Metabolic Analysis of the Asparagine and Glutamine Dynamics in an Industrial CHO Fed-Batch Process

Jimmy Kirsch[1*], Sandra V. Bennun[2*], Adam Mendez[1], Amy S. Johnson[2], Hongxia Wang[3], Haibo Qiu[3], Ning Li[3], Shawn M. Lawrence[2], Hanne Bak[2], Michael J. Betenbaugh[1]

[1]Department of Chemical and Biomolecular Engineering, Johns Hopkins University, Baltimore, MD 21218, USA

[2]Regeneron Pharmaceuticals, Inc, Preclinical Manufacturing and Process Development Tarrytown, NY 10591, USA.

[3]Regeneron Pharmaceuticals, Inc, Analytical Chemistry Group, Tarrytown, NY 10591, USA.

*These authors contributed equally

Correspondence can be sent to Sandra Bennun at sandra.bennun@regeneron.com

1. Abstract

Chinese Hamster Ovary (CHO) cell lines are grown in cultures with varying asparagine and glutamine concentrations, but further study is needed to characterize the interplay between these amino acids. By following ^{13}C-glucose, ^{13}C-glutamine, and ^{13}C-asparagine tracers using metabolic flux analysis (MFA), CHO cell metabolism was characterized in an industrially relevant fed-batch process under glutamine supplemented and low glutamine conditions during early and late exponential growth. For both conditions MFA revealed glucose as the primary carbon source to the tricarboxylic acid (TCA) cycle followed by glutamine and asparagine as secondary sources. Early exponential phase CHO cells prefer glutamine over asparagine to support the TCA cycle under the glutamine supplemented condition, while asparagine was critical for TCA activity for the low glutamine condition. Overall TCA fluxes were similar for both conditions due to the trade-offs associated with reliance on glutamine and/or asparagine. However, glutamine supplementation increased fluxes to alanine, lactate and

enrichment of glutathione, N-Acetyl-Glucosamine (NAG) and pyrimidine-containing-molecules. The late exponential phase exhibited reduced central carbon metabolism dominated by glucose, while lactate reincorporation and aspartate uptake were preferred over glutamine and asparagine. These ^{13}C studies demonstrate that metabolic flux is process time dependent and can be modulated by varying feed composition.

Keywords: Chinese hamster ovary cells, glutamine, asparagine, glucose, fed-batch, ^{13}C tracers, metabolism, Metabolic Flux Analysis (MFA), pyrimidine synthesis, glutathione.

2. Introduction

The biopharmaceutical market is expected to surpass USD500 billion by 2023 (Rader & Langer, 2018). A large number of these products are generated by Chinese hamster ovary (CHO) cells, the host organism of choice in the biopharmaceutical industry (Walsh, 2014). Efforts to understand how these cells process nutrients will increase our capacity to design medium to optimize production of recombinant proteins. One key aspect of CHO production is glutamine and asparagine metabolism, representing major carbon and nitrogen sources for the cell. Glutamine is a critical component, for growth, energy, redox, signaling, and pH regulation of mammalian cell metabolism (Borys, Linzer, & Papoutsakis, 1994; Dyring, Hansen, & Emborg, 1994; Wahrheit, Nicolae, & Heinzle, 2014; Xu, Dai, Graf, Martel, & Russell, 2014). In addition to being used to synthesize protein and fuel the TCA cycle, glutamine also functions as a carbon and nitrogen source for pyrimidines and their derivatives (Hayter et al., 1991; Holden, Thoden, & Raushel, 1999; D. Mathur et al., 2017). Nucleotides from pyrimidines and purines are precursors for nucleic acid synthesis and nucleotide sugars, which are substrates for glycosylation (Evans & Guy, 2004; McAtee, Templeton, & Young, 2014; Nyberg et al., 1999; Rijcken, Overdijk, Vandeneijnden, & Ferwerda, 1993).

Glutamine supplementation can alter the specific growth rate, the maximum viable cell density, titer, and the pH profile (Dean & Reddy, 2013; Wahrheit et al., 2014; Xu et al., 2014; F. Zhang, Sun, Yi, & Zhang, 2006). However, due to its high demand, glutamine is often the first amino acid to be depleted from the medium (Ahn & Antoniewicz, 2012; Chen & Harcum, 2005; Nyberg, Balcarcel, Follstad, Stephanopoulos, & Wang, 1999; Schneider, Marison, & von Stockar, 1996). When fed to CHO cells, some glutamine is channeled into the tricarboxylic acid (TCA) cycle after conversion to glutamate, which coincides with either the generation of free ammonia or excess alanine acting as a sink for ammonia (Dean & Reddy, 2013). Ammonia accumulation at higher concentrations can be detrimental for cell growth, production, and quality (Altamirano, Illanes, Becerra, Cairo, & Godia, 2006; Genzel, Ritter, Konig, Alt, & Reichl, 2005; Gupta et al., 2017; Kurano, Leist, Messi, Kurano, & Fiechter, 1990). As a result, the benefits of glutamine addition to the culture can be limited. Finding the optimal level and time point for feeds can be challenging for a fed-batch process due to the balance of requirements for cell growth against cell line sensitivity to toxic quantities of ammonia.

Similar to glutamine, asparagine is of paramount importance for cellular metabolism and can sometimes fulfill a similar role to glutamine, serving as an alternative nitrogen and carbon source for the cell and can be consumed in addition to or in place of glutamine (Altamirano et al., 2006; Dean & Reddy, 2013; Duarte et al., 2014; Fomina-Yadlin et al., 2014; Genzel et al., 2005; Xu et al., 2014; L.-x. Zhang et al., 2016). Asparagine can also be depleted during fed-batch culture (Hansen & Emborg, 1994; Hayter et al., 1991; Kishishita et al., 2015; Xu et al., 2014). In cancer cells, asparagine along with other non-essential amino acids can restore glutamine-deprived cell survival even with limited or reduced TCA cycle activity (Pavlova et al., 2018; J. Zhang et al., 2014).

As glutamine and asparagine can feed the TCA, their consumption rates can exceed their stoichiometric demands, therefore, a better understanding of how these amino acids are used and interplay as substrates for growth and maintenance in CHO cell cultures would be valuable. In this study

a CHO cell expression platform is cultured under fed-batch mode to understand over batch time how asparagine and glutamine metabolism is modulated in glutamine supplemented and low glutamine conditions using ^{13}C labeling approaches. All conditions contained glutamine in the basal medium, with additional glutamine added in both the basal medium and feeds for the supplemented condition only. To characterize the specific contributions of asparagine, glutamine, and glucose to CHO cell metabolism during early and late exponential phase for the low glutamine and glutamine supplemented conditions, CHO cells were fed with ^{13}C-glucose, ^{13}C-glutamine, and ^{13}C-asparagine tracers at two appropriate points during the exponential growth phase. These tracers were followed through tracer enrichment analysis and ^{13}C metabolic flux analysis (^{13}C MFA). CHO cells utilized all three resources to support central carbon metabolism with characteristic patterns of utilization and distribution rates that changed from early exponential to late exponential phases. Glutamine and asparagine played key roles during early exponential phase, while glucose utilization was relevant throughout the process. The cells prefer glutamine over asparagine for the glutamine supplemented conditions while asparagine contributes significantly to the TCA cycle under the low glutamine condition. These amino acids also contribute to other pathways including pyrimidine, NAG, and glutathione synthesis. However, the utilization of both amino acids becomes less prominent in the late exponential growth phase as glucose, aspartate, and lactate play increasingly important roles in carbon metabolism.

3. Methods

3.1 Cell Culture

A recombinant monoclonal antibody producing CHO cell line that grows in suspension with a proprietary chemically defined medium was used for this study. After expansion in a seed train, the cells were inoculated in 250 ml shake flasks and cultured in fed-batch mode for 14 days in a humidified incubator. Bolus proprietary chemically defined feeds were administrated during the culture with glucose fed daily. The glucose, glutamine and asparagine ^{13}C tracer studies were fed depending on the

early or late exponential phase focus of the study **(Figure 1A)**. The early exponential labeling period started with labeled basal media at inoculation time (day 0) and continued with labeled glucose feeds (day 1) until samples were taken on day 2. Tracers were added to the basal medium. The late exponential labeling period starts with a labeled feed on day 4 and ends when samples were taken on day 5. Parallel cell cultures were prepared for each tracer [U-^{13}C] Asparagine, [U-^{13}C] Glutamine, and a glucose stock containing a mixture of [U-^{13}C] Glucose and [1,2-^{13}C] Glucose. For each culture with its corresponding tracer modified in the media or feeds, sampling was performed over a brief time interval to confirm that the system reached a quasi-steady-state (Crown, Long, & Antoniewicz, 2016).

3.2 Determination of specific consumption and production rates

The Bioprofile Flex analyzer, Bioprofile CDV and BP400 (Nova Biomedical, Waltham MA) were used to measure glucose, lactate, ammonia and cell number. Ultra-performance liquid chromatography (UPLC) with an AccQ•Tag Ultra C18 column was used to collect amino acid concentration data based upon absorption at 260nm. Antibody titer was determined by high performance chromatography (HPLC) using POROS 20µm ProteinA ID cartridge on an Agilent HPLC column with samples detected at 280 nm absorbance.

Specific extracellular consumption and production rates of metabolites were obtained by calculating the derivatives of the cumulative concentration profiles using a central difference method and dividing by the average cell density:

$$q_C = \frac{dC}{X\,dt}$$

Where: q_C is the specific consumption rate of C [mmol/cell/time], C is the cumulative concentration [mmol] and X the average cell density [cell/mL]. The specific production rate of biomass was calculated using a 3-parameter nonlinear equation for purposes of smoothing. Tabulated values for the fluxes calculated, normalized to the glucose input flux in low glutamine condition, are included in **Table S1**.

3.3 Quenching, extraction, and derivatization of intracellular metabolites

Extraction and processing of Gas Chromatography Mass Spectrometry (GCMS) samples were adapted from Ahn *et al.* (Ahn & Antoniewicz, 2012; Ahn, Crown, & Antoniewicz, 2016) and for Liquid Chromatography Mass Spectrometry (LCMS) were adapted from Lu *et al.* (Lu, Bennett, & Rabinowitz, 2008). Samples of cells were taken 48 hours after addition of the tracers for the early exponential phase and after 24 hours for the late exponential phase. Approximately ten million cells were collected from the cell culture and cold quenched.

3.4 GCMS and LCMS analysis

GCMS analysis was performed on a Shimadzu GCMS-QP2010 with a Rtx-5MS (30m x 0.25um x 0.25mm i.d.) capillary column. The injection temperature was 200℃ for metabolites, samples were run on splitless mode, and pressure was held at 65.2kPa. The column flow was 1.00mL/min and the ion source temperature was 200℃.

LCMS analysis was conducted on Acuity I-Class UPLC (Waters, Milford, MA) coupled to a Q-Exactive™ HF mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Cellular metabolites were separated on a SeQuant ZIC-pHILIC column (2.1x 100mm, 5µm) (EMD Millipore, Billerica, MA). The column temperature was set at 25℃ and the flow rate was 0.15mL/min. A 25-minute gradient was used for metabolites separation. Metabolites were detected in negative mode full scan analysis with resolution at 120,000.

3.5 Enrichment calculation of GCMS and LCMS data

GCMS and LCMS chromatograms were analyzed and integrated for intracellular metabolites using MATLAB. Integrated peaks for the m+0, m+1, *etc*. isotopologues were converted into fractions of the total integration. Data was corrected for natural abundance using INCA (Young, 2014). Enrichments were determined using a weighted average of the m+1, m+2…m+n isotopologues to determine the percentage of ^{13}C in the measured pool as follows:

$$Enrichment = \sum_{n=1}^{N} \frac{n M_n}{N}$$

Where n is the number of ^{13}C in a compound, N is the total number of carbons in a compound, and M_n is the percentage of the compound with n ^{13}C. This results in a weighted average of all the possible isotopologues. Standard errors for enrichments were less than 1% except for lactate and malate measurements of ^{13}C-glucose experiments, which were less than 2%.

3.6 MFA analysis and network model of CHO metabolism

^{13}C-Metabolic flux analysis (^{13}C -MFA) was performed using INCA (Young, 2014). INCA applies an elementary metabolite unit (EMU) to estimate fluxes and minimize residuals (Antoniewicz, Kelleher, & Stephanopoulos, 2007; Young, Walther, Antoniewicz, Yoo, & Stephanopoulos, 2008). GCMS data was used to calculate network fluxes under a steady state assumption. Standard error tabulated from the GCMS data were applied in INCA. A network of CHO carbon metabolism was adapted from previous studies (Nargund, Qiu, & Goudar, 2015) as seen in **Table S2**. The equation for the CHO biomass and antibody was created using a combination of in-house analysis and distributions previously reported (Sheikh, Forster, & Nielsen, 2005; Zupke, Sinskey, & Stephanopoulos, 1995). Fluxes and tolerances were calculated based on a 95% confidence interval of the compiled data.

4. Results and Discussion

To characterize the metabolic role of glucose, glutamine, and asparagine, ^{13}C-MFA was performed on an industrially relevant CHO cell line during the early (day 0 to day 2) and late exponential (day 4 to day 5) growth phases of a fed-batch process. The incorporation of isotopically labeled carbon from these amino acids and glucose into the intracellular metabolites of central carbon metabolism and pathways related to nucleotides, glutathione, and NAG was analyzed. Two different glutamine levels were fed to a recombinant CHO cell line in a collection of shake flask fed-batch cultures. The “low

glutamine" condition had 2 mM of glutamine in the basal medium but lacked glutamine in the feeds while the "glutamine supplemented" condition included 4 mM of glutamine added to the basal medium and each of the fed-batch feeds (**Figure 1A**).

4.1. Glutamine supplementation upregulates glycolysis in the early exponential phase but does not prevent slowdown of glycolysis when transitioning into late exponential phase

Cell culture profiles of viable cell density, titer, and ammonia for the low glutamine and glutamine supplemented conditions are shown in **Figure 1B**. Growth rates and nutrients extracellular rates were determined throughout the fed-batch process and used in our subsequent MFA analysis (**Table S2**). Regardless of glutamine supplementation, the cell growth rates were relatively similar, with an early exponential growth rate of approximately $0.05h^{-1}$ (day 2) and a late exponential growth rate of approximately $0.016h^{-1}$ (day 5) for both conditions.

Glutamine supplementation increased the average peak viable cell density and ammonia levels of the culture ($p<0.05$) but did not alter the final titer significantly ($p>0.05$) (**Figure 1B**). For comparison purposes, the glucose uptake in the low glutamine condition during the early exponential phase was normalized on a molar basis to 100 unit/cell-day, and other fluxes are reported relative to this value (**Table S1, Figure 2**). For the glutamine supplemented condition in the early exponential phase, normalized glucose consumption increased to 112 unit/cell-day while lactate production increased to 73 unit/cell-day compared to 58 unit/cell-day in the low glutamine condition. Increases in lactate and ammonia (**Figure 1B**) were also accompanied by increases in alanine, suggesting a more active overflow metabolism with higher glucose consumption under the glutamine supplemented condition.

During the late exponential phase (day 5), glucose consumption rate decreased to 30 unit/cell-day in both conditions (**Table S1, Figure 4**). The 70% decline in glucose utilization coincided with the metabolism switching to lactate uptake for both the low glutamine and glutamine supplemented

conditions. Lactate consumption also differed for the two different conditions, with a lactate consumption of 4.8 unit/cell-day for the low glutamine condition and 6.7 unit/cell-day in the glutamine supplemented condition. Increases in consumption and production rates correlated with slightly increased peak cell density and enhanced ammonia production in the glutamine supplemented condition (**Figure 1B)**. The abundance of nitrogen likely drives CHO cells to convert more pyruvate to alanine as a sink for the intracellular nitrogen (**Table S1**), which has been observed previously (Chen & Harcum, 2005; McAtee Pereira, Walther, Hollenbach, & Young, 2018; Ozturk, Riley, & Palsson, 1992; Schneider et al., 1996). The metabolism of glutamine and alanine are connected by the glutamine-pyruvate transaminase reaction, in which glutamine is metabolized to alpha-ketoglutarate and the amine group produced is transferred to pyruvate, generating alanine. Through these transaminase reactions, such as that facilitated by glutamine-pyruvate and glutamate-aspartate transaminase, CHO cells can eliminate some of the excess ammonia generated, and perhaps produce a more favorable cell culture environment.

4.2 Glutamine is preferred over asparagine to support the TCA Cycle in the early exponential phase

^{13}C-Metabolic Flux Analysis (^{13}C-MFA) was performed using GCMS data for ^{13}C-glucose, ^{13}C-glutamine, and ^{13}C-asparagine incorporated into the media (Day 0) for both the low glutamine and the glutamine supplemented conditions. Intracellular labeling data was combined with measured extracellular flux data (**Table S1)** and then analyzed by the Inca MFA algorithm (Young, 2014), which minimized residuals to generate intracellular flux maps for the low glutamine and the glutamine supplemented conditions**.**

During the early exponential phase, approximately half of the glucose entering the cells was channeled through pyruvate into the TCA cycle for both conditions (represented as percentages in **Figure 2A** and **2B**). Much of the remaining glucose consumed was converted into lactate and alanine,

with higher fluxes of these metabolites observed for the glutamine supplemented condition (**Figure 2B**). Other key secondary metabolites that enter the TCA cycle included asparagine for both conditions and glutamine in the supplemented case. While 55% and 47% of the glucose-derived carbon entered the TCA cycle (calculations detailed in the Supplemental Methods SM.1) in the low glutamine and the glutamine supplemented conditions respectively, asparagine and glutamine (for the glutamine supplemented condition) still provided a substantial influx into the central carbon metabolism. For the low glutamine condition, glutamine and glutamate carbon contribution to the TCA cycle was negligible while asparagine and aspartate contributed 14% of the carbon entering the TCA relative to the contribution via acetyl-CoA (ACA or AcCoA). This percentage is calculated from the ratio of fluxes entering the TCA cycle, weighted by the number carbon atoms in the source molecule. A detailed description of how this relation can be calculated is found in the Supplemental Methods SM.2. In the glutamine supplemented condition, glutamine and glutamate provided 30% of the carbon relative to the glucose carbon contribution, while asparagine and aspartate contributed only 5% of the carbon relative to the Acetyl-CoA contribution. Therefore, asparagine by way of aspartate (7.9 molar unit/cell-day, **Figure 3A**) was the second largest contributor of carbon to the TCA cycle for the low glutamine condition while glutamine by way of glutamate was the second largest supplement to the TCA (13 unit/cell-day, **Figure 3B**) in the glutamine supplemented condition. Furthermore, the asparagine/aspartate flux into the TCA cycle (2.8 unit/cell-day, **Figure 3B**) decreased in the glutamine supplemented condition relative to the low glutamine condition. Despite, CHO cells can use both glutamine and asparagine to support the TCA cycle along with glucose, glutamine was clearly preferred over asparagine as indicated for the glutamine supplemented condition.

Interestingly, asparagine functions as a significant secondary resource for TCA metabolism in the low glutamine case while aspartate notably switches between excretion and consumption for the two conditions. For the low glutamine condition, aspartate was consumed (1.2 unit/cell-day **Figure 3A**),

while in the glutamine supplemented condition aspartate was excreted (4.0 unit/cell-day-**Figure 3B**). Therefore, for the glutamine supplemented case, a significant fraction of the asparagine consumption leading to aspartate does not ultimately feed into the TCA as some aspartate produced left the cell or was consumed for other metabolic requirements. The excretion of aspartate for these conditions may also benefit the cell by preventing further nitrogen generation. This aspartate excretion resulted in a net decrease in flux into the TCA cycle from asparagine and aspartate (**Figure 2**). Overall, the net changes in glutamine and asparagine fluxes into the TCA cycle in the glutamine supplemented condition (**Figure 3**) together with glucose input resulted in similar TCA cycle fluxes for both conditions in the early exponential phase. Given comparable TCA cycle fluxes, the upregulated glycolytic flux for the glutamine supplemented condition may provide the cells with more rapid ATP production. Interestingly, asparagine is critical in supporting growth in some cancer cells under glutamine limited conditions, which also indicates that the flux of asparagine and aspartate, in addition glutamine and glucose, can be a critical metabolic pathway for cell expansion across cell types (Pavlova et al., 2018).

4.3 Aspartate is the main amino acid flux into the TCA Cycle in late exponential phase

^{13}C-MFA was also performed during the late exponential phase to generate intracellular flux maps for the low glutamine and glutamine supplemented conditions (**Figure 4A and 4B).** Glucose consumption was reduced to approximately 30% of that observed in early exponential phase (**Figure 4A and 4B**). Another difference from early exponential growth was that lactate served as a secondary carbon source for metabolism. Due to the greater production of lactate in the glutamine supplemented condition in the early exponential phase, more lactate was available for reuptake during the late exponential phase. Reuptake of lactate has been widely observed in previous CHO cell culture studies (Altamirano et al., 2006; Duarte et al., 2014; Freund & Croughan, 2018).

In addition, a higher fraction of glucose (and lactate) derived carbon entered the TCA cycle for both low glutamine and glutamine supplemented conditions (>90%) during the late exponential phase (**Figure 4A and 4B**) compared to (~50%) for the early exponential phase (**Figure 3A and 3B**), perhaps in part, to the generation of fewer byproducts, including serine, alanine, and glycine. Asparagine continued to provide supporting flux into TCA cycle metabolism. However, aspartate also become a key contributor of flux into the TCA cycle for both low glutamine and glutamine supplemented cases (**Figure 5**). In contrast, fluxes around glutamate and glutamine were minor and directed away from the TCA cycle in both conditions (**Figure 5**).

Interestingly, CHO cells preferentially utilized aspartate over either asparagine and glutamine in the late exponential phase for both conditions (**Figure 5A and 5B**). Asparagine fluxes were reduced by more than a factor of three during the late exponential compared to the early exponential phase for both conditions (**Figure 5A and 5B, Figure 3**), but the overall fractional contribution from asparagine and aspartate to the TCA was higher than during the early exponential phase. Indeed, for the low glutamine condition, aspartate and asparagine together contributed fully 40% of the carbon relative to the glucose/lactate contribution via Acetyl-CoA. Also, in the glutamine supplemented condition, aspartate and asparagine contributed 33% of the carbon relative to the glucose contribution. In contrast, glutamate and glutamine drew a small amount of carbon from the TCA cycle (1.4 molar unit/cell-day) for the low glutamine condition (**Figure 5A**) and a negligible amount for the glutamine supplemented condition **(Figure 5B)**.

4.4 Intracellular metabolite enrichment confirms that glucose is the predominant input to the TCA in the early and late exponential phases

To further examine changes in cellular metabolism at different days and conditions, the enrichment of select metabolites derived from the ^{13}C-glucose, ^{13}C-glutamine, and ^{13}C-asparagine

labeling experiments was estimated for the early exponential phase (**Figure 6**) and the late exponential phase (**Figure 7**). ^{13}C-glutamine labeling enrichment was detected in glutamate, malate, citrate, and aspartate along with a small amount in lactate and alanine (glutamine supplemented only) during the early exponential period (**Figure 6**). Further, the contribution of ^{13}C-glutamine in all these compounds was greater in glutamine supplemented condition. ^{13}C-asparagine enrichment was noticeable in aspartate, citrate, malate, and glutamate, with a very limited level of tracer noted in other compounds (**Figure 6**). When additional glutamine was added, the ^{13}C-asparagine enrichment in citrate, malate, and aspartate declined, indicating the preferential usage of glutamine in the TCA cycle and its intermediates and by-products.

During the late exponential phase, ^{13}C-glucose enrichment remained predominant while the enrichment from ^{13}C-glutamine and ^{13}C-asparagine declined for many metabolites in both conditions (**Figure 7**). Lactate, pyruvate, alanine, glycine, and serine were dominated by ^{13}C-glucose enrichment over ^{13}C-glutamine and ^{13}C-asparagine. Glucose also contributed a substantial percentage to citrate, malate, and glutamate, but a smaller percentage of aspartate. This is consistent with the flux analysis which indicates that unlabeled aspartate represents a principal contributor to the TCA after glucose in the late exponential phase.

Limited amounts of ^{13}C-asparagine were detected in aspartate, citrate, glutamate, and lactate (in the glutamine supplemented condition). Its reduction relative to the early exponential phase is likely due to the increased uptake of aspartate in both the low glutamine and glutamine supplemented conditions (**Figure 5A and 5B**). ^{13}C-glutamine noticeably labeled glutamate but principally in the glutamine supplemented conditions. Only trace amounts of ^{13}C-glutamine were detected in citrate and lactate. The enrichment results support the predominance of ^{13}C-glucose tracer incorporation in all the

measured metabolites, and the major role that glucose plays in supporting the TCA cycle during both phases, especially during the late exponential phase.

4.5 Glutamine supplementation increases intracellular enrichment of nitrogen containing molecules in the early exponential phase

The role that glutamine and asparagine play in other metabolic pathways was also examined using LCMS during the early exponential phase when glutamine and asparagine incorporation into other metabolites was elevated. Significant ^{13}C-glutamine and ^{13}C-asparagine labeling of metabolites was detected for pyrimidines, glutathione, and N-acetyl-glutamate (NAG) pathways in both low glutamine and glutamine supplemented conditions (**Figure 8**). The pyrimidine synthesis pathway utilizes the initial precursors carbamoyl phosphate and aspartate to begin synthesis of other subsequent precursors and products (**Figure 8A**). Carbon enters the pyrimidine synthesis pathway through aspartate (Deepti Mathur et al., 2017). Therefore, glutamine and asparagine must first be converted to aspartate. In addition, the initial step of this pathway is glutamine dependent, due to the key role of glutamine as a nitrogen provider. Although glutamine does not contribute carbon directly, perhaps its increased availability may help to drive pathway activity in glutamine supplemented cultures by contributing some aspartate generation in the supplemented condition (**Figure 6**). Noticeable ^{13}C enrichment from ^{13}C -asparagine and ^{13}C-glutamine was detected for N-carbamoyl-aspartate, UMP, UTP, deoxyuridine, and dTTP, all of which showed increased enrichment from ^{13}C -glutamine for the glutamine supplemented condition (**Figure 8B**). Furthermore, the production of nucleotides, critical to cell expansion, is impacted by glutamine supplementation as shown by the enrichment of identified metabolites in the pyrimidine pathway which is consistent with previous findings (Burleigh et al., 2011). We also observed enrichment in nucleotide sugars including UDP-glucoronate and UDP-GlcNAc for the glutamine supplemented cultures. This role of glutamine for production of UDP sugars has been noted previously for other mammalian cell lines (D. Mathur et al., 2017; Taschwer et al., 2012), and UDP-GlcNAc levels can have a significant impact on glycosylation of IgG and other cellular glycoproteins (Fan et al., 2012; Hills, Patel,

Boyd, & James, 2001). Enrichment from ^{13}C-asparagine exhibited some fluctuations but remained consistent for most compounds in both conditions.

Glutathione is an antioxidant tripeptide synthesized from glycine, cysteine, and glutamate. As glutamine was supplemented, the fraction of enriched glutathione increased three-fold (**Figure 8C**). Since the enrichment from ^{13}C-asparagine slightly decreased, most of this increase came from the ^{13}C-glutamine. Cultures in which glutamine is elevated have been known to exhibit an increase in the levels of glutathione (Amores-Sanchez & Medina, 1999; Hong, Rounds, Helton, Robinson, & Wilmore, 1992; Sies, 1999). Glutathione itself has been found to be an antioxidant that controls reactive oxygen species and inhibits apoptosis (al-Rubeai & Singh, 1998; Amores-Sanchez & Medina, 1999; Franco & Cidlowski, 2009; Sies, 1999) as well as regulating disulfide bonds in proteins (Chakravarthi & Bulleid, 2004; Chiosa, Niculescu, Bonciocat, & Stancu, 1965; Sies, 1999). Providing glutamine to cultures may thus provide beneficial impacts well beyond TCA cycle metabolism such as facilitating the generation of glutathione and other metabolites that can provide resistance against apoptosis and alter the protein folding and redox environment (Orellana et al., 2015). This may help explain, at least in part, the slight increase in cell densities observed in the VCC measurements of the glutamine supplemented condition.

In addition, the metabolite, N-acetyl-glutamate (NAG), derived from glutamine as shown in **Figure 8D** was enriched from ^{13}C-asparagine with a significant boost in enrichment following ^{13}C-glutamine supplementation (**Figure 8D**). NAG serves as an activator of the initial steps of the urea cycle, which are identical to those in pyrimidine synthesis (Anderson, 1981; Holden et al., 1999; Levenberg, 1962; Makoff & Radford, 1976) although NAG is not known to promote the pyrimidine pathway. Increased expression of urea cycle enzymes in the CHO cells is known to result in reduced accumulation of ammonium ions in the culture medium (Chung et al., 2003). Overall, glutathione and pyrimidine pathway enrichment along with NAG increases under glutamine supplemented medium may provide

supplemental benefits to cell growth and robustness beyond the direct effects of glutamine's impact on the TCA cycle.

5. Conclusions

^{13}C MFA was used to examine the distribution of glucose, glutamine, and asparagine at different points of the exponential growth phase for a low glutamine condition with glutamine in the basal medium only compared to a condition with glutamine supplementation in the feeds in addition to the basal medium for an industrially relevant fed-batch process. While glucose is the primary carbon source into the TCA cycle during the exponential phase, glucose uptake rates and fluxes decrease from the early to the late exponential phase. However, the percent contribution of carbon from glucose to the TCA cycle increases at later times. In contrast, glutamine and asparagine are the preferred secondary carbon sources during the early exponential growth phase. Glutamine was favored over asparagine in the glutamine supplemented condition, while asparagine was more important for the low glutamine condition. In addition, for the glutamine supplemented condition, lactate, alanine and ammonia fluxes were elevated during the early exponential phase. Flux data indicates a dynamic change in the glucose to amino acid ratio channeled into the TCA as the culture progresses from early to late exponential phase with glutamine input into TCA cycle activity becoming negligible and asparagine uptake dropping 3-fold regardless of glutamine supplementation. Interestingly, aspartate was preferentially utilized during the late exponential phase for both the low glutamine and glutamine supplemented cultures perhaps due to either increased excretion during the early exponential phase or its potential preference as a substrate for the slower-growing late exponential cells. This suggest the importance of consistent aspartate supplementation in the late exponential phase when glutamine is being produced and asparagine is highly consumed.

While TCA fluxes appeared comparable for glutamine supplemented and low glutamine conditions, enrichment data and ^{13}C MFA indicates that the glutamine supplemented condition leads to greater lactate production in the early exponential phase, resulting in a larger lactate reservoir. Furthermore, complementary LC analysis during the early exponential phase indicated notable enrichment of N-Acetyl-Glucosamine, glutathione and various pyrimidines, leading to enrichment of nucleotides and nucleotide sugars such as UDP-GlcNAc in glutamine supplemented conditions. While glutamine supplementation is not essential for sufficient cell growth, its presence may impact cellular energetics via TCA cycle intermediates but also potentially other pathways that may affect reactive oxygen species and apoptosis via glutathione, and glycosylation via sugar nucleotides such as UDP−GlcNAc and other pyrimidine pathway derivatives. Therefore, supplementing cultures with the proper glutamine concentrations that balance the benefits of glutamine availability versus by-product accumulation such as lactate and ammonia may ultimately be beneficial for cell cultures.

Acknowledgments

The authors would like to thank Dr Phil Mortimer for his support with GCMS and all the PMPD analytics group for amino acid and monoclonal antibody titer measurements, in special to John Reeves and Rebecca Lawlor.

References

Ahn, W. S., & Antoniewicz, M. R. (2012). Towards dynamic metabolic flux analysis in CHO cell cultures. *Biotechnol J, 7*(1), 61-74. doi:10.1002/biot.201100052

Ahn, W. S., Crown, S. B., & Antoniewicz, M. R. (2016). Evidence for transketolase-like TKTL1 flux in CHO cells based on parallel labeling experiments and C-13-metabolic flux analysis. *Metabolic Engineering*, *37*, 72-78. doi:10.1016/j.ymben.2016.05.005

al-Rubeai, M., & Singh, R. P. (1998). Apoptosis in cell culture. *Curr Opin Biotechnol*, *9*(2), 152-156.

Altamirano, C., Illanes, A., Becerra, S., Cairo, J. J., & Godia, F. (2006). Considerations on the lactate consumption by CHO cells in the presence of galactose. *J Biotechnol*, *125*(4), 547-556. doi:10.1016/j.jbiotec.2006.03.023

Amores-Sanchez, M. I., & Medina, M. A. (1999). Glutamine, as a precursor of glutathione, and oxidative stress. *Mol Genet Metab*, *67*(2), 100-105. doi:10.1006/mgme.1999.2857

Anderson, P. M. (1981). Purification and properties of the glutamine- and N-acetyl-L-glutamate-dependent carbamoyl phosphate synthetase from liver of Squalus acanthias. *J Biol Chem*, *256*(23), 12228-12238.

Antoniewicz, M. R., Kelleher, J. K., & Stephanopoulos, G. (2007). Elementary metabolite units (EMU): a novel framework for modeling isotopic distributions. *Metabolic Engineering*, *9*(1), 68-86. doi:10.1016/j.ymben.2006.09.001

Borys, M. C., Linzer, D. I., & Papoutsakis, E. T. (1994). Ammonia affects the glycosylation patterns of recombinant mouse placental lactogen-I by chinese hamster ovary cells in a pH-dependent manner. *Biotechnol Bioeng*, *43*(6), 505-514. doi:10.1002/bit.260430611

Burleigh, S. C., van de Laar, T., Stroop, C. J., van Grunsven, W. M., O'Donoghue, N., Rudd, P. M., & Davey, G. P. (2011). Synergizing metabolic flux analysis and nucleotide sugar metabolism to understand the control of glycosylation of recombinant protein in CHO cells. *BMC Biotechnol*, *11*, 95. doi:10.1186/1472-6750-11-95

Chakravarthi, S., & Bulleid, N. J. (2004). Glutathione is required to regulate the formation of native disulfide bonds within proteins entering the secretory pathway. *J Biol Chem*, *279*(38), 39872-39879. doi:10.1074/jbc.M406912200

Chen, P., & Harcum, S. W. (2005). Effects of amino acid additions on ammonium stressed CHO cells. *J Biotechnol*, *117*(3), 277-286. doi:10.1016/j.jbiotec.2005.02.003

Chiosa, L., Niculescu, V., Bonciocat, C., & Stancu, C. (1965). The protective action of N-acetyl and N-carbamyl derivatives of glutamic and aspartic acids against ammonia intoxication. *Biochem Pharmacol*, *14*(11), 1635-1643.

Chung, M. I., Lim, M. H., Lee, Y. J., Kim, I. H., Kim, I. Y., Kim, J. H., . . . Kim, H. J. (2003). Reduction of ammonia accumulation and improvement of cell viability by expression of urea cycle enzymes in Chinese hamster ovary cells. *Journal of microbiology and biotechnology*, *13*(2), 217-224.

Crown, S. B., Long, C. P., & Antoniewicz, M. R. (2016). Optimal tracers for parallel labeling experiments and (13)C metabolic flux analysis: A new precision and synergy scoring system. *Metabolic Engineering*, *38*, 10-18. doi:10.1016/j.ymben.2016.06.001

Dean, J., & Reddy, P. (2013). Metabolic analysis of antibody producing CHO cells in fed-batch production. *Biotechnol Bioeng*, *110*(6), 1735-1747. doi:10.1002/bit.24826

Duarte, T. M., Carinhas, N., Barreiro, L. C., Carrondo, M. J., Alves, P. M., & Teixeira, A. P. (2014). Metabolic responses of CHO cells to limitation of key amino acids. *Biotechnol Bioeng*, *111*(10), 2095-2106. doi:10.1002/bit.25266

Dyring, C., Hansen, H. A., & Emborg, C. (1994). Observations on the influence of glutamine, asparagine and peptone on growth and t-PA production of Chinese hamster ovary (CHO) cells. *Cytotechnology*, *16*(1), 37-42. doi:10.1007/BF00761777

Evans, D. R., & Guy, H. I. (2004). Mammalian pyrimidine biosynthesis: fresh insights into an ancient pathway. *J Biol Chem*, *279*(32), 33035-33038. doi:10.1074/jbc.R400007200

Fan, L., Kadura, I., Krebs, L. E., Hatfield, C. C., Shaw, M. M., & Frye, C. C. (2012). Improving the efficiency of CHO cell line generation using glutamine synthetase gene knockout cells. *Biotechnology and Bioengineering*, *109*(4), 1007-1015.

Fomina-Yadlin, D., Gosink, J. J., McCoy, R., Follstad, B., Morris, A., Russell, C. B., & McGrew, J. T. (2014). Cellular responses to individual amino-acid depletion in antibody-expressing and parental CHO cell lines. *Biotechnol Bioeng*, *111*(5), 965-979. doi:10.1002/bit.25155

Franco, R., & Cidlowski, J. A. (2009). Apoptosis and glutathione: beyond an antioxidant. *Cell Death Differ*, *16*(10), 1303-1314. doi:10.1038/cdd.2009.107

Freund, N. W., & Croughan, M. S. (2018). A Simple Method to Reduce both Lactic Acid and Ammonium Production in Industrial Animal Cell Culture. *Int J Mol Sci*, *19*(2). doi:10.3390/ijms19020385

Genzel, Y., Ritter, J. B., Konig, S., Alt, R., & Reichl, U. (2005). Substitution of glutamine by pyruvate to reduce ammonia formation and growth inhibition of mammalian cells. *Biotechnol Prog*, *21*(1), 58-69. doi:10.1021/bp049827d

Gupta, S. K., Srivastava, S. K., Sharma, A., Nalage, V. H. H., Salvi, D., Kushwaha, H., . . . Shukla, P. (2017). Metabolic engineering of CHO cells for the development of a robust protein production platform. *PLoS One*, *12*(8), e0181455. doi:10.1371/journal.pone.0181455

Hansen, H. A., & Emborg, C. (1994). Influence of ammonium on growth, metabolism, and productivity of a continuous suspension Chinese hamster ovary cell culture. *Biotechnol Prog*, *10*(1), 121-124. doi:10.1021/bp00025a014

Hayter, P. M., Curling, E. M., Baines, A. J., Jenkins, N., Salmon, I., Strange, P. G., & Bull, A. T. (1991). Chinese hamster ovary cell growth and interferon production kinetics in stirred batch culture. *Appl Microbiol Biotechnol*, *34*(5), 559-564.

Hills, A. E., Patel, A., Boyd, P., & James, D. C. (2001). Metabolic control of recombinant monoclonal antibody N−glycosylation in GS−NS0 cells. *Biotechnology and Bioengineering*, *75*(2), 239-251.

Holden, H. M., Thoden, J. B., & Raushel, F. M. (1999). Carbamoyl phosphate synthetase: an amazing biochemical odyssey from substrate to product. *Cell Mol Life Sci*, *56*(5-6), 507-522.

Hong, R. W., Rounds, J. D., Helton, W. S., Robinson, M. K., & Wilmore, D. W. (1992). Glutamine preserves liver glutathione after lethal hepatic injury. *Ann Surg*, *215*(2), 114-119.

Kishishita, S., Katayama, S., Kodaira, K., Takagi, Y., Matsuda, H., Okamoto, H., . . . Aoyagi, H. (2015). Optimization of chemically defined feed media for monoclonal antibody production in Chinese hamster ovary cells. *J Biosci Bioeng*, *120*(1), 78-84. doi:10.1016/j.jbiosc.2014.11.022

Kurano, N., Leist, C., Messi, F., Kurano, S., & Fiechter, A. (1990). Growth behavior of Chinese hamster ovary cells in a compact loop bioreactor. 2. Effects of medium components and waste products. *J Biotechnol*, *15*(1-2), 113-128.

Lao, M. S., & Toth, D. (1997). Effects of ammonium and lactate on growth and metabolism of a recombinant Chinese hamster ovary cell culture. *Biotechnol Prog*, *13*(5), 688-691. doi:10.1021/bp9602360

Levenberg, B. (1962). Role of L-glutamine as donor of carbamyl nitrogen for the enzymatic synthesis of citruline in Agaricus bisporus. *J Biol Chem*, *237*, 2590-2598.

Lu, W., Bennett, B. D., & Rabinowitz, J. D. (2008). Analytical strategies for LC−MS-based targeted metabolomics. *Journal of chromatography B*, *871*(2), 236-242.

Makoff, A. J., & Radford, A. (1976). Glutamine utilization in both the arginine-specific and pyrimidine-specific carbamoyl phosphate synthase enzymes of eurospora crassa. *Mol Gen Genet*, *149*(2), 175-178.

Mathur, D., Stratikopoulos, E., Ozturk, S., Steinbach, N., Pegno, S., Schoenfeld, S., . . . Cantley, L. C. (2017). PTEN regulates glutamine flux to pyrimidine synthesis and sensitivity to dihydroorotate dehydrogenase inhibition. *Cancer discovery*, *7*(4), 380-390.

Mathur, D., Stratikopoulos, E., Ozturk, S., Steinbach, N., Pegno, S., Schoenfeld, S., . . . Parsons, R. (2017). PTEN Regulates Glutamine Flux to Pyrimidine Synthesis and Sensitivity to Dihydroorotate Dehydrogenase Inhibition. *Cancer Discov*, *7*(4), 380-390. doi:10.1158/2159-8290.CD-16-0612

McAtee, A. G., Templeton, N., & Young, J. D. (2014). Role of Chinese hamster ovary central carbon metabolism in controlling the quality of secreted biotherapeutic proteins. *Pharmaceutical Bioprocessing*, *2*(1), 63-74. doi:10.4155/Pbp.13.65

McAtee Pereira, A. G., Walther, J. L., Hollenbach, M., & Young, J. D. (2018). (13) C Flux Analysis Reveals that Rebalancing Medium Amino Acid Composition can Reduce Ammonia Production while Preserving Central Carbon Metabolism of CHO Cell Cultures. *Biotechnol J*. doi:10.1002/biot.201700518

Nargund, S., Qiu, J., & Goudar, C. T. (2015). Elucidating the role of copper in CHO cell energy metabolism using (13)C metabolic flux analysis. *Biotechnol Prog*, *31*(5), 1179-1186. doi:10.1002/btpr.2131

Nyberg, G. B., Balcarcel, R. R., Follstad, B. D., Stephanopoulos, G., & Wang, D. I. (1999). Metabolism of peptide amino acids by Chinese hamster ovary cells grown in a complex medium. *Biotechnol Bioeng*, *62*(3), 324-335.

Orellana, C. A., Marcellin, E., Schulz, B. L., Nouwens, A. S., Gray, P. P., & Nielsen, L. K. (2015). High-antibody-producing Chinese hamster ovary cells up-regulate intracellular protein transport and glutathione synthesis. *J Proteome Res*, *14*(2), 609-618. doi:10.1021/pr501027c

Ozturk, S. S., Riley, M. R., & Palsson, B. O. (1992). Effects of ammonia and lactate on hybridoma growth, metabolism, and antibody production. *Biotechnol Bioeng*, *39*(4), 418-431. doi:10.1002/bit.260390408

Pavlova, N. N., Hui, S., Ghergurovich, J. M., Fan, J., Intlekofer, A. M., White, R. M., . . . Zhang, J. (2018). As Extracellular Glutamine Levels Decline, Asparagine Becomes an Essential Amino Acid. *Cell Metab*, *27*(2), 428-438 e425. doi:10.1016/j.cmet.2017.12.006

Rader, R. A., & Langer, E. S. (2018). Biopharma Manufacturing Markets. Retrieved from https://www.contractpharma.com/issues/2018-05-01/view_features/biopharma-manufacturing-markets/

Rijcken, W. R. P., Overdijk, B., Vandeneijnden, D. H., & Ferwerda, W. (1993). Pyrimidine Nucleotide-Metabolism in Rat Hepatocytes - Evidence for Compartmentation of Nucleotide Pools. *Biochemical Journal*, *293*, 207-213.

Schneider, M., Marison, I. W., & von Stockar, U. (1996). The importance of ammonia in mammalian cell culture. *J Biotechnol*, *46*(3), 161-185.

Sheikh, K., Forster, J., & Nielsen, L. K. (2005). Modeling hybridoma cell metabolism using a generic genome-scale metabolic model of Mus musculus. *Biotechnol Prog*, *21*(1), 112-121. doi:10.1021/bp0498138

Sies, H. (1999). Glutathione and its role in cellular functions. *Free Radic Biol Med*, *27*(9-10), 916-921.

Taschwer, M., Hackl, M., Hernandez Bort, J. A., Leitner, C., Kumar, N., Puc, U., . . . Borth, N. (2012). Growth, productivity and protein glycosylation in a CHO EpoFc producer cell line adapted to glutamine-free growth. *J Biotechnol*, *157*(2), 295-303. doi:10.1016/j.jbiotec.2011.11.014

Wahrheit, J., Nicolae, A., & Heinzle, E. (2014). Dynamics of growth and metabolism controlled by glutamine availability in Chinese hamster ovary cells. *Appl Microbiol Biotechnol*, *98*(4), 1771-1783. doi:10.1007/s00253-013-5452-2

Walsh, G. (2014). Biopharmaceutical benchmarks 2014. *Nat Biotechnol*, *32*(10), 992-1000. doi:10.1038/nbt.3040

Xu, P., Dai, X. P., Graf, E., Martel, R., & Russell, R. (2014). Effects of glutamine and asparagine on recombinant antibody production using CHO-GS cell lines. *Biotechnol Prog*, *30*(6), 1457-1468. doi:10.1002/btpr.1957

Young, J. D. (2014). INCA: a computational platform for isotopically non-stationary metabolic flux analysis. *Bioinformatics*, *30*(9), 1333-1335. doi:10.1093/bioinformatics/btu015

Young, J. D., Walther, J. L., Antoniewicz, M. R., Yoo, H., & Stephanopoulos, G. (2008). An elementary metabolite unit (EMU) based method of isotopically nonstationary flux analysis. *Biotechnol Bioeng*, *99*(3), 686-699. doi:10.1002/bit.21632

Zhang, F., Sun, X., Yi, X., & Zhang, Y. (2006). Metabolic characteristics of recombinant Chinese hamster ovary cells expressing glutamine synthetase in presence and absence of glutamine. *Cytotechnology*, *51*(1), 21-28. doi:10.1007/s10616-006-9010-y

Zhang, J., Fan, J., Venneti, S., Cross, J. R., Takagi, T., Bhinder, B., . . . Thompson, C. B. (2014). Asparagine plays a critical role in regulating cellular adaptation to glutamine depletion. *Mol Cell*, *56*(2), 205-218. doi:10.1016/j.molcel.2014.08.018

Zhang, L.-x., Zhang, W.-y., Wang, C., Liu, J.-t., Deng, X.-c., Liu, X.-p., . . . Tan, W.-s. (2016). Responses of CHO-DHFR cells to ratio of asparagine to glutamine in feed media: cell growth, antibody production, metabolic waste, glutamate, and energy metabolism. *Bioresources and Bioprocessing*, *3*(1), 5.

Zupke, C., Sinskey, A. J., & Stephanopoulos, G. (1995). Intracellular flux analysis applied to the effect of dissolved oxygen on hybridomas. *Appl Microbiol Biotechnol*, *44*(1-2), 27-36.

Figure Captions

Figure 1. Cell culture profiles for CHO cells in low glutamine and glutamine supplemented conditions. A process design schematic (A) for the addition of ^{13}C labeled tracers (glucose, glutamine, or asparagine) for each experiment. The glutamine supplemented condition had additional glutamine added to the basal medium and glutamine feeds which were absent for the low glutamine condition. All other feeds remained the same. For day 2 samples, for tracer analysis, tracers were added to the basal at day 0 and glucose feeds at day 1. Samples were taken before feeds were added on day 2. For day 5 samples, tracers were added to the feeds on day 4. (B) Shows normalized plots of viable cell count, antibody titer, and ammonia concentration as a function of culture time in days over the course of the CHO fed-batch process. Values were normalized to the maximum profile concentration. Multiple curves are replicates, values are given for the low glutamine (green) and the glutamine supplemented (red) conditions over the 14-day culture period.

Figure 2. Flux maps for early exponential phase.

Early exponential phase flux maps of central carbon metabolism for the low glutamine condition (A) and glutamine supplemented condition (B). Flux values along with standard errors are normalized to glucose uptake flux of the low glutamine condition and are given in unit/cell-day with standard errors. Approximately half of the glucose derived carbon (orange percentages) reaches the TCA cycle. A detailed description of this calculation is given in the Supplemental Methods. Blue values indicate reactions which showed statistically significant differences between conditions, while black values indicate no statistically significant differences. Glutamine supplementation drives glutamine uptake into the TCA cycle, upregulates glycolysis and increases alanine production that acts as an ammonia sink.

Figure 3. Glutamine and asparagine metabolic flux maps during early exponential phase.

Flux maps of glutamine and asparagine metabolism for the low glutamine condition (A) and glutamine

supplemented condition (B). Flux values are normalized to glucose uptake flux of the low glutamine condition and are given in unit/cell-day with standard errors. When glutamine is supplemented, it is highly consumed increasing the net flux of amino acids into the TCA cycle even though aspartate is being excreted. More asparagine is consumed in the low glutamine condition compared to the glutamine supplemented condition. Blue values indicate reactions which showed statistically significant differences between the conditions, while black values indicate no significance.

Figure 4. Flux maps for late exponential phase.

Late exponential phase flux maps of central carbon metabolism for the low glutamine condition (A) and glutamine supplemented condition (B). Flux values are normalized to glucose uptake flux of the low glutamine case of the early exponential phase and are given in unit/cell-day with standard errors. Glucose uptake was reduced to approximately 30% of that from the early exponential phase, indicating overall slowed metabolism during the late exponential phase. More than 90% of the total glucose uptake goes into the TCA (orange percentages) and glutamine supplementation may increase lactate consumption. A detailed description of this calculation is given in the Supplemental Methods. Blue values indicate reactions which showed statistically significant differences between the conditions, while black values indicate no significance.

Figure 5. Glutamine and asparagine metabolism flux maps for late exponential phase.

Late exponential phase flux maps of glutamine and asparagine metabolism for the low glutamine condition (A) and glutamine supplemented condition (B). Flux values are normalized to the glucose uptake flux of the low glutamine case and are given in unit/cell-day with standard errors. Amino acid utilization and net flux into the TCA are similar in both conditions. Glutamine and asparagine supplement the TCA cycle to a lesser extent than in the early exponential phase, while more aspartate is

fed directly into the TCA cycle. Blue values indicate reactions which showed statistically significant differences between the conditions, while black values indicate no significance.

Figure 6. Intracellular metabolite enrichment during early exponential phase.

Enrichment in select free metabolites from ^{13}C-glucose (black), ^{13}C-glutamine (blue) and ^{13}C-asparagine (yellow) for the early exponential phase is shown in the inset graphs. Enrichments are shown for the low glutamine (Low) and glutamine supplemented (Sup) conditions. Lactate and alanine are mainly formed from ^{13}C-glucose. With glutamine supplementation there is a shift of glutamine into and asparagine out of the TCA cycle (*e.g.* malate).

Figure 7. Late exponential phase intracellular metabolite Enrichment.

Enrichment in select free metabolites from ^{13}C-glucose (black), ^{13}C-glutamine (blue) and ^{13}C-asparagine (yellow) experiments for the late exponential phase is shown in the inset graphs. Enrichments are shown for the low glutamine (Low) and glutamine supplemented (Sup) conditions. Glucose enrichment of metabolic products is predominant. There is less glutamine and asparagine labeling compared with the early exponential phase. Glutamine only noticeably labels glutamate. Asparagine labeling is generally low, a result of aspartate being utilized over asparagine. There is a slight total enrichment increase in the glutamine supplemented conditions.

Figure 8. Early exponential phase enrichment of intracellular nucleotides, nucleotide-derivatives, and glutathione.

Reaction network for pyrimidine synthesis originating from aspartate and glutamine (A). Reversible reactions and direction reactions are specified based on KEGG pathways. Enrichments for the early

exponential phase are shown for the low glutamine (Low) and glutamine supplemented (Sup) conditions. Experimental ^{13}C-glutamine (blue) and ^{13}C-asparagine (yellow) data are shown for in select free nucleotides and nucleotide derivatives (B). Glutathione is synthesized from the amino acids cysteine, glycine, and glutamate (C). N-Acetyl Glutamate (NAG) is synthesized from Acetyl-CoA and glutamate from (D).