutarate to glutamate to glutamine was positive. This raises the possibility that the synthesis of glutamine occurred at this state to maintain the flux from glutamine to biomass.
Figure 4.25 Schematic of the changing direction of glutamine fluxes at each steady state.

Arrows indicate the direction of flux, while the black arrow at state 1 indicates that the mean flux between glutamine and glutamate is zero. (TCA cycle = Tricarboxylic acid cycle, αKG = α-ketoglutarate, Glu = glutamate, Gln = glutamine).
A significant decrease in the flux from pyruvate to alanine was detected at state 2 (Figure 4.12). The concentration of ammonium also decreased significantly at this steady state (Figure 4.7). It has previously been found that transamination of glutamate and pyruvate to alanine and α-ketoglutarate by alanine aminotransferase occurs when CHO cells are incubated with excess glutamine (Street et al., 1993). This is a method of detoxifying ammonium, since maintaining the amino group within alanine prevents release of an additional ammonium molecule, by the direct deamination of glutamate by glutamate dehydrogenase (Schneider et al., 1996). The increased flux to alanine with glutamine supplementation at state 1 indicated that this CHO cell line might be carrying out some level of ammonium detoxification at high ammonium concentrations. Alternatively, at high glucose consumption and lactate production rates, the metabolism of cells in culture has been found to 'overflow' towards unnecessary production of amino acids, such as alanine. This overflow metabolism might explain the increased flux towards alanine under these conditions.

In conclusion, at non-limiting glucose concentrations, very minor changes to metabolism occurred when glutamine was removed from culture medium.

4.4.3 Metabolic fluxes at steady states 3 and 4

Upon reintroduction of 8 mM glutamine at the third steady state, the circulating concentration of glucose dropped to approximately 0 mM (Figure 4.4). The decrease in glucose concentration was accompanied by a decrease in lactate production, which translated into a decrease in the flux from glucose to pyruvate to lactate (Figures 4.6 and 4.12). However, the flux from pyruvate to acetyl-CoA was unchanged (Figure 4.12). It has recently been shown that a decrease in glucose consumption rate occurs at reduced residual glucose concentrations in a continuous culture of BHK cells (Zielke et al., 1978; Cruz et al., 1999). The reduced flux towards lactate indicates that the metabolism of glucose is more efficient at this steady state. While this switch in metabolism was likely caused by the adaptation to the altering glutamine concentrations, the reasons for this were unclear. However, at this third steady state, there was a significant increase in the number of viable cells (Figure 4.1). This was achieved by increases in cell number during the adaptation phase of state 3, and suggested that the switch to more efficient metabolism also resulted in a more efficient use of this energy, since it was directed towards producing new cells.
A major difference in the fluxes around glutamine, glutamate and α-ketoglutarate were seen at steady state 3 (Figure 4.14). In contrast to the null flux from glutamine to glutamate at state 1, in this case there was a direct flux from glutamine to glutamate and α-ketoglutarate (Figure 4.25). Literature indicates that under limiting glucose concentrations, glutamine consumption can increase to fuel the TCA cycle, which ensures that there is no reduction in energy generation (Ljunggren and Haggstrom, 1994; Wong et al., 2005; Maranga and Goochee, 2006). This is a likely explanation of the consumption of glutamine to fuel the TCA cycle at this steady state.

While the consumption rate of glutamine did not change between states 1 and 3, the concentration of ammonium was at its highest in state 3 (Figure 4.7 and 4.9). This was also observed when PER.C6 cells were grown under glucose limited conditions, and indicated that glutamate deamination occurred rather than transamination, in order to generate more energy (Maranga and Goochee, 2006). In these experiments, the flux from pyruvate to alanine was significantly increased relative to the previous state, but the mean flux was comparable to that observed in state 1 (Figure 4.12). As a result, it is probable that glutamate was being transaminated to alanine and α-ketoglutarate at a similar degree to state 1, but an additional amount was being directly deaminated to α-ketoglutarate, which resulted in the elevated ammonium concentration. This would provide a balance between energy generation by direct deamination and ammonium detoxification by transamination.

A significant reduction in the flux from asparagine to aspartate and oxaloacetate was also observed at state 3 (Figure 4.14). A decrease in this flux pathway toward the TCA cycle resulted in an increase in the production of aspartate and asparagine. Aspartate is necessary for the formation of purine and pyrimidine nucleotides, so, formation at this state could be an indirect result of the change in glutamine status. However, the synthesis of aspartate is also considered to be a mechanism of ammonium detoxification, in a method similar to the formation of alanine from pyruvate. In this case, aspartate transaminase catalyses the conversion of glutamate and oxaloacetate to α-ketoglutarate and aspartate, maintaining the amino group within aspartate (Street et al., 1993). As a result, the increase in flux towards aspartate and asparagine may be due to its requirement in nucleotides, or as an additional method to minimise the toxicity of ammonium.

The reduction in flux from aspartate to oxaloacetate caused a significant impact on the subsequent fluxes through the TCA cycle. The flux from oxaloacetate to α-ketoglutarate
was significantly reduced during this steady state (Figure 4.13). Maintaining this deficit throughout the cycle is likely to result in significant impacts on the cell since this cycle is a major hub for the oxidation of carbohydrates, fatty acids and amino acids alongside the generation of numerous biosynthetic precursors. However, this was prevented by the formation of additional amounts of \( \alpha \)-ketoglutarate by the conversion of glutamine to glutamate (Figure 4.14). As a result, the flux of glutamine to glutamate and \( \alpha \)-ketoglutarate at this steady state was required for the optimal operation of the TCA cycle, and thus for optimal cellular metabolism. The input at the level of \( \alpha \)-ketoglutarate caused a significant increase in the two subsequent measured fluxes \( J_{\text{akg-succ}} \) and \( J_{\text{succ-fum}} \), while the final two fluxes to oxaloacetate were returned to previous levels (Figure 4.13).

The glutamine was removed from culture in state 4, which results in a doubly restricted culture due to the limiting glucose concentrations. Despite this, only minor changes to metabolism occurred – the fluxes to alanine, asparagine and aspartate, as well as the fluxes within the TCA cycle, were comparable to those determined at states 1 and 2 (Figures 4.12 through 4.14). Glutamine was again produced by flux from the TCA cycle at the level of \( \alpha \)-ketoglutarate, causing a decrease in ammonium concentrations (Figure 4.17 and 4.14). However, there were more dramatic effects on the amount of intracellular nucleotides and sugar nucleotides, and on the glycosylation of HCG. This is the subject of further discussion in Section 4.4.6.

**4.4.4 Glutamine and productivity**

Although the calculated specific productivity of HCG did not change significantly during the culture time, the decreasing trend led to a significant decrease in the calculated flux for its synthesis from amino acids at steady states 3 and 4 (Figure 4.15 and 4.17). The reduction in glucose consumption and lactate production at these steady states may also have impacted on the productivity (Figure 4.5 and 4.6). The productivity of tPA in a continuous cultivation of CHO cells has been found to vary with the concentration of glucose within the feed medium (Altamirano et al., 2000; Altamirano et al., 2001). This is in agreement with the trend observed here. However, a reduction in productivity is an expected consequence of the extended time in culture. In one reported instance, after 60 days of culture of a CHO cell line, the productivity was seen to decrease to 70% of its initial value (Pallavicini et al., 1990). Although a significant decrease in flux to HCG was observed, the lack of statistical change in the specific and volumetric productivity indicated that this trend was not as severe as other reported examples. Investigations have
shown that the loss in productivity can be as a result of an increase in the fraction of the non-producing cell population (Frame and Hu, 1990). This topic is explored further in Chapter 6, where an aliquot of cells from the last day of each continuous culture was re-inoculated in shake flasks. As a result, the growth, metabolism and productivity of this cell line was assessed after at least 60 generations of growth in varying culture conditions.

4.4.5 Glutamine and cell cycle progression

With the loss of glutamine at state 2, the percentage of cells in the G1 phase increased, while a corresponding decrease in G2/M was observed (Figure 4.19). However, it has been shown that CHO cells incubated in F-10 medium in the absence of glutamine and isoleucine are halted at G1 after just 30 hours. Isoleucine is required for the synthesis of glutamine, so these cells were incapable of synthesising glutamine for the formation of nucleotides required for DNA synthesis. However, re-introduction of both these amino acids restarted the cell cycle (Ley and Tobey, 1970; Wu, 2009). This fact has been exploited as a method to synchronise the cell cycle of cultured cancer cells, which have been found to consume large quantities of glutamine (Ellison et al., 2007). Since this experimental cultivation merely limited glutamine without impeding its synthesis from glutamate and other sources, a small increase in the percentage of cells at G1 was expected, and was observed.

However, at steady states 3 and 4, the proportion of cells in the G1 phase increased once more, and notably, there was no significant difference between the percentages determined at each state. Similarly, this was accompanied by a decrease in the percentage of cells in the G2/M phase (Figure 4.19). The link between the rate of glycolysis and cell cycle progression has been investigated by decreasing the concentrations of fructose-2,6-bisphosphate, by two separate mechanisms. Fructose-2,6-bisphosphate, which is a potent regulator of the glycolytic enzyme phosphofructokinase, is synthesised by phosphofructokinase-2 (PFK-2), and degraded by fructose-2,6-bisphosphatase (FBPase-2). Both overexpression of FBPase-2 and silencing of PFK-2 reduced the concentration of ATP and lactate, and resulted in a increase in the proportion of cells in the G1 phase of the cell cycle (Perez et al., 2000; Calvo et al., 2006). In a similar manner, this experiment caused a reduction in the flux through glycolysis while increasing the proportion of cells in the G1 phase of the cell cycle (Figure 4.19).
4.4.6 *Glutamine, sugar nucleotides and the glycosylation of HCG*

Statistical analysis has shown significant increases in the proportion of UDP-Gal, CTP and UTP and a decrease in UDP-GlcNAc at state 4 (Figures 4.20 and 4.21). For all of these structures, a trend of increasing amounts from state 1 to 4 was noted (with significance differences observed between some of the steady states), while UDP-GlcNAc decreased only at state 4.

As mentioned in the introduction, the synthesis of UTP, as well as its conversion to CTP, requires glutamine (Figure 1.12). Additionally, the final conversion of Gal-1-P to UDP-Gal, the activated donor for galactose incorporation to N-linked glycans, requires UTP. So there is a logical link between intracellular UTP, CTP and UDP-Gal content. Glutamine limitation is known to increase the concentration of intracellular nucleotides (Nyberg *et al.*, 1999). However, the reason for the trend of increasing proportion of these structures during culture is harder to decipher. UTP and CTP are both necessary precursors for the synthesis of DNA, and as such are key molecules for the maintenance of a cell. It is possible that the flux from glutamine to these nucleotides needs to be preserved, and even increased, with the variations in glutamine status during cultivation.

Glutamine also has a direct impact on the synthesis of UDP-GlcNAc. It has been well established that under *in vitro* conditions, both ammonium and glutamine are incorporated into UDP-GlcNAc by the action of glucosamine-6-phosphate isomerase and glucosamine-6-phosphate synthetase, respectively (GPI, GPS). Consequently, a significant increase in the UDP-GNAc pool is observed (Rijcken *et al.*, 1995a; Grammatikos *et al.*, 1998; Gawlitzek *et al.*, 1999; Valley *et al.*, 1999). In addition, the accumulation of the UDP-GNAc pool requires the consumption of glucose, since the initial substrate in the pathway, fructose-6-phosphate, is formed in glycolysis (Ryll *et al.*, 1994). As a result, the accumulation of this pool is doubly restricted at low concentrations of ammonium and glucose, and in the absence of glutamine. This decrease in the UDP-GNAc pool in response to both glucose and glutamine limitation has been demonstrated in CHO cells (Nyberg *et al.*, 1999). This process was also observed at state 4 in this culture (Figure 4.21).

Isoelectric focusing failed to detect changes in glycosylation at any state, as measured by changes in the calculated average pi (Figure 4.22 and 4.23). However, a more detailed and sensitive analysis of the N-linked glycan sugars attached to HCG yielded more insight (Figure 4.23). The significant changes were observed at state 4, where increases in peaks 3,
4 and 8 were accompanied by decreases in peaks 10 through 15. Peaks 3 and 4 were composed of homogeneous pools of A1G1 and M5, respectively. This indicated a decrease in the proportion of neutral / under processed glycans. Peak 8 was also found to increase, and contained a pool of A2G2 and FA2G1S1. Peak 10, which was found to decrease, was formed of A2G2S2 and FA2G2S1. So it seems that the changes in these two peaks were related - by increasing A2G2 in peak 8 and decreasing A2G2S2 in peak 10. Peaks 12 and 13, which contained a mixture of A3G2S2, A3G3S1 and FA3G3S1, A3G3S2, respectively showed an unusual, non-significant increase in state 3, followed by a significant decrease in state 4. Peaks 11, 14 and 15 showed a significant decrease at state 4 and were composed of A3G2S1, FA2G2S2 (peak 11), A3G3S3, FA3G3S2 (peak 14) and FA3G3S3 (peak 15). Since peaks 10 and 11 were two of the three largest peaks in the pool, the decrease in the percentage area at state 4 is significant. As a result, the changes in glycosylation at state 4 can be described overall as an increase in the proportion of neutral / under processed glycans and decrease in sialylation and antennarity. Additionally, Table 4.3 illustrates that the fucosylation of HCG decreases at this steady state.

The changing proportion of the nucleotide and sugar nucleotides can explain these changes in glycosylation. It has previously been shown that an increase in the UDP-GNAc pool caused by elevations in ammonium concentrations can either result in an increase (Grammatikos et al., 1998; Cayli et al., 1999; Valley et al., 1999) or a decrease in the antennarity of N-linked glycans (Yang and Butler, 2002). In this case, the observed decrease in triantennary structures at state 4 corresponded with a decrease in the UDP-GlcNAc concentration. So, a lack of substrate for GlcNAc transferases, which catalyse the addition of branches of sugars via the addition of N-acetylglucosamine, would lead to a decrease in antennarity.

A large number of papers show that increased ammonium causes a decrease in sialylation (Thorens and Vassalli, 1986; Andersen and Goochee, 1995; Rijcken et al., 1995a; Gawlitzek et al., 1998; Grammatikos et al., 1998; Gawlitzek et al., 2000; Yang and Butler, 2000b; Yang and Butler, 2000c; Yang and Butler, 2002; Gillmeister et al., 2009). Despite the increase in CTP, which is a precursor for sialic acid synthesis, sialylation was found to decrease in the absence of glutamine in state 4. However, it has previously been noted that an increase in CTP does not necessarily lead to increased CMP-NeuAc levels (Rijcken et al., 1995a). This may have occurred here, but this fact could not be verified since the technique used to assay intracellular concentrations of nucleotide and nucleotide sugars
was unable to detect CMP-NeuAc in CHO extracts. However, UDP-GlcNAc is also a precursor in the synthesis of CMP-NeuAc. The observed decrease in this sugar nucleotide would also cause a reduction in the amounts of intracellular CMP-NeuAc, leading to decreased sialylation.

A fed-batch cultivation of CHO cells overexpressing IFNγ has shown similar results. Limiting either glucose or glutamine concentrations to less than 1 mM impaired the transition from N-linked mannose glycan structures to complex structures, decreased the efficiency of sugar addition to bi-antennary and tri-antennary structures and decreased the sialylation of expressed IFNγ. This effect was most pronounced at limited glucose concentrations. The authors postulate that the initial lack of complex structures coupled with a theoretical reduction in UDP-GlcNAc would cause these effects on glycosylation (Wong et al., 2005). A more recent publication has shown that the expression of the genes encoding sialyltransferase III and VI, as well as UDP-GlcNAc-2-epimerase are reduced with low glutamine feeding of fed-batch cultures of CHO cells (Wong et al., 2010a).

This work has shown that under glucose and glutamine limited conditions, a decrease in the intracellular concentration of UDP-GlcNAc resulted in a reduction in the sialylation and antennarity of N-linked glycans.

4.4.7 *Steady state multiplicity*

Despite the fact that the same dilution rate and feed concentration was used in states 1, 3 and 2, 4, a major difference was seen in the cell metabolism and glycosylation between the states. At states 3 and 4, the residual concentration of glucose was significantly reduced to a mean value of 0 mM, and the flux from glucose to pyruvate and lactate decreased similarly. This led to alterations in the metabolism of glutamine and of the TCA cycle at these states. This concept of different steady states at the same cultivation conditions is known as steady state multiplicity. It has been shown that the conditions preceding a steady state can alter the resulting metabolism. Cells grown in a fed-batch manner of cultivation preceding the continuous phase of culture were found to have a much lower internal metabolic fluxes at steady state due to the more efficient production and use of energy (Europa et al., 2000; Gambhir et al., 2003). The mechanisms by which these alternative states are reached has also been explored, and significant decreases on the genomic and proteomic level of the enzymes of central metabolism were observed (Korke
et al., 2004). It is likely that this multiplicity arose as a result of the cellular adaptation to the loss of glutamine, although the reasons for this require further exploration.

4.4.8 Conclusions

In a CHO culture cultivated with non-limiting glucose concentrations, varying glutamine had no impact on the N-linked glycosylation (steady states 1 and 2). Minor metabolic adaptations occurred during these states to accommodate the lack of glutamine. In this case, an increase in alanine production occurred at state 1, either as a result of inefficient metabolism or in order to detoxify excess ammonium. At state 2, glutamine was synthesised from α-ketoglutarate.

The adaptation to the lack of glutamine at state 2 induced a case of steady state multiplicity. Despite the fact that the cells were cultivated at the same operating parameters (dilution rate and feed concentration) in states 3 and 4, a different metabolism resulted. Most significantly, the flux of glucose to pyruvate and lactate decreased at both states. At state 3, the number of viable cells was increased, while ammonium concentrations increased. This induced synthesis of aspartate as well as alanine, which caused a significant decrease in the TCA cycle flux from oxaloacetate to α-ketoglutarate. As a result, glutamine was converted to glutamate and α-ketoglutarate in order to rescue the TCA cycle. The lack of glutamine and limiting glucose at state 4 caused a significant decrease in intracellular UDP-GlcNAc. As a result, the proportion of triantennary structures decreased. Despite the increase in CTP, a decrease in sialylation was observed, which was probably caused by the decrease in UDP-GlcNAc, since this is a precursor for the synthesis of CMP-NeuAc. In conclusion, significant changes glycosylation caused by glutamine are only detected in a condition of glucose limitation.

The cell cycle distribution was found to alter with the glutamine status. An increase in the proportion of cells in the G1 phase was seen in state 2, with an accompanying decrease in G2/M. At states 3 and 4, the reduction in glucose flux caused a further increase in the percentage of cells in G1.

A decrease in the flux towards production of HCG was noted in states 3 and 4. This may have been caused by the increased number of generations of the cell line, or as a result of the decrease in glucose consumption at this states.
Chapter 5

Investigating the Effect of Varying Dilution Rate on CHO Metabolism and HCG Glycosylation in a Continuous Mode of Culturing
5.1 Introduction

Altering the rate of protein transit through the Golgi by a variety of methods can result in significant differences in the N-linked glycosylation. Inhibiting the process of protein synthesis with use of cycloheximide has been found to increase the occupancy of the potential N-linked glycosylation sites of secreted prolactin in C127 cells (Shelikoff et al., 1994). Reducing the culture temperature has also been found to decrease the specific growth rate alongside an inhibition of Golgi transit (Wang et al., 1991; Ahn et al., 2008). The cellular metabolism has also been observed to decrease at low culture temperatures (Jenkins and Hovey, 1993; Chuppa et al., 1997; Moore et al., 1997; Bollati-Fogolin et al., 2005; Berrios et al., 2007). An increase in the volumetric and specific productivity is usually observed at low temperatures despite these metabolic changes (Jenkins and Hovey, 1993; Kaufmann et al., 1999; Yoon et al., 2005; Galbraith et al., 2006; Trummer et al., 2006; Ahn et al., 2008). In a similar manner, reducing the ATP content by inhibiting oxidative phosphorylation has also been found to decrease the rate of Golgi transit (Datema and Schwarz, 1981). Use of 2-deoxyglucose to inhibit glycolysis has been found to inhibit the formation of lipid linked oligosaccharides (Kurtoglu et al., 2007).

Varying the dilution rate in a continuous culture set up is an ideal method to study the effect of varying growth rate, and thus the rate of protein transit through the Golgi, on the metabolism and glycosylation of this CHO cell line. Decreasing the dilution rate has been shown to reduce the viability of cells due to an apoptosis-induced increase in the death of cells (Miller et al., 1988; Frame and Hu, 1991; Hayter et al., 1993; Lee et al., 1995; Majid et al., 2007). However, a minimal growth rate is necessary for cell survival, as illustrated by the observation that at low dilution rate the growth rate deviates from the dilution rate (Miller et al., 1988; Hayter et al., 1993; Martens et al., 1993). The metabolism of cells tends to be more efficient at low dilution rates, as demonstrated by the decrease in glucose and glutamine consumption which are also accompanied by decreases in lactate production (Miller et al., 1988; Hayter et al., 1993; Follstad et al., 2000). An increase in the percentage of cells in the G1 phase of the cell cycle with proportional decrease of cells in the S phase has also been noted at decreasing dilution rates (Martens et al., 1993). The productivity of recombinant proteins is seen to increase at low dilution rates (Miller et al., 1988; Frame and Hu, 1991; Martens et al., 1993).

Varying the dilution rate has been found to impact on N-linked glycosylation. A decrease in the proportion of glycosylated recombinant interferon-γ is accompanied by a
proportional increase in the non-glycosylated fraction at dilution rates lower than 0.3/d for a CHO cell cultivation (Hayter et al., 1993). However, this occurred alongside glucose and glutamine limitation, which suggests that the effect may not be entirely as a result of the dilution rate in this case. Galactosylation of recombinant IgG was also significantly reduced at low dilution rates (Majid et al., 2007).

Aim

This chapter examines the effect of varying the dilution rate on CHO metabolism, cell cycle distribution, intracellular concentrations of nucleotides and sugar nucleotides and the glycosylation of HCG.

5.2 Methods

5.2.1 Medium preparation and preculture

Basal CD-CHO medium supplemented with 8 mM glutamine was prepared in 10 L batches by the service group of P & MSc - CCPD (Process & Manufacturing Sciences - Cell Culture Process Development) in MSD, Oss, the Netherlands. The medium also contained HT supplement (100 μM sodium hypoxanthine, 16 μM thymidine). Cells were precultured in 250 ml and 1 L shake flasks while maintaining the cell density between 0.3 and 2 x 10^6 cells/ml. After 18 days of preculture, sufficient cells were generated to inoculate duplicate bioreactors according to the conditions detailed in Section 2.8. These experiments were carried out in the small scale cell culture process development lab, MSD, Oss, the Netherlands.

5.2.2 Experimental culturing and analyses

Three days after the inoculation of cells in bioreactors, the perfusion of medium began at a rate of 0.3/d for one day before reducing to 0.2/d. Once a steady state had been attained (state 1, as defined in Section 2.8.1) the dilution rate was increased in one step to 0.5/d, by increasing the flow rate of culture out of the system. By alternating the dilution rate between these two values, four steady states were examined. It was intended that returning the cultures to 0.2/d and 0.5/d in the second instance would provide duplicate results for analysis. The time points of these changes in dilution rate and deviations of any parameters from setpoint are described in Table 5.1. At steady state, the growth rate equals the dilution rate, and as such, these experiments study varying growth rate.
Each day, 10 ml of culture was withdrawn from the bioreactor and a number of different analyses were carried out. The viability and number of viable cells was established with use of the CEDEX AS\textsuperscript{20}, the concentrations of ammonium, glucose, glutamate, glutamine and lactate were assayed with use of the Nova Bioprofile 100 plus and BGA parameters (pH, pO\textsubscript{2}, and pCO\textsubscript{2}) were determined with the Bayer Rapidlab 248. Excess sample was centrifuged at room temperature for 5 minutes at 1000 g and subsequently filtered through a 0.2 μm filter. These cell-free supernatants were stored at -20°C for further analysis.

Once a steady state had been reached, extra samples were taken for analysis of extracellular amino acid concentrations (Section 2.11), cell cycle distribution (Section 2.13) and intracellular nucleotide and sugar nucleotide content (Section 2.14). Additionally, a new harvest line was attached to the bioreactor to collect cell suspension for purification of HCG and subsequent assessment of the glycosylation of HCG by IEF and analysis of N-linked glycan structures (Sections 2.15, 2.19 and 2.20, respectively). This was carried out for two consecutive days during the steady state period.

Steady states were defined for the following time periods; days 38 – 41 (state 1), days 66 – 69 (state 2), days 100 – 103 (state 3), days 127 – 130 (state 4A) and days 137-140 (state 4B). The metabolism of the cells was assessed over these periods using metabolic flux analysis, where fluxes were predicted using the previously described metabolic flux model and the calculated rates of changes in the consumption and production of key metabolites. Additional analysis of cell cycle distribution, intracellular nucleotide and sugar nucleotide content and supernatant harvest for glycosylation analysis was carried out on the final two days only.
<table>
<thead>
<tr>
<th>Day</th>
<th>A Remark</th>
<th>Day</th>
<th>B Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Perfusion of medium begins at 0.2/d</td>
<td>3</td>
<td>Perfusion of medium begins at 0.2/d</td>
</tr>
<tr>
<td>4</td>
<td>D decreased in one step to 0.2/d</td>
<td>37</td>
<td>Temp. reduction (min. 34°C)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>38</td>
<td>Culture level was 1.5L</td>
</tr>
<tr>
<td>41</td>
<td>D increased in one step to 0.5/d</td>
<td>41</td>
<td>D increased in one step to 0.5/d</td>
</tr>
<tr>
<td>52-54</td>
<td>Culture level was 1.5L</td>
<td>52-56</td>
<td>Culture level was 1.5L</td>
</tr>
<tr>
<td>60</td>
<td></td>
<td>60</td>
<td>Low pO₂ flow (15%)</td>
</tr>
<tr>
<td>69</td>
<td>D decreased in one step to 0.2/d</td>
<td>69</td>
<td>D decreased in one step to 0.2/d</td>
</tr>
<tr>
<td>103</td>
<td>D increased in one step to 0.5/d</td>
<td>103</td>
<td>D increased in one step to 0.5/d</td>
</tr>
<tr>
<td>108</td>
<td>Culture level was 1.6L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>117</td>
<td>Culture level was 1.5L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>130</td>
<td>Culture stopped</td>
<td>130</td>
<td>Temp. reduction (min. 35°C)</td>
</tr>
<tr>
<td>140</td>
<td></td>
<td>140</td>
<td>Culture stopped</td>
</tr>
</tbody>
</table>

Table 5.1 Timeframe for continuous experiments with varying dilution rate.

A and B denote the name of the individual culture. Purposely induced changes are given in bold, while unintended deviations from set point are shown with normal text formatting. The impact of the deviations is discussed in the Section 5.3.9. Due to the large number of deviations in both cultures between days 52 and 56, the results of state 2 are treated individually (termed states 2A and 2B). The culture level set point was 1.7 L and D represents the dilution rate.
5.3 Results

5.3.1 Reducing the dilution rate decreases cell viability and alters the viable cell number

The number of viable cells increased at the start of cultivation and stabilised from day 6 to reach a mean value of $9.1 \pm 0.2 \times 10^6$ cells/ml at state 1 (Figure 5.1). As a result of the lag in change of the specific growth rate as a response to the increased dilution rate on day 41, a washout of cells occurred. This caused a reduction in the cell number, with the minimum value reached on day 53 for the A culture, and day 47 for the B culture. On day 45, a growth rate of 0.5/d was achieved. Due to the problems in maintaining the culture level in state 2, a difference in the number of viable cells at steady state was observed for the duplicate cultures (termed state 2A and 2B). The reason for the problems in maintaining culture level is discussed in Section 5.3.9. The mean viable cell number decreased significantly relative to the first steady state for the A culture, while this value was statistically increased for the B culture (to $6.4 \pm 0.1 \times 10^6$ cells/ml and $10.9 \pm 0.4 \times 10^6$ cells/ml, respectively, $p<0.001$ for both, Figure 5.1).

The dilution rate was reduced on day 69 and the viable cell number decreased gradually for both cultures after this time. This was likely to have occurred due to the length of time required for the specific growth rate to stabilise at 0.2/d, approximately 5 days in this case. At state 3, an identical steady state was reached by both cultures, as regards viable cell number (at $5.7 \pm 0.2 \times 10^6$ cells/ml, not significantly different from state 2A, Figure 5.1).

On day 103, the dilution rate was increased to 0.5/d. Two days after this, the specific growth rate approached the new dilution rate. The specific growth rate exceeded the setpoint until day 109, which resulted in an increase in the viable cell number as a response to the higher dilution rate. Both cultures reached a similar mean viable cell number at state 4, although these values were not attained at the same time point ($5.9 \pm 0.1 \times 10^6$ cells/ml, Figure 5.1). This value was not significantly different from the previous steady state.

The percentage viability of the cells was above 97% at states 2A, 2B and 4 (Figure 5.1). However, the cell viability decreased at low dilution rate (states 1 and 3). Additionally, there was a significantly reduced mean value at state 3 ($p<0.001$, $89.2 \pm 0.4\%$ at state 3 compared to $94.0 \pm 0.4\%$ at state 1). The calculated specific growth rate stabilised at values close to the setpoint at each steady state (Figure 5.2). The dilution rate was controlled accurately with a close agreement of the setpoint and measured values throughout cultivation (Figure 5.3).
Figure 5.1 Reducing the dilution rate lowers the viability of cells while also changing the viable cell number.

Each day, a sample of culture supernatant was withdrawn from the bioreactor and 500 µl was diluted 1:1 with trypsin for automated determination of the viable cell number and percentage viability using the CEDEX AS^o. The viable cell numbers of duplicate cultures (VC A and VC B) are shown. The percentage viability for both cultures is plotted on the secondary y-axis (% Viability A and % Viability B). The vertical dashed lines indicate the time points at which the dilution rate was changed from an initial value of 0.2/d to 0.5/d, 0.2/d and 0.5/d, as described in Table 5.1. The dilution rate is defined as the flow rate per bioreactor volume and the green boxes indicate the steady states examined. The change in the number of viable cells at each steady state was assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. No significant difference was found between states 2A, 3 and 4 (Significance not indicated, but p < 0.001).
Following the daily determination of viable cell numbers (Figure 5.1), the specific growth rate was calculated for duplicate cultures (A and B) according to Section 2.9.2 The vertical dashed lines indicate the time points at which the dilution rate was changed from an initial value of 0.2/d to 0.5/d, 0.2/d and 0.5/d, as described in Table 5.1. The green boxes indicate the steady states examined. The change in the specific growth rate at each steady state was assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. No significant difference was found between states 2A, 2B and 4 or states 1 and 3 (Significance not indicated, but p < 0.001).
Duplicate cultures were established and were allowed to run in batch mode for three days. At this time, the perfusion of medium was begun at a rate of 0.3/d for one day, before decreasing to 0.2/d. This was achieved with the use of Watson Marlow 101U/R pumps for pumping new medium into the reactor (the feed), as well as culture supernatant out of the bioreactor (the harvest). The harvest pump ran continuously (and is therefore considered leading), while the feed pump was turned on when the culture level fell below a set point achieved with the use of level sensors. The harvest dilution rate was calculated daily for both cultures (A and B) by weighing the harvest reservoir. The vertical dashed lines indicate the time points at which the dilution rate was changed from an initial value of 0.2/d to 0.5/d, 0.2/d and 0.5/d, as described in Table 5.1. This change in dilution rate was achieved by altering the flow rate of the harvest pump. The green boxes indicate the steady states examined. The change in the dilution rate at each steady state was assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. No significant difference was found between states 2A, 2B and 4 or states 1 and 3 (significance not indicated, but p < 0.001).
The rate of glucose consumption and lactate production reduced at low dilution rate

CD-CHO medium routinely contains a concentration of approximately 35 mM glucose. Due to rapid consumption by the cells in the initial days of cultivation, the residual concentration reached 0 mM on day 7 (Figure 5.4). With a few exceptions, the concentration remained at this level for the duration of culture. The increases in extracellular glucose that occurred around day 50, and on days 108 and 117 were a consequence of the difficulties in maintaining a culture level of 1.7 L (see Section 5.3.9 also).

The concentration of lactate increased from the beginning of culture to a value of approximately 30 mM on day 6 (Figure 5.4). After this time, the concentration decreased until a steady state value of 0.4 ± 0.2 mM was reached (state 1). With the increase in dilution rate to 0.5/d on day 41, there was an immediate increase in the extracellular lactate concentration, with the lactate concentration reaching approximately 26 mM four days after the switch. The concentration continued to increase from this time, but at a slower rate. As a result of the problems with maintaining the culture level, there was a significant difference in the extracellular lactate concentration at this steady state. A concentration of 30.7 ± 0.6 mM was reached for the A culture, while a concentration of just 13.0 ± 0.5 mM was attained for the B culture (state 2). The decrease in dilution rate on day 69 caused a rapid decrease in lactate concentration and the steady state value of 0.0 ± 0.0 mM was reached within 5 days of the shift (state 3). At state 4, the increase in dilution rate caused an increase in lactate concentration in a similar fashion to state 2. The highest lactate concentration of 35.4 ± 1.1 mM was reached at this steady state.

The specific rate of consumption of glucose exceeded 3 pmol/VC/day at the beginning of cultivation due to the batch mode of cultivation (Figure 5.5). At state 1, the mean rate stabilised at 0.79 ± 0.08 pmol/VC/day. There was an increase in the rate of glucose supply to the cells that was effected by the increased dilution rate at state 2. This resulted in a significant increase in the glucose consumption rate, although the steady state values differed for each replicate as a result of the culture level deviations (p<0.001). A significantly higher rate of glucose consumption occurred in the A culture at 2.43 ± 0.02 pmol/VC/day while a value of 1.47 ± 0.04 pmol/VC/day was determined for the B culture (p<0.001). The rate of consumption decreased on day 69 as an immediate response to the decrease in dilution rate at the beginning of state 3.
Duplicate continuous cultures were established and were allowed to proceed in batch mode for the first three days. The perfusion of medium began at this point. A sample of culture supernatant was removed from the bioreactor each day, and analysis of extracellular concentrations of glucose and lactate were carried out with the use of a Nova Bioprofile 100 plus. The vertical dashed lines indicate the time points at which the dilution rate was changed from an initial value of 0.2/d to 0.5/d, 0.2/d and 0.5/d, as described in Table 5.1. The glucose and lactate concentrations determined daily for duplicates A and B are shown with dashed and full lines, respectively. Meanwhile, glucose concentrations are shown in green and lactate concentrations shown in purple. The green boxes indicate the steady states examined. The change in the glucose and lactate concentration at each steady state was assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. No significant difference was found in the concentration of glucose at any steady state, but there was a significant difference between the concentrations of lactate at steady states 2A, 2B and 4 (significance not indicated, but p < 0.01).
Duplicate cultures were established and the extracellular concentration of glucose was assayed daily with the Nova Bioprofile 100 plus. The viable cell number was also determined each day, as described in Section 5.2.2. The specific consumption rate of glucose in pmol per viable cell per day was calculated according to Section 2.9.2, and plotted against the time in culture (A and B). The vertical dashed lines indicate the time points at which the dilution rate was changed from an initial value of 0.2/d to 0.5/d, 0.2/d and 0.5/d, as described in Table 5.1. The green boxes indicate the steady states examined. The change in the glucose consumption rate at each steady state was assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. With the exception of states 2A and 4, no significant differences were found between each steady state (significance not indicated, but p < 0.05).
Despite the fact that state 3 was operated at the same dilution rate as state 1, there was a significant increase in the glucose consumption rate to $1.08 \pm 0.04$ pmol/VC/day when compared to state 1 ($p<0.05$). At state 4, a similar level of glucose consumption occurred in both replicates, despite the difference in timing of the steady states. The mean glucose consumption rate was comparable to that determined for the 2A culture ($2.55 \pm 0.08$ pmol/VC/day, Figure 5.5).

The cell specific lactate production rate begins at values above 4 pmol/VC/day and decreases from this time in culture (Figure 5.6). On day 8, a mean value of approximately 0 pmol/VC/day was reached, and remained at this value until a steady state was achieved (where the mean value is $0.00 \pm 0.04$ pmol/VC/day, Figure 5.6). This demonstrated that the decrease in lactate concentration from day 6 onwards was a result of the cessation of lactate production, and subsequent washout of residual lactate by the process of perfusion. After the increase in dilution rate on day 41, the production of lactate began immediately and rates continued to increase thereafter. However, as a result of the culture level deviations, the duplicate cultures reached different steady state lactate production rates. A higher rate was observed for the A culture at $2.49 \pm 0.16$ pmol/VC/day, and just $0.65 \pm 0.006$ pmol/VC/day for the B culture ($p<0.001$). The reduction of the dilution rate to 0.2/d on day 69 caused a rapid decrease in the lactate production rate. A mean value of $0.00 \pm 0.00$ pmol/VC/day was reached for both cultures, in a similar manner to state 1. Consequently, despite the differences in state 2, both replicates settled into the same steady state 3 regarding metabolism of lactate. At state 4 the highest production rate of lactate was reached at a mean value of $3.29 \pm 0.19$ pmol/VC/day ($p<0.01$).

5.3.3 Glutamine and glutamate consumption decreases at low dilution rate
Analysis of extracellular metabolites was carried out by use of the Nova Bioprofile 100 and using HPLC analysis (by SAFC Biosciences, Section 2.11), and resulted in an overlap of data points for glutamine and glutamate. There was a broad agreement of the concentrations determined by both methods, although minor variations were detected at states 3 and 4 (Table 5.2). The same trends of changes were evident, regardless of the analysis method employed. In addition, the calculated rate of consumption of glutamine and glutamate for both methods were similar. Since the HPLC method of quantification was more accurate, these values were used for steady state metabolic flux analysis.
Duplicate cultures were established and the extracellular concentration of lactate was assayed daily with the Nova Bioprofile 100 plus. The viable cell number was also determined daily as described in Section 5.5.2. The specific production rate of lactate in pmol per viable cell per day was calculated according to Section 2.9.2, and plotted against the time in culture (A and B). The vertical dashed lines indicate the time points at which the dilution rate was changed from an initial value of 0.2/d to 0.5/d, 0.2/d and 0.5/d, as described in Table 5.1. The green boxes indicate the steady states examined. The change in the lactate production rate at each steady state was assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. With the exception of state 1 and 3, all states were significantly different (significance not indicated, but p < 0.05).
Table 5.2 Concentrations and consumption rates of glutamine and glutamate as determined by HPLC.

<table>
<thead>
<tr>
<th></th>
<th>1 (0.2/d)</th>
<th>2A (0.5/d)</th>
<th>2B (0.5/d)</th>
<th>3 (0.2/d)</th>
<th>4 (0.5/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Gln]</td>
<td>2.2 ± 0.2</td>
<td>2.5 ± 0.1</td>
<td>3.0 ± 0.1</td>
<td>0.8 ± 0.0</td>
<td>1.9 ± 0.0</td>
</tr>
<tr>
<td>[Glu]</td>
<td>3.3 ± 0.0</td>
<td>2.7 ± 0.0</td>
<td>2.5 ± 0.0</td>
<td>4.2 ± 0.1</td>
<td>3.1 ± 0.1</td>
</tr>
<tr>
<td>qGln</td>
<td>0.11 ± 0.01</td>
<td>0.35 ± 0.03</td>
<td>0.17 ± 0.01</td>
<td>0.21 ± 0.01</td>
<td>0.37 ± 0.01</td>
</tr>
<tr>
<td>qGlu</td>
<td>-0.04 ± 0.00</td>
<td>-0.08 ± 0.00</td>
<td>-0.03 ± 0.00</td>
<td>-0.09 ± 0.01</td>
<td>-0.13 ± 0.01</td>
</tr>
</tbody>
</table>

These values show an agreement with values determined using the Nova Bioprofile 100 plus (Figures 5.7 and 5.8). Concentrations were determined by SAFC Biosciences and are given in mM, while specific rates are given in pmol/VC/day. Mean values of the four days previous to the switch in condition for both cultures ± the SEM of the value are quoted. States 1 and 3 were operated at a dilution rate of 0.2/d, while this rate was increased to 0.5/d at states 2 and 4.
After the initial phase of glutamine consumption, a steady state concentration of 2.6 ± 0.0 mM was reached (state 1, Figure 5.7). The glutamine concentration decreased significantly at state 3 to 1.6 ± 0.1 mM (p<0.001). Ammonium concentrations were significantly higher at increased dilution rates (p<0.001). There was a statistically higher concentration of ammonium at state 3, when compared to state 1 (7.9 ± 0.4 mM at state 1, and 10.8 ± 0.2 mM at state 3, p<0.001, Figure 5.7). The concentrations at states 2A, 2B and 4 were considered to be similar.

A concentration of 2.6 ± 0.0 mM glutamate was reached at state 1, but no significant changes were noted in this concentration when compared to states 2A and 2B (Figure 5.8). At state 3, the concentration increased significantly to 3.1 ± 0.1 mM (p<0.05). The concentration decreased at state 4, but the mean value was significantly higher than states 2A and 2B (p<0.05).

The specific consumption rate of glutamine stabilised at 0.13 ± 0.05 pmol/VC/day at state 1 (Figure 5.9) The problems in maintaining culture level during state 2 led to significantly different glutamine consumption rates at steady state, where a significantly increased value was reached at state 2A (p<0.05, no statistical difference observed between this state and state 1). The mean glutamine consumption rate obtained at state 3 is considered to be statistically similar to that at state 1. At state 4, the consumption rate of glutamine increased and was considered to be statistically similar to state 2A. Consequently, the glutamine consumption rate decreases at low dilution rate.

Since the mean values of all calculated glutamate consumption rates at steady state were negative, glutamate was produced during the cultivation time (Figure 5.10). The production rate was significantly increased at state 4 relative to all other states, with the exception of state 2A (p<0.05, a consumption rate of -0.10 ± 0.01 pmol/VC/day was noted at state 4). As a result, the high dilution rate states 2A and 4 were considered to have similarly increased rates of glutamate production.

The rate of ammonium production at steady state 1 was 0.14 ± 0.04 pmol/VC/day and no significant difference was noted between this mean value and those determined at states 2A and 2B (Figure 5.11). At state 3, the production rate increased and was considered to be significantly higher than state 1 (p<0.001). The mean rate at state 4 was considered to be statistically similar to those determined for state 2A and 2B.
Duplicate cultures were established and were allowed to proceed in batch mode for the first three days. The perfusion of medium began at this point. A sample of culture supernatant was removed from the bioreactor each day, and analysis of extracellular concentrations of glutamine and ammonium were carried out with the use of a Nova Bioprofile 100 plus. The vertical dashed lines indicate the time points at which the dilution rate was changed from an initial value of 0.2/d to 0.5/d, 0.2/d and 0.5/d, as described in Table 5.1. The glutamine and ammonium concentrations determined daily for duplicates A and B are shown with dashed and full lines, respectively. Meanwhile, glutamine concentrations are shown in green and ammonium concentrations shown in purple. The green boxes indicate the steady states examined. The change in concentration at each steady state was assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism (significance not indicated, but p < 0.05).
Duplicate cultures were established and were allowed to proceed in batch mode for the first three days. The perfusion of medium began at this point. A sample of culture supernatant was removed from the bioreactor each day, and analysis of the extracellular concentration of glutamate was carried out with the use of a Nova Bioprofile 100 plus (A and B). The vertical dashed lines indicate the time points at which the dilution rate was changed from an initial value of 0.2/d to 0.5/d, 0.2/d and 0.5/d, as described in Table 5.1. The green boxes indicate the steady states examined. The change in the concentration at each steady state was assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. The concentration of glutamate was significantly higher at state 3 (significance not indicated, but p < 0.05).
Duplicate cultures were established and the extracellular concentration of glutamine was assayed daily with the Nova Bioprofile 100 plus. The viable cell number was also determined daily as described in Section 5.2.2. The specific consumption rate of glutamine in pmol per viable cell per day was calculated according to Section 2.9.2, and plotted against the time in culture (A and B). The vertical dashed lines indicate the time points at which the dilution rate was changed from an initial value of 0.2/d to 0.5/d, 0.2/d and 0.5/d, as described in Table 5.1. The green boxes indicate the steady states examined. The change in the glutamine consumption rate at each steady state was assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. There was no significant difference between the rate at states 2A and 4, or 2B and 3 (significance not indicated, but p < 0.05).
Duplicate cultures were established and the extracellular concentration of glutamate was assayed daily with the Nova Bioprofile 100 plus. The viable cell number was also determined daily as described in Section 5.2.2. The specific consumption rate of glutamate in pmol per viable cell per day was calculated according to Section 2.9.2, and plotted against the time in culture (A and B). The vertical dashed lines indicate the time points at which the dilution rate was changed from an initial value of 0.2/d to 0.5/d, 0.2/d and 0.5/d, as described in Table 5.1 The green boxes indicate the steady states examined. The change in the glutamate consumption rate at each steady state was assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism (significance not indicated, but p < 0.05).
Figure 5.11 The specific ammonium production rate increases at states 3 only.

Duplicate cultures were established and the extracellular concentration of ammonium was assayed daily with the Nova Bioprofile 100 plus. The viable cell number was also determined daily as described in Section 5.2.2. The specific production rate of ammonium in pmol per viable cell per day was calculated according to Section 2.9.2, and plotted against the time in culture (A and B). The vertical dashed lines indicate the time points at which the dilution rate was changed from an initial value of 0.2/d to 0.5/d, 0.2/d and 0.5/d, as ammonium production rate at each steady state was assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism (significance not indicated, but p < 0.05).
5.3.4 Flux through glycolysis and the TCA cycle decreases at low dilution rate

The changes in intracellular metabolic flux at each steady state were assessed with use of metabolic flux analysis (Section 2.12), and results are illustrated in Figures 5.12 through 5.16. The changes in glucose and lactate metabolism were mirrored by differences in the $J_{\text{glc-pyr}}$ and $J_{\text{pyr-iac}}$ fluxes. The reduced glucose consumption rates observed at state 2A and 4 translated into decreased $J_{\text{glc-pyr}}$ fluxes of $0.558 \pm 0.005$ and $0.594 \pm 0.019$, respectively (Figure 5.12). All other fluxes were significantly different, with an overall decrease in the flux at low dilution rate. The flux from pyruvate to lactate, $J_{\text{pyr-iac}}$, was significantly different at all steady states, with the exception of states 1 and 3, where statistically similar values were determined. There was also a significant reduction in this flux at low dilution rate. The term $\Delta L/\Delta G$, which is a measure of the number of moles of lactate produced per mole of glucose consumed, followed the same pattern of changes as the $J_{\text{pyr-iac}}$ flux.

The flux from pyruvate to the TCA cycle via acetyl-CoA ($J_{\text{pyr-acc}}$) was significantly reduced to $0.070 \pm 0.001$ at state 1 only (Figure 5.12). At all other states this flux was approximately 30% higher.

A negative flux from malate to pyruvate was noted at all states, indicating flux occurred from pyruvate to malate. This flux was significantly increased at state 3 to $-0.068 \pm 0.005$, although no statistical difference was noted when compared to state 2B (Figure 5.12).

The flux from pyruvate to alanine was significantly higher at increased dilution rate. However, the flux at state 2A was significantly increased above that of state 2B to $0.62 \pm 0.001$. No significant difference was noted in this flux between states 1 and 3 (Figure 5.12).

Serine fluxed towards the synthesis of glycine and pyruvate at each steady state. The flux towards glycine decreased at low dilution rates, while a similar flux was noted at states 2A and 4. A significant increase in the flux to pyruvate was observed at state 3, when compared to state 1, which was operated at the same dilution rate. No significant differences were noted in this flux between state 4 and either state 2A or 2B (Figure 5.12).

A minor flux from pyruvate to cysteine was observed during culture, and a significant change was noted between states 3 and 4. At state 4, the mean flux was $0.001 \pm 0.001$ which suggests pyruvate is synthesised from cysteine at this state only (Figure 5.12).
Figure 5.12 Decreasing the dilution rate reduces the flux from glucose to pyruvate, and subsequently from pyruvate to lactate and alanine.

Duplicate continuous cultures proceeded through four steady states with varying dilution rate, where state 1 = 0.2/d, state 2A = 0.5/d for culture A, state 2B = 0.5/d for culture B, state 3 = 0.2/d and state 4 = 0.5/d. A sample of culture supernatant was made cell-free and frozen at -20°C before assay of the extracellular concentrations of amino acids (Section 2.11). The results were combined with other metabolic parameters in a metabolic flux analysis model and key fluxes were predicted (Section 2.12). The change in the fluxes at each steady state was assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism, significance not indicated. DL/DG is a measure of the moles of lactate produced per mole of glucose consumed. J represents the flux and abbreviations are noted elsewhere. $J_{\text{pyr-accoa}}$, $J_{\text{pyr-ala}}$, $J_{\text{gly-ser}}$, $J_{\text{ser-pyr}}$, and $J_{\text{cys-pyr}}$ are plotted on the secondary y-axis for clarity. Results are expressed as the mean ± SEM (error bars). At points where no error bar is shown, the SEM was within the size of the border (*=p<0.05, **=p<0.01, ***p<0.001).
There was a significant decrease in all the fluxes within the TCA cycle at low dilution rate ($J_{\text{oaakg}}, J_{\text{akg-succ}}, J_{\text{succ-fum}}, J_{\text{fum-mal}}, J_{\text{mal-oaa}}$, Figure 5.13). Additionally, a significant decrease was noted in $J_{\text{succ-fum}}$ and $J_{\text{fum-mal}}$ at state 3, when compared to state 1. With the exception of $J_{\text{mal-oaa}}$, no significant differences were noted between state 4 and either state 2A or 2B (in this case, state 2B was determined to be significantly higher than state 4). $J_{\text{aa-TCA}}$ is a composite flux that sums the total fluxes from amino acids into the TCA cycle. This flux was significantly increased to $-0.069 \pm 0.006$ at state 3, although no statistical difference was noted when compared to state 2B.

The flux from acetyl-CoA to lysine, isoleucine, leucine and tyrosine was significantly increased at state 3 ($-0.043 \pm 0.003$, $J_{\text{acoa}}$, Figure 5.14). No significant differences were noted between states 2A, 2B and 4 and no dilution rate dependent changes were observed in this flux.

The flux from glutamine was positive at all steady states and indicates that this metabolite was always consumed to some degree in order to fuel the TCA cycle ($J_{\text{gin-giu}}$). This flux was significantly higher at state 3 when compared to state 1, which was operated at the same dilution rate ($0.028 \pm 0.004$ at state 3, and Figure 5.14). The flux at state 2A was comparable to that determined for state 4, since a significantly lower flux was observed at state 2B. No significant changes were noted in $J_{\text{glu-akg}}$, but the sum of the fluxes from the amino acids histidine, arginine and proline to glutamate were significantly reduced at state 4 to $0.004 \pm 0.002$ (compared to $0.011 \pm 0.001$ at state 1, $J_{\text{aa-glu}}$).

A significant increase in the fluxes from methionine, isoleucine, threonine and valine to succinyl-CoA was observed at state 3, when compared to state 1. Similarly, an increase in the flux from phenylalanine to tyrosine and subsequently to fumarate was observed at state 3, when compared to state 1 (Figure 5.14).

The fluxes from asparagine to aspartate and oxaloacetate were increased at high dilution rate, with no significant difference noted between states 2A and 4 for $J_{\text{asn-asp}}$. State 2B was additionally considered to be similar to state 4 for $J_{\text{asp-oaa}}$. In both cases, the flux at state 3 was significantly higher than that at state 1 (Figure 5.14).
Figure 5.13 Decreasing the dilution rate decreases the fluxes within the TCA cycle.

Duplicate continuous cultures proceeded through four steady states with varying dilution rate, where state 1 = 0.2/d, state 2A = 0.5/d for culture A, state 2B = 0.5/d for culture B, state 3 = 0.2/d and state 4 = 0.5/d. A sample of culture supernatant was made cell-free and frozen at -20°C before assay of amino acids (Section 2.11). The results were combined with other metabolic parameters in a metabolic flux analysis model and key fluxes were predicted (Section 2.12). The change in the fluxes at each steady state was assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Significance is not indicated, but is discussed in Section 5.3.4. J represents the flux and abbreviations are noted elsewhere. J_{aa-xcA} is the sum of the fluxes into the TCA cycle, i.e. those from glutamate to α-ketoglutarate, aspartate to oxaloacetate, tyrosine to fumarate and the sum of the amino acid fluxes to acetyl-CoA and succinyl-CoA. Results are expressed as the mean ± SEM (error bars). At points where no error bar is shown, the SEM was within the size of the border (*=p<0.05, **=p<0.01, ***p<0.001).
Duplicate continuous cultures proceeded through four steady states with varying dilution rate, where state 1 = 0.2/d, state 2A = 0.5/d for culture A, state 2B = 0.5/d for culture B, state 3 = 0.2/d and state 4 = 0.5/d. A sample of culture supernatant was made cell-free and frozen at -20°C before assay of amino acids (Section 2.11). The results were combined with other metabolic parameters in a metabolic flux analysis model and key fluxes were predicted (Section 2.12). The change in the fluxes at each steady state was assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Significance is not indicated, but is discussed in Section 5.3.4. J represents the flux and abbreviations are noted elsewhere. \( J_{\text{aaglu}} \) is the sum of the fluxes from histidine, arginine and proline to glutamate, \( J_{\text{aasucc}} \) is the sum of the fluxes from methionine, isoleucine, threonine and valine to succinyl-CoA, and \( J_{\text{aaccoa}} \) is the sum of the fluxes from lysine, isoleucine, leucine and tyrosine to acetyl-CoA. Results are expressed as the mean ± SEM (error bars). At points where no error bar is shown, the SEM was within the size of the border (*=p<0.05, **=p<0.01, ***p<0.001).
Statistical analysis shows that there was a significant increase in the flux from amino acids, glucose and glutamine towards the production of biomass at states 2A, 2B and 4 (Figure 5.15). This demonstrates that at higher dilution rates, all fluxes towards biomass were increased. In the case of the flux from amino acids towards synthesis of biomass, the flux approximately halves at low dilution rate (from 0.069 ± 0.010 at state 1 to 0.156 ± 0.010 at state 2B). The greatest contribution towards biomass flux was derived from amino acids, with an average of 70% of the total flux attributed to this source.

In determining the total amount of amino acid flux at each steady state, it was noted that the total production of amino acids was exactly balanced with the total consumption at state 3 (Figure 5.15). Neglecting this specific result, an increase in dilution rate resulted in an increase in the total production and consumption of amino acids (states 2 and 4). For example, the net amino acid production increases from 0.056 ± 0.005 at state 1 to 0.136 ± 0.014 at state 2B.

The flux from amino acid metabolism towards CO$_2$ production followed a similar trend to those that were determined for flux towards biomass (Figure 5.15). This flux was increased at higher dilution rates, with similar values observed at states 2A, 2B and 4 (approximately 0.10 ± 0.00), while a reduced mean flux of 0.065 ± 0.000 was determined at state 1.

HCG synthesis from amino acid constituents was calculated with the flux termed J$_{aa-HCG}$. The most significant increase in amino acid flux towards production of HCG is noted at state 3, where the flux was almost two times higher than at all other states (0.005 ± 0.000). Possible reasons for this increased flux are explored in Section 5.4.4. There was also a significant increase in the flux from state 1 to 2, from 0.002 ± 0.000 to 0.003 ± 0.000 at both state 2A and 2B. The flux at state 4 was reduced to similar levels as those determined for state 1 (0.002 ± 0.000, Figure 5.15).

An overall schematic of the changes in metabolic fluxes was constructed from the data illustrated in Figures 5.12 to 5.15 (Figure 5.16). The schematic was based on textbook metabolic pathway drawings, as illustrated in Figure 2.4. Results of statistical analysis and scale were omitted for clarity; however, related flux pathways were shown at similar scales.
Figure 5.15 Reducing the dilution rate decreases all fluxes towards synthesis of biomass and the production of CO$_2$.

Duplicate continuous cultures proceeded through four steady states with varying dilution rate, where state 1 = 0.2/d, state 2A = 0.5/d for culture A, state 2B = 0.5/d for culture B, state 3 = 0.2/d and state 4 = 0.5/d. A sample of culture supernatant was made cell-free and frozen at -20°C before assay of amino acids (Section 2.11). The results were combined with other metabolic parameters in a metabolic flux analysis model and key fluxes were predicted (Section 2.12). The change in the fluxes at each steady state was assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism, significance not indicated. The flux from amino acids to HCG is plotted on the secondary y-axis for clarity. J represents the flux and abbreviations are noted elsewhere. The determination of the relative contributions towards the biomass flux is detailed in Section 2.12, as is the flux towards HCG. The amino acid fluxes were calculated by summing the specific rates of all amino acids used, with the exception of cysteine and proline, while the flux towards CO$_2$ was assumed to have derived from amino acid metabolism only. Results are expressed as the mean ± SEM (error bars). At points where no error bar is shown, the SEM was within the size of the border (*=p<0.05, **=p<0.01, ***p<0.001).
Figure 5.16 At low dilution rate there is a significant reduction in the flux towards synthesis of alanine, aspartate, CO$_2$, biomass, and, in the flux through glycolysis and the TCA cycle.

Duplicate continuous cultures proceeded through four steady states with varying dilution rate, where state 1 = 0.2/d, state 2A = 0.5/d for culture A, state 2B = 0.5/d for culture B, state 3 = 0.2/d and state 4 = 0.5/d. A sample of culture supernatant was made cell-free and frozen at -20°C before assay of amino acids (Section 2.11). The results were combined with other metabolic parameters in a metabolic flux analysis model and key fluxes were predicted (Section 2.12). Results of statistical analysis and scale are omitted for clarity; however, related flux pathways are shown at similar scales. Relative values are shown in figures 5.12, 5.13, 5.14 and 5.15. The flux between pyruvate and acetyl-CoA and alanine are also shown in blue boxes at a different scale in order to illustrate the relative changes at each steady state. AA denotes amino acids, and abbreviations are noted elsewhere. Results are expressed as the mean ± SEM (error bars). At points where no error bar is shown, the SEM was within the size of the border.
5.3.5 The rate of production of HCG increases at state 3 only

Figure 5.17 illustrates how the extracellular HCG content was significantly increased at a dilution rate of 0.2/d to values of $1085 \pm 85$ IU/ml at state 1 and $1345 \pm 28$ IU/ml at state 3 ($p<0.05$). However, this is likely to be an effect of the increased dilution rate, which causes the system to flush out more often and results in lower total extracellular HCG content. If the extracellular HCG content at states 2A and 2B were assumed to be similar, no significant difference was noted between states 2 and 4.

However, the specific productivity of HCG was significantly higher at state 3 only, at a value of $55.1 \pm 3.9$ µIU/VC/day ($p<0.05$, Figure 5.18). This occurred due to the lower number of viable cells at steady state 3.

5.3.6 Cell cycle progression does not alter with dilution rate

No significant difference was noted in the proportion of cells in the G1 phase of the cell cycle at any steady state. The percentage of cells in the S phase of the cell cycle at state 2A was statistically lower than that at state 3 (14.7 ± 1.3% in state 2A and 19.1 ± 1.3% in state 3). Additionally, there was an increase in the percentage of cells in the G2/M phase of the cell cycle in states 2A and 4, when compared to state 1 only (15.7 ± 0.8% at state 1, and 21.3 ± 1.3% at state 3, Figure 5.19)

5.3.7 The intracellular content of nucleotides and sugar nucleotides decreases at low dilution rate, with the exception of state 3

There were no dilution rate dependent changes in the intracellular content of any of the nucleotide or sugar nucleotide compounds tested. However, there was a significant increase in the amount of every compound at state 3 when compared to the previous state with the same dilution rate, state 1 (Figure 5.20 and 5.21). If this increase at state 3 is neglected, a trend of increasing content at increased dilution rate became obvious. In each case the mean value was increased at states 2A, 2B and 4. The trend was most obvious with UDP-Gluc/GDP-Man, UTP, CTP, ATP and UDP-GalNAc (Figure 5.20 and 5.21). The smallest increase was typically seen between states 1 and 4, with the exception of UDP-GlcNAc, where state 2B was most similar to state 1. While statistical analysis does not confirm this trend for all states and compounds, nine compounds were significantly different at some states (the exceptions were UDP-Gal, UDP and ADP). The reasons for the increase in intracellular sugar nucleotide content at steady state 3 are explored in Section 5.4.4.
Figure 5.17 Decreasing dilution rate increases the extracellular HCG content.

Duplicate cultures were established, and a volume of culture supernatant was taken daily from the bioreactor. This supernatant was made cell-free by centrifuging at 1000 g for five minutes and filtering through a 0.2 μm filter. Samples were stored at -20°C before analysis of various samples for HCG content by ELISA. The vertical dashed lines indicate the time points at which the dilution rate was changed from an initial value of 0.2/d to 0.5/d, 0.2/d and 0.5/d, as described in Table 5.1. The green boxes indicate the steady states examined. The change in the extracellular HCG content at steady states 1, 3 and 4 was assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism (significance not indicated).
A sample was taken from duplicate cultures each day was treated and stored according to Section 5.2.2. At the time points shown, the extracellular HCG content was assayed by ELISA (Figure 4.12). The specific productivity of HCG by these cells in µIU per viable cell per day was calculated according to Section 2.9.2 (A and B). The vertical dashed lines indicate the time points at which the dilution rate was changed from an initial value of 0.2/d to 0.5/d, 0.2/d and 0.5/d, as described in Table 5.1. The green boxes indicate the steady states examined. The change in the specific production of HCG at steady states 1, 3 and 4 was assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism (significance not indicated).
Figure 5.19 No dilution rate dependent changes are seen in the proportion of cells in each phase of the cell cycle.

Duplicate continuous cultures proceeded through four steady states with varying dilution rate, where state 1 = 0.2/d, state 2A = 0.5/d for culture A, state 2B = 0.5/d for culture B, state 3 = 0.2/d and state 4 = 0.5/d. 2 x 10⁶ cells were harvested in triplicate for both cultures on two consecutive days at each steady state. The percentage of cells in each phase of the cell cycle was determined by flow cytometry analysis (Section 2.13) and the mean value ± SEM was calculated. These values were compared across steady states 1, 2A, 2B, 3 and 4. Statistical analysis was performed by one-way ANOVA followed by post-hoc Bonferroni analysis using Prism (*=p<0.05, **=p<0.01, ***p<0.001).
Figure 5.20 The intracellular content of nucleotides and sugar nucleotides increases at state 3 and at increasing dilution rate.

Duplicate continuous cultures proceeded through four steady states with varying dilution rate, where state 1 = 0.2/d, state 2A = 0.5/d for culture A, state 2B = 0.5/d for culture B, state 3 = 0.2/d and state 4 = 0.5/d. 3 x 10^6 cells were harvested in duplicate for both cultures on two consecutive days at each steady state. The amount of ADP, UDP-Gal, UDP-GalNAc, UDP, GDP, AMP and CTP was determined via RP-HPLC (Section 2.14) and the mean value was expressed in fmol per viable cell ± SEM. These values were compared across steady states 1, 2A, 2B, 3 and 4. Statistical analysis was performed by one-way ANOVA followed by post-hoc Bonferroni analysis using Prism, *=p<0.05, **=p<0.01, ***p<0.001.
Duplicate continuous cultures proceeded through four steady states with varying dilution rate, where state 1 = 0.2/d, state 2A = 0.5/d for culture A, state 2B = 0.5/d for culture B, state 3 = 0.2/d and state 4 = 0.5/d. 3 x 10^6 cells were harvested in duplicate for both cultures on two consecutive days at each steady state. The amount ATP, GTP, UDP-GlcNAc and UDP-Gluc/GDP-Man was determined via RP-HPLC (Section 2.14) and the mean value was expressed in fmol per viable cell ± SEM. These values were compared across steady states 1, 2A, 2B, 3 and 4. Statistical analysis was performed by one-way ANOVA followed by post-hoc Bonferroni analysis using Prism, *=p<0.05, **=p<0.01, ***p<0.001.

**Figure 5.21 The intracellular content of nucleotides and sugar nucleotides increases at state 3 and at increasing dilution rate.**
5.3.8 Low dilution rates result in decreased sialylation, antennarity and fucosylation of HCG and increased proportions of neutral glycans

There were no distinctive changes in the IEF pattern of HCG at each steady state examined, as illustrated by the representative samples shown in Figure 5.22. Mirroring this fact, no significant differences were noted for the calculated average pl at each steady state (Figure 5.23). However, due to the obvious trend of decreased average pl at high dilution rate, additional statistical analyses were carried out. A two-tailed, unpaired T-test revealed that the average pl at state 2A was significantly lower than that at state 3 (p < 0.05). In addition, a more detailed structural analysis of the N-linked glycans attached to HCG showed that there were significant differences in peaks 2, 3, 4, 5, 7, 10, 11, 12, 13, 14 and 15 during the cultivation time.

Peaks 3 and 4 were composed of A1G1 and M5, respectively (See Table 2.7 and Section 2.20.9 for details of N-linked glycan abbreviations). There was a significant increase in the percentage of A1G1 at states 1, 2B and 3, with values of 3.77 ± 0.23% at state 1 and 2.15 ± 0.14% at state 2 (Figure 5.24). A similar trend of changes was seen for the percentages of M5 at each steady state, although statistical significance was only noted between states 3 and 4. This indicates that reducing the dilution rate increased the proportion of some neutral glycans. Peaks 10 through 15 showed a similar trend of changes at each steady state. In peaks 10, 11, 14 and 15 there was a statistical difference between states 1 and 4, 2A and 3, and 3 and 4. Additionally, state 1 was significantly lower than state 2A for peaks 11 and 15. There was a significant difference between states 3 and 4 for peaks 12 and 13. In addition, state 2A was higher than 3 for peak 13 (Figure 5.24). These peaks contained the largest proportion of sialylated structures, and comprised the total pool of triantennary structures. Consequently, a decrease in dilution rate caused an overall reduction in sialylation and antennarity. Peak 2 contains A2, and the statistically highest proportion of this was found at state 2B. Additionally, the proportion of A2G1 / A1G1S1 was significantly higher at state 2B, when compared to state 4 only. Significantly increased proportions of FA2G1 were detected at state 2B, when compared with states 1, 3 and 4 (Figure 5.24).

The overall changes in key structural features were estimated at each steady state. Table 5.3 illustrates that at low dilution rates, there was an increase in the proportion of neutral structures, while sialylated, triantennary and fucosylated structures decreased. The proportion of diantennary structures was unchanged.
Figure 5.22 Varying dilution rate has no impact on the IEF pattern of HCG.

Duplicate continuous cultures proceeded through four steady states at varying dilution rates, where state 1 = 0.2/d, state 2A = 0.5/d for culture 2A, state 2B = 0.5/d for culture 2B, state 3 = 0.2/d, and state 4 = 0.5/d. Culture supernatant was harvested on two consecutive days at each steady state, and isoelectric focusing was carried out on HCG purified at each of these points. This figure shows a representative IEF profile of HCG at each steady state, where M denotes the migration pattern of the pI marker (from GE healthcare) while numerals 1 through 4 denote the steady state examined (corresponding to the states described above).
Duplicate continuous cultures proceeded through four steady states at varying dilution rates, where state 1 = 0.2/d, state 2 = 0.5/d, state 3 = 0.2/d, and state 4 = 0.5/d. Culture supernatant was harvested on two consecutive days at each steady state, and isoelectric focusing was carried out on HCG purified at each of these points. The average pl of HCG for each steady state (1, 2A, 2B, 3 and 4) was determined with reference to a pl marker by densitometry analysis of the IEF pattern shown in Figure 5.21. Results are expressed as the mean ± SEM (error bars). Statistical analysis was performed by one-way ANOVA followed by post-hoc Bonferroni analysis using Prism, however no differences were noted.
Figure 5.24 Increasing the dilution rate increases the sialylation and triantennary structures on HCG while decreasing the proportion of neutral structures.

Duplicate continuous cultures proceeded through four steady states at varying dilution rates, where state 1 = 0.2/d, state 2A = 0.5/d for culture 2A, state 2B = 0.5/d for culture 2B, state 3 = 0.2/d, and state 4 = 0.5/d. Culture supernatant was harvested on two consecutive days at each steady state, and the structures of the N-glycans attached to purified HCG were determined (Section 2.20). Results are expressed as the mean ± SEM (error bars). Statistical analysis was performed by one-way ANOVA followed by post-hoc Bonferroni analysis using Prism, *=p<0.05, **=p<0.01, ***p<0.001. (See Table 2.7 and Section 2.20.9 for details of N-linked glycan abbreviations).
Table 5.3 Estimates of N-linked glycosylation structural changes at various steady states.

These values were determined by summing the percentage area of every peak that contained any given structure (for example, the proportion of neutral structures is estimated by addition of the mean percentage area of peaks 1 through 5 and 7 through 9, since each of these peaks has a neutral component). States 1 and 3 were operated at a dilution rate of 0.2/d, while this rate was increased to 0.5/d at states 2 and 4.
5.3.9 Impacts of deviations from setpoint

There were two temperature deviations due to a malfunctioning waterbath attached to the B culture, on days 37 and 130 (Table 5.1). In both these cases, the temperature was below 36°C for a maximum of 1.2 hours, and the minimum temperature reached was 34°C. Due to the short duration and small degree of change in temperature, these deviations were not considered to be significant.

On numerous occasions, the culture level deviated from the 1.7 L setpoint. Table 2.2 illustrated that the rate of oxygen consumption increased 54% when the rate of dilution was increased from a value of 0.2/d to 0.5/d. This was matched by an increase in the frequency of oxygen sparging to the bioreactor (data not shown). However, the oxygen valves were not of the type designed to accommodate pure oxygen over long culture periods, and corrosion occurred within the valve. This resulted in the formation of debris that impeded the flow of oxygen to the bioreactor resulting in the formation of a foam layer at the top of the culture. Due to the continuous contact of the level sensor with the foam, the sensor did not detect the decrease in culture volume caused by the rate-setting harvest pump, and fresh medium was not supplied at the necessary rate to maintain 1.7 L. Increasing the flow rate of medium into the bioreactor until the correct volume was reached rectified this problem. In addition, five volume changes were allowed before a steady state was defined.

On day 38, the B culture level was just 1.5 L, and this resulted in a small increase in the lactate production rate on day 39 (Figure 5.6). However, there were no sharp differences observed in the steady state reached at this point, so the deviation was considered to have had no effect.

During the adaptation period of state 2, additional difficulties in maintaining the culture level were experienced for both cultures. These problems continued for three consecutive days for the A culture from days 52 through 54, and for five consecutive days for the B culture (days 52 through 56). As a result of the prolonged duration of these deviations, the cultures settled into distinct steady states, and significant differences were noted between the cultures at this steady state.

On day 60, the B culture experienced low pO₂ flow, with the saturation falling below 40% for one hour, and minimum value of 15% reached. However, metabolism of cells is only
considered to be significantly compromised at values of less than 1% O₂ (Okazaki and Maltepe, 2006). Consequently, the short duration and small degree of shift leads to the conclusion that this deviation was of no consequence.

Additional decreases in culture level occurred on days 108 and 117 of the A culture. However, 5 volume changes were allowed after these deviations and before a steady state was considered. The resulting steady state was the same as that determined for the B culture, so the deviation was considered to be insignificant.

5.4 Discussion

5.4.1 Cell death at low dilution rate

As expected, there was a decrease in the culture viability at low dilution rate (Figure 5.1). This is in agreement with published literature (Miller et al., 1988; Frame and Hu, 1991; Hayter et al., 1993; Lee et al., 1995; Follstad et al., 2000). Some papers have shown that the decrease in cell viability occurs as a result of apoptosis-induced cell death at these conditions (Majid et al., 2007). While the reasons for this were not extensively investigated here, an increase in the number of dead cells was noted at low dilution rate (data not shown). As a result, it seems likely that the reduction in cell viability was mediated by an increase in cell death caused by apoptosis.

No difference was noted between the dilution rate setpoint and the resulting specific growth rate. Many investigations have shown that there is a deviation between these two values at low dilution rate and suggests that a minimal growth rate is necessary for maintenance and survival of the cells (Miller et al., 1988; Hayter et al., 1993; Martens et al., 1993; Follstad et al., 2000). However, in this case it seems that the low dilution rate used was sufficiently higher than the minimal growth rate to cause no such effects.

With use of flow cytometry, it has been shown that the mean cell volume increases with the dilution rate (Martens et al., 1993). The average diameter of the cells was estimated at each steady state by use of the CEDEX\textsuperscript{AS20}, which calculates this parameter alongside determination of the viable cell number and percentage viability (data not shown). However, no dilution rate dependent effects were noted, which suggests that this technique may not be sensitive enough to detect any changes.
5.4.2 Dilution rate dependent effects on metabolic fluxes

At all steady states, the residual glucose concentration was approximately 0mM, indicating that under these conditions, this metabolite was considered to be limiting. Despite the differences observed in state 2 (which are discussed in further detail in Section 5.5.3), some key observations can be made about the differences in metabolic flux, intracellular concentrations of nucleotides and sugar nucleotides and glycosylation of HCG at varying dilution rate. Further steady state dependent changes are explored in Section 5.4.4.

As mentioned in Section 5.4.1, the cell viability decreased at low dilution rate. The rate of glucose consumption decreased, and lactate production ceased to result in a concentration of 0mM at steady state (Figure 5.4, 5.5 and 5.6). When these metabolic fluxes were examined, a similar decrease in $J_{\text{glc-pyr}}$ and $J_{\text{pyr-lac}}$ was observed. There was also a reduction in the extracellular concentration of ammonium at low dilution rate (Figure 5.7).

The internal TCA cycle fluxes, i.e. $J_{\text{ooa-akg}}$, $J_{\text{akgsucc}}$, $J_{\text{succ-fum}}$, $J_{\text{fum-mal}}$ and $J_{\text{mal-ooa}}$ were significantly reduced at 0.2/d (Figure 5.13). Additionally, the fluxes from pyruvate to alanine and from asparagine to aspartate to oxaloacetate were also reduced at this dilution rate (Figure 5.14).

There was a significant decrease in the flux from amino acids, glucose and glutamine towards the synthesis of biomass at low dilution rate (Figure 5.15). The production of CO$_2$ from amino acid metabolism was also predicted to reduce at 0.2/d, however this can be more accurately considered as a reduction in the rate of oxygen consumption (due to the metabolic flux model assumption that the measured oxygen consumption rate equals the rate of carbon dioxide evolution (Figure 5.15 and Table 2.2). The content of the amino acid pool was also significantly reduced ($J_{\text{a(tot)}}$, Figure 5.15).

The specific productivity and flux to HCG was significantly increased at state 3 (Figure 5.15 through 5.18). This is in contrast to literature, which suggests that the specific productivity of recombinant proteins should increase at low dilution rates (Miller et al., 1988; Linardos et al., 1991; Hayter et al., 1993; Martens et al., 1993).

In summary, at a dilution rate of 0.2/d, the glycolytic, TCA cycle fluxes and the fluxes to alanine and asparagine/aspartate (which are generally considered as "overflow" metabolites) were reduced. The overall flux to biomass and the total number of amino
acids was reduced. As a result, reducing the dilution rate resulted in a decreased cellular metabolism, and this metabolism can be considered to be more efficient due to the decrease in the extracellular concentration of lactate. This is in agreement with literature (Miller et al., 1988; Frame and Hu, 1991; Hayter et al., 1993; Martens et al., 1993; Follstad et al., 2000).

The cell cycle distribution was determined at each steady state, and while some statistically relevant differences were noted, no significant dilution rate dependent changes were found (Figure 5.19). This is in contrast with expectations (Martens et al., 1993). However, a continuous culture study using a hybridoma cell line at varying dilution rates, showed no change in cell cycle distribution at low dilution rate (Al-Rubeai et al., 1992). This indicates that the results observed here might not be unusual.

The glycosylation of HCG was examined at each steady state by IEF and structural analysis of the N-linked glycans using NP-HPLC (Figures 5.22 through 5.24). The detailed structural analysis illustrated that decreasing the dilution rate led to an increase in the proportion of neutral structures and a decrease in the sialylated, triantennary and fucosylated structures (Figure 5.24, Table 5.3). Calculation of the average pi following IEF failed to detect any changes in glycosylation (Figure 5.22 and 5.23). These results are in agreement with those determined for CHO and hybridoma cell lines, where a decrease in the site occupancy of IFN-γ and the galactosylation of IgG respectively, was observed at low dilution rates (Hayter et al., 1993; Majid et al., 2007). In a similar manner to Hayter et al, this experiment was carried out under conditions where glucose was a limiting substrate. Consequently, these alterations in glycosylation may be caused by a reduced glycolytic flux, as discussed previously (Chapter 3, (Ryll et al., 1994)). Alternatively, the reduction in cell viability at low dilution rate may cause the release of cellular glycosidases to the culture supernatant, which would degrade the glycan structures (Gramer and Goochee, 1993). However, overexpression of bcl-2, which suppresses apoptosis-induced cell death at low dilution rates, was also found to cause a decrease in the galactosylation of IgG. This indicates that the glycosylation changes were not induced in a manner related to cell-death in this case (Majid et al., 2007).

Analysis of key intracellular nucleotides and sugar nucleotides yielded some insights into the mechanisms of the glycosylation changes observed in these cultures. At steady state 1, there was a significant decrease in the intracellular content of each compound studied.
(Figure 5.20 and 5.21). (These values were increased at state 3, but there were no differences in glycosylation between states 1 and 3. This is discussed further in Section 5.4.4). This suggests that the decrease in sialylation and antennarity was brought about, in part, by a reduction in the amount of substrate for the synthesis reactions, i.e. CTP for sialylation and UDP-GlcNAc for antennarity.

This phenomenon of more complete glycosylation at high dilution and specific growth rate can also be explained from a metabolic control analysis perspective. Experiments have shown that in order to increase the flux through a pathway towards a certain product, the enzyme activities and substrate concentration for every reaction in the pathway needs to be increased (Niederberger et al., 1992). These experiments showed an increase in levels of intracellular nucleotides and sugar nucleotides at high dilution rates, while the elevation of cell growth rate was expected to cause an increase all cell activities and processes, including enzyme activity (Figure 5.20 and 5.21). According to this principle, an increase in the sialylated, fucosylated and triantennary structures is expected at high dilution rates.

5.4.3 Distinct metabolic states at steady state 2

As a result of the problems encountered in maintaining the correct culture volume with the first increase of dilution rate to 0.5/d, two distinct steady states were achieved for the duplicate cultures. Culture 2A experienced level deviations on three consecutive days of culture, while this occurred for a total of five days for culture 2B (Table 5.1). As a result, an increase in the glucose consumption rate and lactate production rate led to higher $J_{\text{glc-pyr}}$, $J_{\text{pyr-lac}}$ and $\Delta L/\Delta G$ for culture 2A. The flux from glutamine to glutamate was also significantly higher, and the resulting increase in $J_{\text{pyr-ala}}$, $J_{\text{asn-asp}}$ and $J_{\text{asp-oaa}}$ suggests the additional glutamate was transaminated to alanine and asparagine. The consumption rates of glutamine and glutamate were also increased at state 2A, and this translated into a statistically higher $J_{\text{glu-glu}}$, lower $J_{\text{aa-glu}}$ and a trend of higher $J_{\text{glu-akg}}$. For all of these parameters, the values attained for culture 2A were most similar to those determined for state 4. All fluxes within the TCA cycle were similar. Additionally, the proportion of glycans at steady state 2A was comparable to that at state 4.

This illustrates that the steady state reached at state 2A was comparable to state 4. Despite the variation in steady state reached, both cultures attained the same state 3, indicating that this particular set of deviations was overcome.
The fact that there were an increased number of days with non-glucose limitation at state 2A was likely to have led to this alteration in metabolism, despite this occurrence preceding the level problems.

5.4.4 State 3: A case of increased amino acid synthesis causing an increase in HCG productivity

Despite using the same cultivation conditions, some differences were noted between states 1 and 3. The glucose, glutamine, glutamate and ammonium consumption and production rates increased at state 3. This resulted in an increased extracellular concentration of ammonium and glutamate, while the glutamine concentration was reduced. This translated into an increase in $J_{\text{glic-pyr}}$, $J_{\text{pyr-lac}}$, $J_{\text{gln-glu}}$ and $J_{\text{pyr-accoa}}$. The internal TCA fluxes at succinyl-CoA to fumarate and subsequently to malate were also significantly reduced. This reduction was caused by an increase in the use of succinyl-CoA and fumarate for the synthesis of the amino acids methionine, isoleucine, threonine, valine, tyrosine and phenylalanine (since $J_{\text{aa-succ}}$, $J_{\text{tyr-fum}}$ and $J_{\text{phe-tyr}}$ were significantly increased at state 3, Figure 5.14). A similar increase in the synthesis of lysine, isoleucine, leucine and tyrosine from acetyl-CoA and from pyruvate to cysteine was noted at state 3 (Figure 5.12 and 5.15). It seems likely that cell metabolism should alter at this state to prevent a significant decrease in other key metabolic intermediates such as pyruvate and other constituents of the TCA cycle due to this synthesis of amino acids.

At this steady state, serine flux towards synthesis of pyruvate increased, and the subsequent flux from pyruvate to malate was increased. The TCA cycle was additionally maintained at oxaloacetate by an increase in $J_{\text{asn-asp}}$ and $J_{\text{asp-oaa}}$. A significant increase in the flux from glutamine to glutamate was also noted; however this translated into a trend of increased flux from glutamate to $\alpha$-ketoglutarate. This suggests that this postulated maintenance of cell metabolism at state 3 by alternative metabolism does in fact occur.

Despite the increased synthesis of amino acids, the consumption of amino acids was exactly balanced with this production, which resulted in no net pool of amino acids at this state. However, a two-fold increase in the flux from amino acids towards synthesis of HCG was noted (Figure 5.15). The intracellular concentration of nucleotides and sugar nucleotides were also significantly increased at this state, although the glycosylation was similar to that observed at state 1 (Figure 5.20 and 5.21).
As a result, this state 3 was a much less efficient state as regards the metabolism of glucose and lactate, however the synthesis of amino acids was significantly increased for incorporation into HCG. Consequently, this can be considered as another case of steady state multiplicity, where, within a continuous culture, the same feed composition and dilution rate can lead to more than one type of metabolism. The deviations encountered during state 2 were considered to have had no impact on the formation of this new state 3 since both cultures reached this same state (Section 5.5.3). However, the previous metabolic history is likely to have had an impact on this multiplicity.

The existing literature on steady state multiplicity of a variety of cell lines illustrates a reason for the observed metabolic shift of this culture. By sequentially reducing the dilution rate, the metabolism of a hybridoma cultivation was reported to become more efficient, as determined by the decrease in glycolytic flux and increase in the flux from pyruvate to the TCA cycle. However, the final change in dilution rate returned the culture to the initial high rate of 1/d, but the favourable increased flux from pyruvate to the TCA cycle was retained (Follstad et al., 2000). In another case, the initial establishment phase of a continuous culture was carried out in fed-batch mode (in contrast to the usual batch mode). As a result, the steady state achieved was more efficient as illustrated by the decreased extracellular lactate concentration, flux through the TCA cycle, glucose and glutamine consumption rates. The establishment phase was similarly efficient (Europa et al., 2000). This indicates that cells can be "guided" into more efficient metabolism with the careful control of culture conditions (i.e. feeding or dilution rate).

It seems logical that cells can be similarly "guided" into less efficient metabolic states, for example by the exposure of cells to a period of relative metabolic inefficiency demonstrated here (steady state 2). However, the state achieved here should be considered as more of an altered state, where glucose was metabolised less efficiently, but an increase in amino acid synthesis fuelled a significantly higher flux towards synthesis of HCG, while maintaining glycosylation. As such, this presents an interesting avenue of research to potentially increase the production of any recombinant protein within a relatively efficient metabolism.

5.4.5 Drawing comparisons across continuous cultures
Chapter 4 explored the effect of glutamine variation at a dilution rate of 0.4/d, and this presents the possibility of integrating this data with the present experiments to draw
conclusions regarding the effect of varying dilution rate over an additional data point. To ensure that comparable steady states were used, state 3 from Chapter 4 was examined in comparison to state 1 and 2A from this chapter. In all cases, the residual glucose concentration was 0mM, and 8mM glutamine was supplemented to the culture medium.

The following parameters were plotted as a function of dilution rate: residual concentration and specific consumption or production rates of glucose, lactate, glutamine, glutamate, ammonium and HCG. The fluxes $J_{\text{biomass}}$, $J_{\text{asn-asp-oaa}}$, $J_{\text{aa-co2}}$, $J_{\text{TCA}}$, $J_{\text{pyr-ala}}$ and the glycosylation of HCG were also examined (Figure 5.25 through 5.27).

The trends of changes that were observed at dilution rates of 0.2/d and 0.5/d were strengthened with the addition of the results at 0.4/d. Reducing the dilution rate, and consequently the specific growth rate, caused a significant reduction in both the consumption rate of glucose and the glycolytic flux (Figure 5.25). The concentration of lactate increased as the production rate increased, although there is only a minor difference in the flux from pyruvate to lactate at dilution rates of 0.4/d and 0.5/d. Consequently, $DL/DG$ also stabilised at this point (Figure 5.25).

The flux to biomass increased with the dilution rate, but the use of amino acids for the use in the TCA cycle and the production of $CO_2$ (more significantly the consumption of $O_2$, see Table 2.2) is only significantly decreased at 0.2/d (Figure 5.26B). The flux from pyruvate to alanine and from aspartate to asparagine and oxaloacetate also decreased in a dilution rate dependent manner (Figure 5.26B). The specific productivity and total amount of HCG changed significantly at low dilution rates only, where an increase in the extracellular HCG content is matched by a decrease in the productivity due to an increased viable cell number (Figure 5.26A). Similarly, glycosylation changes were only noted at low dilution rate (Figure 5.27).

This indicates that there is a major downshift in the cellular metabolism at low dilution rates, as seen in the glycolytic, TCA cycle, biomass and HCG fluxes. However, the glycosylation of HCG was affected at this reduced metabolic state, where a significant decrease in the sialylation, fucosylation and proportion of triantennary structures was noted.
Figure 5.25 The consumption and production of glucose, lactate, glutamine and ammonium does not differ at dilution rates of 0.4/d and 0.5/d.

Part A shows the residual concentration of the extracellular metabolites glucose, glutamine, glutamate, ammonium, and lactate. The secondary y-axis was used for plotting lactate concentration at varying dilution rate. Part B shows the change in the consumption and production rate of these metabolites at steady state, with qGlu, qNH₄⁺ and qGln plotted on the secondary y-axis. DL/DG is a measure of the number of moles of lactate produced per mole of glucose consumed.
Figure 5.26 The productivity of CHO cells decreases at a dilution rate of 0.2/d only, while a dilution rate dependent change is seen in many key metabolic fluxes.

Part A shows the change in total and specific productivity of HCG at each dilution rate. For part B, some key metabolic fluxes are shown, where J(biomass) was calculated by summing the fluxes from glucose, glutamine and amino acids for the formation of biomass, J(asn-asp-ooa) is composed of J_{asn-asp} and J_{asp-ooa} and J(TCA) is the sum of the five fluxes J_{oaa-akg}, J_{akg-suc}, J_{suc-fum}, J_{fum-mal} and J_{mal-ooa}.
Figure 5.27 At a dilution rate of 0.2/d, the sialylation, fucosylation, and triantennarity of HCG decreases, while the proportion of neutral structures increases.

Estimates of the proportion of the neutral, sialylated, fucosylated and tri- and di-antennary structures within the HCG glycan pool were made by summing the percentage area of every peak that contained any given structure.
5.4.6 Conclusions

Reducing the dilution rate in a continuous culture resulted in a decrease in the glycolytic and the TCA cycle flux, while the flux towards the typical overflow metabolites, alanine and aspartate was also reduced. The flux from amino acids, glucose and glutamine towards the synthesis of biomass was also significantly reduced. The intracellular content of nucleotides and sugar nucleotides was also decreased, leading to a reduction in the sialylation, fucosylation and antennarity of HCG produced under these conditions.

Technical difficulties in maintaining a culture level of 1.7 L at high dilution rate resulted in distinct steady states at state 2. However, the cultures were good replicates of each other at the third and fourth steady state.

A minor case of steady state multiplicity was also observed. States 1 and 3 were operated at a dilution rate of 0.2/d, but a significant increase in the flux towards synthesis of HCG from amino acids occurred at state 3. This was caused by a significant increase in the synthesis of amino acids from the TCA cycle for incorporation into HCG. However, the glycosylation of HCG was comparable. A significant number of amino acids were synthesised from the TCA cycle for this purpose, and amino acid consumption and production were exactly balanced at this state. The reduction in the efficiency of glucose metabolism at state 3 was an indication that the multiplicity arose as a result of the increased dilution rate and decreased efficiency in this parameter at the preceding state 2.
Chapter 6

Investigating the Effect of Generation Number on CHO Growth, Metabolism and Productivity
6.1 Introduction

The cultivation of mammalian cells for extended periods of time can result in many changes to the inner working of the cells. One way of describing the age of cells in culture is termed the "generation number," which is defined as the number of times a population has doubled. Alternatively, a "passage number" is a measure of how many times a culture has been subcultured into a new vessel. Passaging is typically carried out every two to three generations. The relationship between these terms was illustrated in Figure 1.19.

The length of time in culture has been found to alter the morphology of Caco-2 cells in culture (Yu et al., 1997; Behrens and Kissel, 2003). Both LNCaP and SHE cells have been found to alter their responses to stimuli after an extended number of generations have been accumulated (Langeler et al., 1993; Esquenet et al., 1997; Chang-Liu and Woloschak, 1997). The doubling time of Syrian hamster embryo cells has been found to decrease after 64 passages (Chang-Liu and Woloschak, 1997). Two varying metabolic responses to increased passage number have been observed in hybridoma cells. In one case, an increase in the glucose consumption rate and lactate production rate was accompanied by a decrease in the ammonium production rate, suggesting less efficient metabolism as the cultures aged (Coco-Martin et al., 1993). However, culture of a different hybridoma cell line at increased passage number caused an increase in the maximal viable cell number a decrease in the lactate yield from glucose, but no change in the ammonium yield from glutamine. This indicates more efficient utilisation of substrates at high passage number (Schmid et al., 1990). It is possible that the variation in response is due to the differences in the two cell lines used.

The expression of cellular proteins is altered at high generation number (Briske-Anderson et al., 1997; Yu et al., 1997; Behrens and Kissel, 2003; Youm et al., 2008). However, the effect on recombinant proteins has been most widely studied due to its importance to the biotechnology industry. A reduction in the expression of recombinant proteins at high generation number has been widely reported for CHO cells (Kaufmann and Schimke, 1981; Weidle et al., 1988; Pallavicini et al., 1990; Kim et al., 1998b; Fann et al., 2000). Theoretically, a change at any one of the control points of transcription, translation and post-translational processing could lead to altered productivity levels (Barnes et al., 2003). However, there are three major reported reasons for the observed reduction in productivity. Firstly a decrease in the productivity of each cell has been found to decrease the overall productivity (Kim et al., 1996; Borth et al., 1999). Secondly, the appearance of a non-
producing population of cells has been found in many cases (Frame and Hu, 1990; Lee et al., 1991; Chuck and Palsson, 1992; Bae et al., 1995). Lastly, a loss of the recombinant gene from the cellular genome has also been observed, most notably for CHO cells (Pallavicini et al., 1990; Merritt and Palsson, 1993; Kromenaker and Srienc, 1994; Kim et al., 1998b; Hammill et al., 2000). There are many methods to overcome this decrease in productivity (Barnes et al., 2003).

Aim

This chapter compares the growth, metabolism and productivity of CHO cells within a batch shake flask culture at a variety of generation numbers. Triplicate cultures were inoculated using the cells from each of the continuous bioreactors (Chapters 4 and 5), with an accumulation of 62 to 91 generations. The shake flask culture that was inoculated at 8 mM glutamine in Chapter 3B was used as the low generation control, with an accumulation of just 7 generations from vial thaw.

6.2 Materials and Methods

6.2.1 Medium preparation
Basal CD-CHO medium supplemented with 8 mM glutamine and HT supplement (100 μM sodium hypoxanthine, 16 μM thymidine) was prepared and was routinely stored at 4°C. On the day of experimentation, 150 ml quantities were pre-incubated in 500 ml shake flasks before inoculation of the cell culture. These experiments were carried out in the small scale cell culture process development lab, MSD, Oss, the Netherlands.

6.2.2 Experimental culturing and analysis
On the final day of cultivation of each continuous culture, a sufficient volume of cells was harvested aseptically from each bioreactor culture and spun down to inoculate triplicate shake flasks at a final experimental cell density of 0.5 x 10^6 cells/ml. Consequently, four individual cultures were examined – the duplicate cultures described in Chapter 4, which experienced varying glutamine concentrations (Gln A and Gln B), and, the duplicate cultures from Chapter 5, which experienced varying dilution rates (Dil A and Dil B). The same system of identifying the cultures is carried through from previous chapters, with the addition of ‘Gln’ or ‘Dil’ to identify the previous culture history. The control culture was based on data taken from Section 3.4, where the cells were also grown at 8 mM glutamine, but with a short preculture time. This is illustrated in Figure 6.1.
The culture age in days (from vial thaw) of each culture examined is detailed in Table 6.1. The generation number of all cultivations at the beginning of these experiments was calculated according to the formula detailed in Section 2.9.5 (Table 6.1). At vial thaw, the generation number was defined as zero, and the pre-culture time was included in the calculations.

The cultures were incubated for five days in a Kuhner shaker at 150 rpm, 5% CO$_2$, and 80% RH (Adolf Kuhner AG, Switzerland). Each day, a 3 ml sample was taken from each culture and the viable cell density was determined by use of the CEDEX AS$^{20}$ (Roche Innovatis AG, Germany). Additionally, the concentrations of ammonium, glucose, glutamate, glutamine and lactate were assayed with use of the Nova Bioprofile 100 plus (Nova Biomedical Corporation, USA). The remaining 1 ml of culture supernatant was centrifugated at 2000 g for 5 minutes, filtered through a 0.2 μm filter and stored at -20°C for extracellular HCG content analysis. This analysis was carried out by an ELISA method by the quality unit in MSD, Oss, the Netherlands.

<table>
<thead>
<tr>
<th>Culture Name</th>
<th>Culture History</th>
<th>Number of Generations</th>
<th>Culture Age (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8mM Gln culture from Chapter 3B</td>
<td>7</td>
<td>15</td>
</tr>
<tr>
<td>Gln A</td>
<td>A culture from Chapter 4</td>
<td>65</td>
<td>102</td>
</tr>
<tr>
<td>Gln B</td>
<td>B culture from Chapter 4</td>
<td>62</td>
<td>102</td>
</tr>
<tr>
<td>Dil A</td>
<td>A culture from Chapter 5</td>
<td>85</td>
<td>148</td>
</tr>
<tr>
<td>Dil B</td>
<td>B culture from Chapter 5</td>
<td>91</td>
<td>158</td>
</tr>
</tbody>
</table>

Table 6.1 The previous history, age and number of generations of each culture examined.

Four high generation number continuous cultures were examined – two were previously cultured with varying glutamine concentrations (Gln A and Gln B) and two with varying dilution rate (Dil A and Dil B). The generation number of a culture was calculated from vial thaw, according to the formula in Section 2.9.5. The generation number and culture age were defined as zero at vial thaw.
A) The low generation number culture (control) was inoculated at the experimental conditions after a short preculture, resulting in the accumulation of 7 generations from vial thaw. The data was taken from results shown in Section 3.4. B) As detailed in Chapter 4, duplicate continuous cultures were inoculated from one preculture train and cultured for 90 days with varying glutamine concentrations. On the last day of continuous culture, cells were harvested from each bioreactor in order to inoculate triplicate shake flasks at the experimental conditions described in Section 6.2.2. These cultures had accumulated 62 and 65 generations at the beginning of this cultivation (A and B cultures, respectively). C) In a similar manner to part B, duplicate continuous cultures were inoculated from one preculture train and cultured for up to 140 days with varying dilution rate (Chapter 5). On the last day of continuous culture, cells were similarly harvested from each bioreactor in order to inoculate triplicate shake flasks at the experimental conditions described in Section 6.2.2. These cultures had accumulated 85 and 91 generations at the beginning of this cultivation (A and B cultures, respectively).
6.3 Results

6.3.1 Increased generation number results in lower maximal viable cell number, but increased growth rate on day 2 of batch culture

With the exception of day 5, the number of viable cells in the low generation number cultivation was higher than every other culture ($p<0.05$). On day 5 there was no significant difference between the Dil A culture and the control culture ($6.0 \pm 0.2 \times 10^6$ cells/ml at 7 generations and $5.2 \pm 0.1 \times 10^6$ cells/ml for Dil A culture, Figure 6.2). The number of viable cells at Dil A was significantly higher than its counterpart on days 2 through 5 (i.e. Dil B, $p<0.05$). Similarly, the number of viable cells reached on days 4 and 5 for the Gln B culture was considered to be statistically higher than the corresponding A culture ($p<0.05$). In both these cases, the culture with the lower number of accumulated generations reached a higher viable cell number, although this finding does not extend to overall generation numbers.

The growth rate of each culture was calculated from days 1 through 5, and no significant differences were noted between any of the cultures on days 1, 3 and 5. The growth rate at low generation number on day 2 was significantly lower than all other cultures ($0.7 \pm 0.0$ for the control culture and $0.9 \pm 0.0$ for Gln A culture, $p<0.01$, Figure 6.3). On day 3 there was a significantly higher growth rate for Dil A culture when compared to Dil B (Figure 6.3, $p<0.05$). This was suggested by the observed increase in viable cells in this culture from day 2 to 5 (Figure 6.1). Overall, at increased generation number there was a trend of increased growth rate on days 1 and 2, thereafter a similar rate for all cultures was observed.

6.3.2 The rate of glucose consumption and lactate production increases on days 1 and 2 of high generation number cultures

There was a trend of increased concentration of extracellular glucose at low generation number, although the shift was only statistical when compared to all other cultures on day 2 ($29.7 \pm 0.1$ mM at 7 generations and $26.8 \pm 0.0$ mM for Dil A culture, $p<0.001$, Figure 6.4). Additionally, the Dil A culture had a significantly higher glucose concentration than its corresponding replicate at all time points ($p<0.05$). In a similar manner, the Gln B culture had a statistically higher concentration than the Gln A culture, but on days 3 to 5 only ($p<0.01$).
Figure 6.2 Cells at increased generation number have a decreased maximal viable cell concentration during batch cultivation.

Triplicate shake flasks were incubated at an initial cell density of $0.5 \times 10^6$ cells/ml from each bioreactor on the last day of continuous culture (Chapters 4 and 5). The number of viable cells was measured daily with the use of a CEDEX AS$^{20}$ automated cell counter. The viability remained above 91% for all cultures examined. The plot shows mean values ± the SEM, and values in parentheses indicate the generation number of each culture at the beginning of the experimental time period. The daily differences in viable cell number were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 6.3.1. Dil and gln represents the continuous cultures with varying dilution rate and glutamine concentration respectively.
Figure 6.3 High generation cultures display an increased growth rate on day 2 of cultivation.

Triplicate shake flasks were incubated at an initial cell density of $0.5 \times 10^6$ cells/ml from each bioreactor on the last day of continuous culture (Chapters 4 and 5). The number of viable cells was measured daily with the use of a CEDEX AS$^{20}$ automated cell counter and the growth rate was calculated according to Section 2.9. The control culture (black) shows the growth rate after just 5 generations of pre-culture. The plot shows mean values ± the SEM, and values in parentheses indicate the generation number of each culture at the beginning of the experimental time period. The daily differences in growth rate were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 6.3.1. Dil and gln represents the continuous cultures with varying dilution rate and glutamine concentration respectively.
Figure 6.4 Cultures with high generation number have a significantly lower extracellular concentration of glucose on day 2 of culture.

Triplicate shake flasks were incubated from each bioreactor on the last day of continuous culture (Chapters 4 and 5). The extracellular glucose concentration was measured daily with the use of a Nova Bioprofile 100 plus. The plot shows mean values ± the SEM, and values in parentheses indicate the generation number of a particular culture at the beginning of the experimental time period. The daily differences in the extracellular glucose concentration were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 6.3.2. Dil and gln represents the continuous cultures with varying dilution rate and glutamine concentration respectively.
The glucose consumption rate was significantly reduced at low generation number on days 1 and 2 (with the exception of the comparison with Dil A on day 1, p<0.001, Figure 6.5). The high generation number cultures had a 2 to 5 fold higher consumption rate than this control culture on day 1 (13.7 ± 0.8 pmol/VC/day at Dil B and 2.7 ± 0.1 for the control culture, Figure 6.5). Additionally, the glucose consumption rate for the control culture was statistically lower than all other cultures on day 5 at 0.9 ± 0.00 pmol/VC/day (the value at Gln A is 1.3 ± 0.0 pmol/VC/day at the same time point, p<0.01). The higher extracellular glucose concentration at Gln B when compared to Gln A translated into a significantly lower rate of consumption rate on days 3 and 4 only (p<0.01). In a similar manner, the Dil B culture had a higher consumption rate than its corresponding culture, from days 1 to 4 (p<0.05).

The control culture had the lowest concentration of lactate throughout the cultivation time. On day 5, a concentration of 18.9 ± 0.4 mM was reached for this culture, while the Gln B culture attained a concentration of 22.0 ± 0.1 mM (Figure 6.6). A significantly higher concentration of lactate was present in the Gln A culture on days 4 and 5, when compared to the Gln B culture only (p<0.05). When comparing the two Dil cultures, an initial significant increase in the lactate concentration in the Dil B culture was seen (p<0.05). However, from day 2 onwards there was a trend of decreased concentration in the B culture, which was statistically relevant on days 2 and 5. On day 5, a concentration of 25.2 ± 0.4 mM was determined for the Dil B culture, and 27.1 ± 0.3 mM for the Dil A culture (p<0.01).

The production rate of lactate was significantly lower for the control culture on days 1 and 2 only (p<0.001, Figure 6.7). There was an almost 6-fold increase in qLac with increased generation number on day 1 (2.2 ± 0.1 pmol/VC/day for the control culture, and 12.9 ± 0.7 pmol/VC/day for Gln A culture). With the exception of Gln B culture, the lactate production rate was also significantly lower on day 5 for the control culture (at -0.1 ± 0.1 pmol/VC/day, p<0.001). The lactate production rate was also significantly higher for the Gln A culture on days 4 and 5 when compared to the Gln B culture (p<0.01). The Dil B culture had a statistically higher rate of lactate production than the A culture on days 2 through 4 (p<0.001).
Triplicate shake flasks were incubated from each bioreactor on the last day of continuous culture (Chapters 4 and 5). The extracellular glucose concentration and the viable cell number were measured daily with the use of the Nova Bioprofile 100 plus and the CEDEX AS\textsuperscript{20} automated cell counter. The specific consumption rate of glucose in pmol per viable cell per day was calculated according to Section 2.9.1, and plotted against the time in culture. The plot shows mean values ± the SEM, and values in parentheses indicate the generation number of each culture at the beginning of the experimental time period. The daily differences in the consumption rate of glucose were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 6.3.2. Dil and gln represents the continuous cultures with varying dilution rate and glutamine concentration respectively.
Figure 6.6 The extracellular concentration of lactate is significantly increased on all days of batch culture in high generation number cultures.

Triplicate shake flasks were incubated from each bioreactor on the last day of continuous culture (Chapters 4 and 5). The extracellular lactate concentration was measured daily with the use of a Nova Bioprofile 100 plus. The plot shows mean values ± the SEM, and values in parentheses indicate the generation number of each culture at the beginning of the experimental time period. The daily differences in the extracellular lactate concentration were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 6.3.2. Dil and gln represents the continuous cultures with varying dilution rate and glutamine concentration respectively.
Figure 6.7 High generation number cultures have an increased rate of lactate production on days 1 and 2 of culture.

Triplicate shake flasks were incubated from each bioreactor on the last day of continuous culture (Chapters 4 and 5). The extracellular lactate concentration and the viable cell number were measured daily with the use of the Nova Bioprofile 100 plus and the CEDEX AS\textsuperscript{30} automated cell counter. The specific production rate of lactate in pmol per viable cell per day was calculated according to Section 2.9.1, and plotted against the time in culture. The plot shows mean values ± the SEM, and values in parentheses indicate the generation number of each culture at the beginning of the experimental time period. The daily differences in the rate of lactate production were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 6.3.2. Dil and gln represents the continuous cultures with varying dilution rate and glutamine concentration respectively.
6.3.3 The ammonium production rate of high generation cultures increases at distinct cultivation time points

There was a trend of decreased concentration of extracellular glutamine at higher generation number, although this trend was only significant on day 2 (p<0.001, Figure 6.8). The concentration of glutamine was statistically higher at Dil B at all time points examined, when compared to the Dil A culture (1.4 ± 0.0 mM for the A culture on day 5 and 2.3 ± 0.0 mM for the B culture at this same time point, p<0.01). There was a significantly higher concentration of glutamine for the Gin A culture on days 4 and 5, when compared to the Gin B culture (2.8 ± 0.0 mM for the A culture on day 5 and 1.9 ± 0.1 mM for the B culture, p<0.001).

The specific rate of glutamine consumption was significantly increased at high generation number on day 1 of culture, with the exception of the comparison with the Gin B culture (p<0.001, Figure 6.9). Additionally, there was a significantly higher rate of glutamine consumption for both Dil cultures on day 1, with an 8 fold increase in the rate at Dil B when compared to control values (2.89 ± 0.18 pmol/VC/day for Dil B culture versus 0.36 ± 0.03 pmol/VC/day for the control culture, p<0.001). The significant difference in glutamine concentration between both Dil cultures translated into a similar rate of glutamine consumption throughout the culture. This result was suggested by the observation that the glutamine concentration decreased at a similar rate for both cultures. The statistical decrease in glutamine concentration for the Gin B culture on days 4 and 5 was caused by a significant increase in the glutamine consumption rate at these time points (p<0.05).

Some striking differences are seen in the extracellular concentrations of glutamate during the experimental time course. From days 2 to 5, a significant increase in the glutamate concentrations at high generation number was observed (with the exception of the comparison between Dil A and the control on day 2, p<0.05, Figure 6.10). In this same time period, both the Dil B and Gin B cultures had a significantly increased glutamate concentration when compared to their corresponding duplicates (p<0.05). For example, there was a concentration of 2.1 ± 0.0 mM glutamate on day 5 for the Gin B culture while the Gin B culture had a concentration of 3.2 ± 0.0 mM at the same time point.

The consumption rate of glutamate was calculated from days 1 to 5, and the resulting negative values indicate production of glutamate (Figure 6.11). There was a trend of
Figure 6.8 High generation number cultures have a significantly higher extracellular concentration of glutamine on day 2 of culture.

Triplicate shake flasks were incubated from each bioreactor on the last day of continuous culture (Chapters 4 and 5). The extracellular glutamine concentration was measured daily with the use of a Nova Bioprofile 100 plus. The plot shows mean values ± the SEM, and values in parentheses indicate the generation number of each culture at the beginning of the experimental time period. The daily differences in the extracellular glutamine concentration were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 6.3.3. Dil and gln represents the continuous cultures with varying dilution rate and glutamine concentration respectively.
Figure 6.9 Cells that have previously been cultured at varying dilution rate have a significantly higher rate of glutamine consumption on day 1 of culture when compared to all other cultures.

Triplicate shake flasks were incubated from each bioreactor on the last day of continuous culture (Chapters 4 and 5). The extracellular glutamine concentration and the viable cell number were measured daily with the use of the Nova Bioprofile 100 plus and the CEDEX AS automated cell counter. The specific consumption rate of glutamine in pmol per viable cell per day was calculated according to Section 2.9.1, and plotted against the time in culture. The plot shows mean values ± the SEM, and values in parentheses indicate the generation number of each culture at the beginning of the experimental time period. The daily differences in the consumption rate of glutamine were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 6.3.3. Dil and gln represents the continuous cultures with varying dilution rate and glutamine concentration respectively.
Figure 6.10 Cultivation of high generation number cells results in a significantly increased glutamate concentration.

Triplicate shake flasks were incubated from each bioreactor on the last day of continuous culture (Chapters 4 and 5). The extracellular glutamate concentration was measured daily with the use of a Nova Bioprofile 100. The plot shows mean values ± the SEM, and values in parentheses indicate the generation number of a particular culture at the beginning of the experimental time period. The daily differences in the extracellular glutamate concentration were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 6.3.3. Dil and gln represents the continuous cultures with varying dilution rate and glutamine concentration respectively.
Figure 6.11 The glutamate consumption rate does not change in a generation number dependent manner.

Triplicate shake flasks were incubated from each bioreactor on the last day of continuous culture (Chapters 4 and 5). The extracellular glutamate concentration and the viable cell number were measured daily with the use of the Nova Bioprofile 100 plus and the CEDEX AS$^{20}$ automated cell counter. The specific consumption rate of glutamate in pmol per viable cell per day was calculated according to Section 2.9.1, and plotted against the time in culture. The plot shows mean values ± the SEM, and values in parentheses indicate the generation number of each culture at the beginning of the experimental time period. The daily differences in the consumption rate of glutamate were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 6.3.3. Dil and gln represent the continuous cultures with varying dilution rate and glutamine concentration respectively.
increased production at high generation number on the early days of cultivation, with similar rates for all cultures observed at the end stages. On days 2 and 3, there was a significant increase in the production of glutamate at Dil B, when compared to Dil A culture only (p<0.05, Figure 6.11). In a similar way, the Gln B culture produced statistically more glutamate than the Gln A culture on days 3 and 4 of cultivation (p<0.001).

At increased generation number, there was a statistically higher concentration of ammonium, with the exception of days 3 and 5, where small insignificances were noted (p<0.05, Figure 6.12). The ammonium concentration was significantly lowered on day 5 for the Gln B culture, when compared to all other high generation cultures (7.1 ± 0.0 mM at this time point, p<0.001). Additionally, the concentration of ammonium reached for the Dil cultures was higher than all others on days 4 and 5 (p<0.001). The higher ammonium concentration at elevated generation number was caused by an increased rate of ammonium production (Figure 6.13). However, this trend was statistically significant on days 2 and 4 only (p<0.01). The rate of production was significantly higher on days 2 and 3 for the Dil A culture when compared to the Dil B culture only (p<0.01). Similarly, there was an increase in the Gln A culture relative to the Gln B culture on day 5 only (p<0.05).

6.3.4 The HCG productivity decreases at late stages of culture

The extracellular HCG content was analysed on a range of time points during the cultivation period. On days 1, 3 and 5, the total HCG content was significantly lower at high generation number (p<0.001, Figure 6.14). A value of 114 ± 1.5 lU/ml was determined for the Dil A culture on day 5, compared to 410 ± 0.0 lU/ml for the control culture. There was no significant difference between the duplicate Gln and Dil cultures on days 3 and 5.

There was no significant difference in the specific productivity of the control culture when compared to all others on day 1 (Figure 6.15). However, on day 3 a value of 26.2 ± 0.9 µIU/VC/day was determined for the control culture and this was considered to be significantly different from all other cultures, with the exception of Gln A and Dil B. On day 5 of culture, the specific productivity at low generation number was significantly higher than all other cultures (p<0.01, at 22.2 ± 0.8 µIU/VC/day). Additionally, there was a significant difference between the specific productivity of the Dil cultures at all time points examined (p<0.01).
Figure 6.12 The extracellular concentration of ammonium increases on days 0, 1, 2 and 4 of high generation number culture.

Triplicate shake flasks were incubated from each bioreactor on the last day of continuous culture (Chapters 4 and 5). The extracellular ammonium concentration was measured daily with the use of a Nova Bioprofile 100 plus. The plot shows mean values ± the SEM, and values in parentheses indicate the generation number of a particular culture at the beginning of the experimental time period. The daily differences in the extracellular ammonium concentration were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 6.3.3. Dil and gln represents the continuous cultures with varying dilution rate and glutamine concentration respectively.
Triplicate shake flasks were incubated from each bioreactor on the last day of continuous culture (Chapters 4 and 5). The extracellular ammonium concentration and the viable cell number were measured daily with the use of the Nova Bioprofile 100 plus and the CEDEX AS\textsuperscript{20} automated cell counter. The specific production rate of ammonium in pmol per viable cell per day was calculated according to Section 2.9.1, and plotted against the time in culture. The plot shows mean values ± the SEM, and values in parentheses indicate the generation number of each culture at the beginning of the experimental time period. The daily differences in the production rate of ammonium were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 6.3.3. Dil and gln represents the continuous cultures with varying dilution rate and glutamine concentration respectively.
Figure 6.14 The HCG content of high generation cultures decreases throughout the culture time.

Triplicate shake flasks were incubated from each bioreactor on the last day of continuous culture (Chapters 4 and 5). Each day, 1 ml of culture supernatant was made cell-free by centrifuging at 1000 g for five minutes and filtering through a 0.2 μm filter. Samples were stored at -20°C before analysis of extracellular HCG content by ELISA. This was carried out by the quality unit, MSD, Oss, the Netherlands. The plot shows mean values ± the SEM, and values in parentheses indicate the generation number of each culture at the beginning of the experimental time period. The differences in the extracellular HCG content at each time point were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 6.3.4. Dil and gln represents the continuous cultures with varying dilution rate and glutamine concentration respectively.
Figure 6.15 The specific productivity of HCG differs significantly on days 3 and 5 of high generation culture (with the exception of the comparison of Gln A and Dil B on day 3).

Triplicate shake flasks were incubated from each bioreactor on the last day of continuous culture (Chapters 4 and 5). 1 ml of culture supernatant was taken each day and relevant samples were subject to HCG ELISA analysis by the quality unit, MSD, Oss, the Netherlands. Additionally, the number of viable cells was established each day with the use of the CEDEX AS automated cell counter. The specific productivity of HCG by these cells in μIU per viable cell per day was calculated according to Section 2.9. The plot shows mean values ± the SEM, and values in parentheses indicate the generation number of each culture at the beginning of the experimental time period. The differences in the specific productivity of HCG at each time point were assessed by use of one-way ANOVA followed by post-hoc Bonferroni analysis using Prism. Statistical significance is not indicated, but is discussed in Section 6.3.4. Dil and gln represents the continuous cultures with varying dilution rate and glutamine concentration respectively.
6.4 Discussion

6.4.1 Increased generation number and cell growth, metabolism and productivity

A number of common changes were apparent when comparing the low generation number culture (control, 7 generations) and those at high generation number (all other cultures, between 62 and 91 generations). The maximal viable cell number decreased above 62 generations (Figure 6.2). This is in contrast to literature where a significant increase in the number of viable hybridoma cells was noted after 57 passages, which would be a considerably larger number of generations (Schmid et al., 1990). However, in these experiments, the number of viable cells inoculated at high generation number on day zero was significantly lower than those at low generation number. This occurred despite the intention to cultivate all inoculums similarly, and suggests that if a comparable initial viable cell number had been achieved, a different result may have occurred. Despite this, there was a trend of increased growth rate at high generation number in the early days of culture (days 1 and 2, with a significant difference noted on day 2, Figure 6.3).

In a similar manner, there was a trend of increased consumption rate of glucose, glutamine and glutamate with corresponding increase in the production rate of lactate and ammonium at high generation number on days 1 and 2 (Figures 6.5, 6.7, 6.9, 6.11 and 6.13). This corresponded to a relative increase in the extracellular concentrations of the metabolites lactate, ammonium and glutamate and a decrease in glucose and glutamine (Figures 6.4, 6.6, 6.8, 6.10 and 6.12). This suggests that there was an overall increase in the key metabolic rates at increased generation number, which is in agreement with the work of Coco-Martín et al (Coco-Martín et al., 1993). However, these changes only occurred over the first two days of culture, thereafter, rates decreased to values that were comparable to the control cultures. It is possible that the culture conditions were more favourable for cell growth and metabolism at these time points. At the end of all continuous cultivation, the extracellular concentration of lactate exceeded 35 mM, and values above 20 mM have been reported to have toxic effects on cells in culture (Schneider et al., 1996). The concentration of ammonium was relatively low at approximately 1.3 mM for the glutamine continuous cultures, and 4 mM for the dilution rate cultures. This suggests that the reduction in lactate concentration at the beginning of the shake flasks cultivation may have resulted in favourable growth patterns for the high generation cultures.

As expected, there was a significant decrease in the total amount of HCG produced at high generation number (Figure 6.14). This is in line with the body of literature that
demonstrates that production of both cellular and recombinant proteins decreases at extended generation numbers (Kaufmann and Schimke, 1981; Weidle et al., 1988; Pallavicini et al., 1990; Briske-Anderson et al., 1997; Yu et al., 1997; Kim et al., 1998b; Fann et al., 2000; Behrens and Kissel, 2003; Youm et al., 2008). While the mechanisms of this decrease in extracellular HCG content were not investigated as part of this work, the reduction in the specific production rate on days 3 and 5 of culture points to a potential cause (Figure 6.15). In some reported cases, a reduction in the total amount of recombinant protein is caused by a decrease in the specific productivity of each cell in the population (Kim et al., 1996; Borth et al., 1999). Consequently, this is a likely method of action. Other literature suggests that a low-producing population of cells appears within high generation cultures, causing a reduction in the overall recombinant protein content (Frame and Hu, 1990; Lee et al., 1991; Chuck and Palsson, 1992; Bae et al., 1995). Additionally, a loss of the recombinant gene from the cellular genome has been observed, most notably in CHO cells (Pallavicini et al., 1990; Merritt and Palsson, 1993; Kromenaker and Srienc, 1994; Kim et al., 1998b; Hammill et al., 2000). As a result, any one of changes could be the cause of the reduction in production of HCG.

The mechanisms of the reduction in productivity can be established relatively easily. Future work on this topic would involve the use of a flow cytometric assay to determine the proportion of the cell population that produces HCG, versus the non-producers (Merritt and Palsson, 1993). In addition, the loss of the HCG gene from the cellular genome can be visualised with use of a fluorescence in-situ hybridisation technique (Pallavicini et al., 1990). If these techniques do not demonstrate an increase in the proportion of non-producing cells or loss of the recombinant gene, then a decrease in the overall productivity of the cell population can be assumed (Kim et al., 1996).

6.4.2 Differences in growth and metabolic responses as a result of previous cultivation conditions

While there was a clear distinction between the metabolism and productivity of high and generation number cultures, the different high generation cultures displayed a spread of responses. In both the Gln and Dil cultures, a particular pattern of altered growth and metabolism was observed at high generation number. Dil B and Gln A had the highest generation number of each condition at the start of the experimental time. The maximal viable cell number and the highest rate of glucose consumption and lactate production
were observed for these replicates within each condition (Figure 6.2, 6.5 and 6.7). This suggests that as generation numbers accumulated, the metabolism became much less efficient. This inefficient metabolism was also seen for the metabolism of glutamine, glutamate and ammonium in the Dil B culture. Increased consumption of glutamine was accompanied by an increase in the production of glutamate and ammonium (Figure 6.9, 6.11 and 6.13). Additionally, a significant increase in the specific production of HCG was seen at all time points for this culture when compared to the Dil A culture (Figure 6.15). As a result, the difference in response of the two Dil replicates could be attributed to a less efficient metabolism at increased generation number.

For the Gin cultures, the inefficient metabolism of glucose was accompanied by altered metabolism of glutamine, glutamate and ammonium. In this case, there was a difference when compared to the Dil cultures; glutamine was consumed at a significantly lower rate on day 1 of culture (Figure 6.9). The consumption rate was increased relative to that observed in the glutamine continuous cultures, but presumably very high rates of consumption were not seen as a result of the lack of glutamine supplementation in the continuous culture, since time would be required for ‘normal’ glutamine metabolism to resume. These cells had previously adapted to the lack of glutamine in the continuous culture by converting α-ketoglutarate to glutamate and glutamine in order to maintain glutamine supplies for production of nucleotides and nicotinamide coenzymes (Chapter 4). At the higher generation number in Gin A, the rate of glutamine consumption was reduced while the production of glutamate and ammonium was increased at the later stages of cultivation when compared to Gin B (Figure 6.9, 6.11 and 6.13). In this case, the extra generations accumulated for the Gin A culture clearly caused alterations in metabolism.

The reason why the metabolic changes were not completely dependent on the number of generations accumulated is unclear. However, Chapters 4 and 5 illustrated that the history of changes in cultivation conditions can impact on the current cell metabolism. Consequently, it is possible that the differences in metabolism observed for each culture were as a result of the conditions each cell population experienced within continuous cultures.

There was no significant difference in the total amount of HCG produced by either Gln cultures. However, an unusual trend of HCG production was noted for the B culture, with a sharp increase in extracellular content on day 3 (Figure 6.14). It seems likely that the data
points on day 2 was an outlier, since there is a linear pattern of increasing HCG content for all other cultures. This resulted in an unusual pattern of specific productivity, as illustrated in Figure 6.14. However, the general trend of decreasing productivity with increased time in culture was obvious. If the productivity on day 3 is neglected, no significant differences were noted between Gln cultures (Figure 6.15).

6.4.3 Conclusions

Increased generation number (between 62 and 91 generations) caused a transient increase in the growth rate, specific consumption rate of glucose, glutamine and glutamate and in the production rates of lactate and ammonium. After three days in batch shake flask conditions, all of these rates returned to values similar to low generation controls. This suggests that an increase in cell metabolism upon inoculation in fresh CD-CHO medium may have occurred as a response to the reduction in extracellular metabolite concentrations, such as lactate.

A reduction in the total amount of HCG produced is observed at all time points examined. A corresponding decrease in the specific productivity of HCG was seen on days 3 and 5. As a result, the loss in total HCG may be due simply to an overall reduction in productivity, although the reasons for the loss of productivity remains unknown.
Chapter 7

General Discussion
7.1 Discussion

This work aimed to investigate the ways in which altered cellular metabolism influenced the glycosylation of HCG which was recombinantly produced by a CHO cell line. These changes in cellular metabolism were induced by varying key culture parameters such as the cellular growth rate or the concentration of glutamine supplied to culture medium in both a batch and continuous culture set up. When the continuous culture system was coupled with appropriate analytic techniques, the method yielded great insight into the mechanisms behind these changes. Continuous cultivation in the absence of glutamine and with reduced glycolytic flux resulted in reduced sialylation, fucosylation and antennarity of the N-linked glycans attached to HCG while the proportion of neutral structures increased. Assay of the intracellular content of nucleotides and sugar nucleotides revealed that this change in glycosylation was mediated by the decrease in the UDP-GlcNAc content (Chapter 4).

Varying the rate of dilution of culture medium altered the growth rate of these cells, and these reduced dilution rates were also found to decrease the sialylation, fucosylation and antennarity of the N-linked glycans attached to HCG while increasing the proportion of neutral structures. In this case, the intracellular content of all nucleotides and sugar nucleotides was similarly decreased at low dilution rate, indicating that the lack of substrates impeded the glycosylation of HCG (Chapter 5).

These studies used HCG as the model glycoprotein. All existing literature has described how the predominant N-linked glycan structure is biantennary, galactosylated and potentially disialylated and core fucosylated (Endo et al., 1979; Kessler et al., 1979; Weisshaar et al., 1991; Liu and Bowers, 1997; Gervais et al., 2003; Toll et al., 2006) (Figure 1.7). These results were confirmed in this work with the detection of A2G2S1, FA2G2S1, A2G2S2 and FA2G2S2 within the largest peaks of the total N-linked glycan pool (peaks 9, 10 and 11, Figure 2.23. See Table 2.7 for details of N-linked glycan abbreviations). However, there was a significant proportion of underprocessed and hybrid structures, for example, the high mannose structure M5 was detected in high amounts for all cultures. Additionally, a significant percentage of the total pool was composed of the structures A1, A2, A2G1, A2G1S1 and FA2G1 (peaks 1, 2, 4, 6 and 7, Table 2.7). While some hybrid structures have been previously described, a key difference is noted in the types of structures found here. With the exception of M5 and A1, each of these underprocessed structures has a GlcNAc residue attached to both arms of the biantennary structure. This indicates that the potential modification and extension of the secondary arm is not carried out in this case. The reasons for this are unclear. However, in the classic
"stable compartments" model of Golgi maturation, these same N-linked glycan structures are transported from cis-Golgi to medial Golgi and from medial to trans-Golgi. This suggests that these transport vesicles may be secreted under these culture conditions. However, the true mode of Golgi maturation has not been fully elucidated, as evidenced by the recent description of "cisternal maturation" of Golgi vesicles in S. cerevisiae (Losev et al., 2006). However, a number of other factors could also result in the production of underprocessed glycans, including an insufficient intracellular content of the substrates for the enzymes of glycosylation or a reduction in the proportion of the enzymes themselves. While the basis for the secretion of underprocessed glycans is unknown, the potential still exists to fully glycosylate these structures.

To date, the glycosylation of recombinant HCG produced in CHO cells has been reported in just one instance (Gervais et al., 2003). These authors observed a population of triantennary structures, and one tetraantennary structure. Whether these structures were observed due to the analytical technique used (mass spectrometry) or the choice of host is unclear. A small but significant proportion of the N-glycan pool detected here was also triantennary, with variations in the sialylation status (peaks 11 through 15, Figure 2.23). However, no tetraantennary structures were found in this case.

In Chapters 3, 4 and 5 the glycosylation of HCG was assessed by calculating the average pi of HCG from an IEF profile and by detailed structural analysis of the N-linked glycans. In all these cases, no significant differences were observed in the average pi, but statistically relevant changes were seen with more detailed analysis. This suggests that structural analysis of the glycans yields more insight into the glycosylation of HCG due to the sensitivity of the technique.

In Chapter 3, the glycosylation of HCG was monitored over a five-day incubation, and minor time-dependent changes were observed. The proportion of A2 increased significantly for all experimental set-ups, while the percentage of M5 increased for the glutamine-supplemented cultures only (Figures 3.36 to 3.38). No significant changes were noted in the amount of M5 for the 0mM glutamine culture; in addition, these values were significantly higher than the glutamine-supplemented cultures at all time points. The percentage of fully glycosylated IFN-γ has been found to decrease with increased batch culturing time of CHO cells (Curling et al., 1990). These types of changes may be caused by the limitation of glucose or glutamine (Hayter et al., 1992; Nyberg et al., 1999).
However, batch feeding of these nutrients was found to have no effect on the glycosylation effects reported (Castro et al., 1995). Since the concentration of glucose is above 12 mM for all the batch cultures examined, the changes are unlikely to be a consequence of this. However, it is possible that the increased proportions of M5 are as a result of the lack of glutamine supplementation.

A large proportion of this thesis explores the effect of varying glutamine concentrations on the glycosylation of HCG. Chapter 3 described how a five-day batch cultivation at 0 mM glutamine resulted in a reduced percentage of N-linked sialylated, fucosylated and triantennary structures but increased neutral structures (Figures 3.36 to 3.38). These changes were evident from day 1 of culture (Figure 3.36). However, a number of additional changes were also seen during this culture; the growth rate and rate of glucose consumption and lactate production decreased while the culture pH was higher. Since each of these parameters has been found to alter N-linked glycosylation, the changes could not be directly attributed to a lack of glutamine (Hayter et al., 1992; Borys et al., 1993; Wong et al., 2005).

Chapter 4 investigated the effect of varying the glutamine concentration between 8 mM and 0 mM on the glycosylation of HCG in a continuous culture set up. The growth rate was controlled at 0.4/d by the rate of dilution of culture medium. However, no changes in glycosylation were noted at states 1 and 2 (8 mM and 0 mM glutamine, respectively, Figure 4.24). This indicates that glutamine alone does not change the glycosylation of HCG in this cultivation set-up, and since the growth rate was the same for both steady states, the impact of this effect can be neglected. However, at state 4, the flux through glycolysis was reduced in the absence of glutamine, and the same changes in glycosylation were observed (Figure 4.12 and 4.24). This suggests that the changes in batch culture were likely caused by the reduced rate of glucose consumption. Additionally, a significant reduction in the intracellular content of UDP-GlcNAc was observed at state 4 in the continuous culture, which caused the observed changes in glycosylation (Figure 4.21). This suggests the possibility that a similar change occurred in the batch cultivation at 0mM glutamine to cause these observed alterations in glycosylation. In conclusion, at low glucose consumption rates, and in the absence of glutamine supplementation to culture medium, the proportion of sialylated, fucosylated and triantennary N-linked glycan structures attached to HCG decreased, and the proportion of neutral structures increased as a result of the reduced in the intracellular content of UDP-GlcNAc.
Glutamine is required in growing cells for the synthesis of purine and pyrimidine nucleotides, nicotinamide coenzymes and activated sugar nucleotides. Transamination of amino acids inevitably leads to the formation of glutamate and some cells can utilise glutamate to produce glutamine by the action of glutamine synthetase. Cells with inadequate activity of this enzyme and with insufficient amino acids concentrations in culture medium will inevitably suffer negative consequences. For example, the cell viability of BHK cells decreased to 50% just 24 hours after inoculation in the absence of glutamine (Christie and Butler, 1999). An ideal cell culture scenario involves glutamine supplementation to culture medium at low enough concentrations to support the flux towards the synthesis of purines, pyrimidines, nicotinamide co-enzymes and activated sugar nucleotides and should result in low concentrations of ammonium. In addition, glucose should be supplied at a sufficient concentration to prevent nutrient starvation and lactate accumulation. However the method of cultivation and concentration of each metabolite needed needs to be determined empirically for each cell line and recombinant protein. Indeed the high rate of lactate production in all culture set-ups suggests that CHO cell metabolism is predominantly glycolytic, which can occur for a range of reasons including limitation of oxygen or for the regeneration of NAD$^+$. 

Chapter 6 explored the effect of increased generation number on CHO cell metabolism and the productivity of HCG. While the significant decrease in productivity seen in the batch incubations post continuous culture was not unexpected (Barnes et al., 2003), it is interesting to note that there was no reduction in the productivity during the varying dilution rate continuous cultures (Chapter 5). The reasons for this are unclear, however, the statistical increase in HCG productivity at state 3 due to altered amino acid metabolism may have had effect on the subsequent steady state productivity.

Steady state multiplicity is a well known concept that has been observed in many chemical syntheses reactions, and in cultures of E. coli and yeast (Europa et al., 2000). The phenomenon has also been observed in CHO and hybridoma cells (Hayter et al., 1992; Europa et al., 2000; Follstad et al., 2000; Gambhir et al., 2003; Korke et al., 2004). A great deal of research has been carried out in recent times – including a genomic and proteomic screen of cultures with altered metabolism, in order to explore this phenomenon in greater detail (Korke et al., 2004). While the principle of guiding cells towards more efficient metabolism is not new, this illustration of altered glycosylation at these new steady states has not been reported previously.
The use of a continuous culture system paired with appropriate analysis tools yields a powerful method for analysing the effect of many stimuli on cells in culture. With the attainment of a stable cell metabolism at steady state, the impact of varying culture conditions can be neglected. Consequently, this technique can be applied for many other areas of research.

7.2 Future work

The wealth of data accumulated in the course of this work points to the potential to explore the findings in more detail with *in silico* modelling. A mathematical model of N-linked glycosylation could be constructed with use of the existing literature (Umana and Bailey, 1997; Hossler *et al.*, 2007; Krambeck *et al.*, 2009). The model could be used to predict the changes in this pathway that would lead to the glycosylation shifts observed in this work. For example, the model described by Hossler *et al* predicts that increasing the holding time of the N-linked glycans in the Golgi will result in increased terminal processing of the glycans (Hossler *et al.*, 2007). However, the opposite case was found in Chapter 5, where a decreased growth rate caused a reduction in the sialylation, antennarity and fucosylation of HCG and an increase in the proportion of neutral structures. As a result, sufficient interlinking of the experimental data and modelling can lead to insights into the control of the process of N-linked glycosylation. Consequently, the ultimate aim of directing N-linked glycosylation may become a reality. Additionally, the assaying of the intracellular content of nucleotides and sugar nucleotides would lend itself to expanding a basic model towards an understanding of how some peripheral events impact on glycosylation.

Metabolic flux analysis at each steady state resulted in reproducible data that could also be incorporated into a mathematical model which may help to predict changes in metabolism (Nazaret and Mazat, 2008).

The phenomenon of steady state multiplicity was observed in this culture, and while it has previously been demonstrated in CHO cells, the mechanisms by which these changes are induced changes is still poorly understood (Hayter *et al.*, 1992). By exploring this topic in more detail, further applications of this technique may be discovered. Additionally, the significant increase in productivity of HCG at steady state 3 of the varying dilution rate experiments is of potential interest to the biopharmaceutical industry (Chapter 5). Further work could explore whether this change can be readily translated into other cell lines expressing different recombinant proteins.
The reason for the loss of productivity of HCG in this cell line at high generation number was not explored (Chapter 6). In understanding how this occurs, measures can be taken to guide the process of generating recombinant cell lines in the future to result in more stable cell lines.

Additional verification that reduced intracellular concentrations of UDP-GlcNAc causes the changes in N-linked glycosylation of HCG at low glucose consumption rates and limited glutamine concentrations can also be carried out (state 4, Chapter 4). Repeating this cultivation and feeding UDP-GlcNAc at adequate concentrations in state 4 to resume normal glycosylation patterns will conclusively prove this fact.

In a similar manner, it is possible that feeding key sugar nucleotides and lowering the dilution rate of a continuous culture may result in efficient metabolism and increased sialylation, fucosylation and proportions of triantennary N-linked glycans in this system (Chapter 5).

Batch cultivation with different glutamine concentrations also resulted in varied culture pH values (Chapter 3, Figure 3.8 and 3.25). A reduction in culture pH has been found to alter cell metabolism and recombinant protein glycosylation (Borys et al., 1993). The pH of the continuous cultivations was controlled in the bioreactors by CO$_2$ addition and NaOH titration. Consequently, any changes in metabolism and glycosylation occurred independent of pH effects. However, the response of this cell line to varied pH values remains to be tested.

As described in Section 1.2.3, the glycan chain of HCG can impact on the correct folding, assembly, activity and half-life of the protein itself. In order to design the most effective glycoform, maximal sialylation is required, since this modification is important for most of these functions. None of the experiments described in this thesis resulted in any improvements to sialylation (above a baseline value found at 8mM glutamine or a dilution rate of 0.5/d, see Figure 5.27). Literature has described a variety of methods which have been shown to increase the sialylation of recombinant proteins including feeding of N-acetylmannosamine, overexpression of key enzymes in sialic acid synthesis pathway, in vitro re-sialylation of recombinant proteins, inhibition of sialidase activity in the supernatant and incorporation of unnatural sialic acid analogues (Bork et al., 2009). Additionally, increasing the antennarity or incorporating novel N-linked glycosylation sites
into the protein sequence of EPO has been shown to increase its serum half-life and efficacy (Sethuraman and Stadheim, 2006). Any one of these methods could be tested to potentially increase the sialylation of HCG.

There was also a significant proportion of underprocessed glycans within the HCG glycan pool. The reasons for this are unknown, and once they have been identified, methods to complete terminal processing of the N-linked glycans can be designed. Literature has shown that overexpression of key enzymes and transporters in the glycosylation pathway as well as feeding specific precursors can impact on the final glycan structure (Baker et al., 2001; Jeong et al., 2009; Hossler et al., 2009; Wong et al., 2010b). In addition, the glycans could be completed after protein purification by an ex-vivo process (Wong et al., 1994). Consequently, some of these could be undertaken to increase the proportion of fully glycosylated HCG.
Chapter 8

Bibliography


