Investigation on Effects of Lactate Adaptation Using Metabolic Indicators and Amino Acids to Explore Cell Metabolism

Lisa Uy
lisau@g.clemson.edu

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Investigation on Effects of Lactate Adaptation Using Metabolic Indicators and Amino Acids to Explore Cell Metabolism

Lisa Uy
INVESTIGATION ON EFFECTS OF LACTATE ADAPTATION USING METABOLIC INDICATORS AND AMINO ACIDS TO EXPLORE CELL METABOLISM

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Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
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Master of Science
Bioengineering

by
Lisa Uy
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Accepted by:
Dr. Sarah Harcum, Committee Chair
Dr. Brian Booth
Dr. Kim Paul
ABSTRACT

Biopharmaceutical products are drugs that are made from living cells. One protein type of high importance to biopharmaceuticals is monoclonal antibodies. Monoclonal antibodies allow for highly specific, targeted treatment of diseases and detection of molecules related to diseases. With major roles in detecting and/or treating diseases like human immunodeficiency virus (HIV), coronavirus disease 2019 (COVID-19), and some cancers, monoclonal antibodies are in demand. This demand has led to a need for efficient cell culture systems to produce high yields of protein with minimal waste. The most commonly used cell culture system for these high-value proteins is Chinese hamster ovary (CHO) cells. Understanding and modifying CHO cell culture conditions can improve culture productivity.

Previous research by Freund et al. (2018) presented a simple means to adapt cells to elevated lactate concentrations to reduce waste product buildup, increase protein productivity, and improve growth. It was also observed that the lactate-adapted cells had lower lactate accumulation. However, the lactate-adaptation process that Freund et al. (2018) used was stressful, as significant growth retardation was observed during the process. The work presented in this thesis developed a less stressful method to adapt CHO cells to elevated lactate concentrations. The lactate adaptation was achieved by serial passages of the CHO cells into incrementally higher lactate concentrations.

Batch cultures were used to compare the lactate-adapted cell growth characteristics to unadapted cells. Cell densities, metabolic indicators, and amino acid profiles were obtained daily for two lactate-adapted cell lines and unadapted cells. The lactate-adapted
cells were cultured in elevated lactate equal to the final lactate-adaptation levels. Additionally, the unadapted and lactate-adapted cells were cultured in the standard media. It was observed that the elevated lactate was used by the lactate-adapted cells late in the cultures instead of alanine. This alteration in metabolism resulted in lower ammonia levels for the lactate-adapted cultures in elevated lactate compared to both lactate-adapted and the unadapted cultures in standard media. Thus, elevated lactate can have a positive effect on metabolism for lactate-adapted cells.
DEDICATION

This work is dedicated to my parents, Ngy Uy and Shuzhu Chen, for their support and encouragement. Thank you for teaching me how to pursue my goals and always believing I am capable of anything. Your sacrifices in life to ensure that I had more opportunities than you had did not go unnoticed. You taught me diligence, perseverance, and to not ever take anything for granted. I am forever grateful for your love and support that has always been there for me and I know will always be there in the future.
ACKNOWLEDGEMENTS

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CHAPTER ONE

1 INTRODUCTION

1.1 Significance

Biotechnology encompasses the use of recombinant technology for the production of therapeutic proteins and other industrial enzymes. Commonly referred to as biopharmaceuticals, therapeutic proteins are a multibillion-dollar industry and account for over half of the biotechnology market (Kalorama Information, 2018b). Biopharmaceuticals are recombinantly produced by several host organisms with the vast majority being produced by Chinese hamster ovary (CHO) cells (Kalorama Information, 2018a). CHO cell cultures are used to manufacture therapeutic proteins, where the most common type are monoclonal antibodies (mAbs). In addition to therapies, mAbs are used as research tools and diagnostics (Kalorama Information, 2018b). CHO cells have several advantages over other host organisms, including robust growth and human-like glycosylation of proteins (Fischer, Handrick, & Otte, 2015). These attributes allow for scalable production of safe and effective therapeutic proteins for intravenous delivery in humans. Because of the success of CHO cell systems and the high demand for biopharmaceuticals, it is beneficial to better understand the metabolism of CHO cells to improve protein production.
A significant mechanism towards gaining a better understanding of CHO cells is its glucose consumption. High glucose consumption in CHO cell cultures often results in lactate accumulation. Lactate is toxic to cells above concentrations of 40 mM and negatively impacts protein production at concentrations as low as 20 mM (Hauser & Wagner, 1997). Successful industrial processes have lactate production followed by lactate consumption during cell growth (Mulukutla, Yongky, Grimm, Daoutidis, & Hu, 2015). The shift from lactate production to consumption is desirable but not fully understood. Significant research has been conducted to reduce lactate production, but the effectors of the lactate shift are not fully understood. This work investigated lactate metabolism in cells adapted to high lactate (>20 mM) as a means to better understand the mechanism of lactate consumption.

1.2 Organization

This Master’s thesis is divided into four chapters: Chapter 1 outlines the background and motivation for this research and provides the organization of the thesis. Chapter 2 contains a literature review on biopharmaceutical production, CHO cells, CHO cell metabolism, and culture stressors. Chapter 3 contains the materials and methods and experimental results. Chapter 4 summarizes the work and discusses possible future work. The Appendix contains the amino acid concentration profiles for the five culture conditions with the lactate-adapted (LA) and unadapted CHO cells. Additionally, growth profiles are provided for previous work with unadapted CHO cells under lactate stress.
CHAPTER TWO

2 LITERATURE REVIEW

2.1 Biotechnology

Biotechnology is defined as “any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use” (O'Grady, 2013). It is a widespread industry that reaches across several different markets. The field impacts medicine, agriculture, environmental approaches, and foods (Verma, Agrahari, Rastogi, & Singh, 2011). Biotechnology has developed from increasing knowledge of biology and genetics. Historically, conventional biotechnology referred to using animals or other living organisms to achieve outcomes, such as with selective breeding or making bread with yeast (Gupta, Sengupta, Prakash, & Tripathy, 2016). Following the discovery of the function, structure, and replication mechanisms of DNA, it became possible to manipulate genetic material to achieve desired results (Verma et al., 2011). With rapid advancements in molecular biology techniques and capabilities, modern biotechnology became known more specifically for its work with living cells or products from cells. With the goal to improve human health, the biopharmaceutical field is a major subsection of biotechnology that focuses on therapeutics and diagnostics using recombinant DNA technology.
2.2 Biopharmaceutical Industry

The field of biopharmaceuticals emerged from the race to produce recombinant insulin, a vital protein in the body that regulates blood sugar levels. Altered production or response to insulin within the body results in diabetes. Exogenous insulin was found to be an effective treatment for diabetes; therefore, the protein was harvested from animal pancreases for several decades (Quianzon & Cheikh, 2012). It was clear this method for acquiring insulin was not sustainable to meet rapidly increasing demands. Genentech and Eli Lilly worked together to produce recombinant human insulin, which was licensed in 1982 as Humulin (Burrill, 2014; Quianzon & Cheikh, 2012). After Humulin was approved by the United States Food and Drug Administration (FDA), the biopharmaceuticals field rapidly developed.

Biopharmaceutical products are medicines produced using recombinant DNA technology (Kesik-Brodacka, 2018). Recombinant DNA refers to the insertion of genetic material from one organism into a different organism to obtain expression or product formation (Khan, S. et al., 2016). Biopharmaceuticals differ from chemically synthesized medicines due to the larger sizes, higher complexity, and increased specificity, in addition to using a cell to manufacture the product (Rader, 2008). Biopharmaceuticals now includes therapeutic proteins that are novel, also called biobetters (Kesik-Brodacka, 2018).
Monoclonal antibodies (mAbs) are a specific group of therapeutic proteins that make up a large portion of the therapeutic protein market. The structure of mAbs has led to the development of therapeutics that target very specific molecules. MAb therapeutics have been designed to bind to signaling proteins in a patient and eliminate harmful cells and viruses. The development of mAbs has also increased the success for treatment of several cancers (Kalorama Information, 2018b). MAb development continues to be primarily focused on disease treatments.

The biopharmaceutical market continues to grow, with an expected growth over the next five years in part due to coronavirus disease 2019 (COVID-19) (Curran, 2021). Because of uncertainty in the economic market during the COVID-19 pandemic in 2020, the biotechnology market had a 7.42% decrease in industry revenue in the United States (Curran, 2021); however, this observed decrease was mainly attributed to non-biopharmaceutical companies (Curran, 2021). Within the biotechnology market in the United States, human health technologies accounts for 65.2% of the industry revenue at $88.21 billion (Curran, 2021). The human health technologies sector includes biopharmaceuticals along with other genetic technologies such as gene therapy and pharmacogenomics (Curran, 2021). Over the next five years, the biotechnology market is predicted to have a 3.0% annual growth rate (Curran, 2021). Much of the predicted growth is attributed to an increased need of therapeutic proteins, such as mAbs, due to the greater life expectancy in the United States and COVID-19 diagnostics and treatments.
2.2.1 **Chinese Hamster Ovary (CHO) Cells**

Biopharmaceuticals are produced through several different expression systems including bacteria, yeast, and animal cells. Choice of expression system depends on the desired product and the simplest and cheapest means of obtaining that product. Bacterial and yeast systems have the advantages of rapid growth and inexpensive cultivation, but can make proteins that are improperly folded and/or lack critical post-translational modification (Kalorama Information, 2018a). Mammalian cells are most commonly used for biopharmaceuticals to harness the more complex protein synthesis and post-translational modification capabilities of these cells (Lim et al., 2010). Cell lines that have been studied include CHO, Vero (green African monkey kidney cells), HeLa cells (human cervical carcinoma), and HEK293 (human embryonic kidney cells) (Dumont, Euwart, Mei, Estes, & Kshirsagar, 2016; Khan, K. H., 2013).

CHO cells are the most frequently used for host therapeutic protein production. CHO cells originate from cells isolated by Theodore Puck in 1956 (Puck, Cieciura, & Robinson, 1958). The advantages that CHO cells provide over other mammalian hosts include its robust growth in chemically-defined and serum-free media, suspension adaptability, lower susceptibility to human pathogenic viruses, and capability for human-like protein post-translation modifications (Fischer et al., 2015). Approximately 60-70% of all recombinant therapeutic proteins on the market are produced by CHO cells (McAtee, Templeton, & Young, 2014).
2.2.2 CHO Cell Culture Challenges

CHO cell media is expensive, and long-duration cultures can be costly to maintain (Kalorama Information, 2018a). Further, compared with bacterial and yeast expression systems, CHO cells exhibit low productivity and final product concentrations (Vergara et al., 2018). Research on CHO cells continues to develop methods to reduce production costs and increase protein yields. A major impediment to high protein yields is toxic byproduct accumulation, such as from lactate and ammonia. These byproducts largely originate from metabolic pathways, which are not fully understood. Mitigation of inhibitory byproducts such as lactate offers a promising strategy to improve final CHO cell culture titer yield.

2.3 Culture Stressors

Culture stressors are important to identify. Stressors may originate from the culture media, which enhances byproduct accumulation, or from physical issues, such as shear stress or poor oxygenation. Knowledge of cell metabolism mechanisms and identification of stressors are important to developing stressor mitigation measures. Much work has been conducted to identify two stressors related to the culture media formulation, lactate and ammonia (Cruz, Freitas, Alves, Moreira, & Carrondo, 2000; Freund & Croughan, 2018; Zhang, Yi, Sun, & Zhang, 2006). Lactate concentrations are widely connected to glucose consumption, and ammonia buildup is connected to glutamine and other amino acid consumption (Pereira, Kildegaard, & Andersen, 2018).
Glucose is the main energy source for CHO cells. Glucose is present in the batch media, but also needs to be independently fed to cell cultures from concentrated stocks for fed-batch cultures. CHO cell metabolism is inefficient, such that high glucose concentration leads to the formation of waste products (Pereira et al., 2018). Under aerobic conditions, the carbons from glucose are serially metabolized via glycolysis and the tricarboxylic acid cycle (TCA) to generate intermediates, CO$_2$, and energy. The intermediates from glucose metabolism are used to form the amino acids that form biomass and the recombinant protein, such as monoclonal antibodies. An attribute of inefficient metabolism is the generation of lactate from glucose in glycolysis.

Specifically, lactate is produced from pyruvate that is generated during glycolysis. In culture, lactate concentrations exceeding 20 mM result in lower protein productivity, while lactate concentrations exceeding 40 mM are considered inhibitory to growth (Hauser & Wagner, 1997). Usually, lactate production is observed early in the culture, and lactate consumption occurs late in the culture. If this shift does not occur, culture growth and productivity can be hindered (Li, Wong, Vijayasankaran, Hudson, & Amanullah, 2012). Lactate as low as 25 mM has been shown to prevent the shift to lactate consumption, resulting in higher final lactate accumulation (Mulukutla et al., 2015). Because of its adverse effects, it is important to gain a better understanding of lactate accumulation effectors.
Glutamine functions as both a primary nitrogen source for CHO cells and a carbon source. Glutamine enters the TCA cycle via α-ketoglutarate. Chemical degradation and metabolic consumption of glutamine, as well as other amino acids, produces ammonium as a byproduct (Adeva-Andany et al., 2014; Chen & Harcum, 2005; Pereira et al., 2018). Ammonium concentrations as low as 10 mM have been observed to be associated with apoptosis and poor protein production (Andersen & Goochee, 1994). In addition to growth and protein productivity inhibition, elevated ammonia has been observed to negatively affect protein post-translational modifications, which can affect function, stability, and/or immunogenicity (Andersen & Goochee, 1994; Andersen & Goochee, 1995; Cruz et al., 2000). Ammonia and lactate are often monitored together, as decreases in lactate accumulation often coincide with increased ammonia accumulation and vice versa (Li et al., 2012; Zhang et al., 2006).

In addition to direct metabolic effects, lactate and ammonium production are associated with increased osmolality and decreased pH (Slivac, Blajić, Radošević, Kniewald, & Gaurina Srček, 2010). Osmolality increases in response to lactate accumulation due to base additions to control pH (Lao & Toth, 1997; Martínez et al., 2013). Hyperosmotic cultures have been reported to have inhibited growth and lower titers (Alhuthali, Kotidis, & Kontoravdi, 2021). Ammonia accumulation also lowers pH, by interacting with the cell membrane potential (Oguchi, Saito, Tsukahara, & Tsumura, 2006). Increasing the buffer capacity of culture media to mitigate pH changes results in media with higher osmolarity (Ahleboot et al., 2021). Therefore, pH and osmolality in conjunction with lactate and ammonia are highly interdependent.
2.4 Lactate Control Methods

Lactate production followed by lactate consumption is desired for CHO cell cultures. Typically, lactate production occurs during exponential growth, while lactate consumption occurs after glutamine is depleted. One approach to control lactate accumulation has been termed the high-end pH-controlled delivery of glucose (HIPDOG). This approach uses increasing culture pH as an indicator of low glucose due to lactate consumption (Gagnon et al., 2011). The HIPDOG approach feeds glucose in response to pH increases caused by lactate consumption, which is acidic. Then, when lactate production occurs due to high glucose, the pH decreases, and the glucose feed is stopped. With the HIPDOG approach, overall lactate accumulation was reduced, and protein production was higher than control cultures (Gagnon et al., 2011). While this approach reduced lactate accumulation and increased protein production, it required the initial lactate to be slightly elevated (~5 mM).
Since lactate accumulation negatively impacts culture performance, methods to decrease lactate accumulation that can be translated to industrial-sized bioreactors are desired. Another approach to control lactate accumulation was to adapt cells to higher lactate concentrations (Freund & Croughan, 2018). Specifically, Freund adapted cells to elevated lactate in one step. Cells were directly placed in 35 mM lactate until a normal growth rate was reached, about 40 days (Freund & Croughan, 2018). These LA-CHO cells were then cultured in a fed-batch system in 40 mM lactate supplemented media. These LA cells had lower lactate production rates. However, the LA cells were not directly assessed in comparison to unadapted cells in terms of growth and titer, nor were other metabolite concentrations such as amino acids reported. In this thesis work described here, a set of serially lactate adapted cells were generated to investigate the metabolism of LA cells versus unadapted cells.
CHAPTER THREE

3 MATERIALS AND METHODS

3.1 Cell Line

The CHO cell line in this study was donated by the Vaccine Research Center at the National Institute of Health (NIH). The CHO-K1 Clone A11 cell line expresses the monoclonal antibody VRC01, which is an IgG1 anti-HIV mAb.

3.2 Media

CHO cell cultures were grown in ActiPro (Cytiva, Marlborough, MA). Powdered media was formulated according to the manufacturer’s instruction. A 200 mM glutamine stock (Sigma-Aldrich, St. Louis, MO) was added to the media to obtain a final concentration of 6 mM. To generate the lactate containing medias, a high lactate ActiPro media (100 mM lactate) was prepared with the addition of sodium lactate powder (Sigma-Aldrich, St. Louis, MO) to the powdered ActiPro. To obtain the final lactate concentrations required for the lactate-adaptation phase and growth in studies in elevated lactate, standard ActiPro was mixed with 100 mM lactate ActiPro. For example, one part standard ActiPro was mixed with nine parts 100 mM lactate ActiPro to obtain 10 mM lactate ActiPro.
3.3 Lactate-Adaptation Protocol

Cells were cultured in 125 mL baffled, vented shake flasks (VWR®, Radnor, PA) in a 5% CO₂ incubator at 37°C with an agitation speed of 180 rpm (0.75 inch throw). CHO VRC01 cells were rapidly thawed from liquid nitrogen stocks into 30 mL of standard ActiPro and cultured for three days. The unadapted CHO cells were then cultured in the 10 mM lactate ActiPro media. All seeded densities targeted 5 x 10⁵ cells/mL. For the cells to be considered lactate-adapted to a particular concentration, three criteria were used: 1) the culture growth rate in the elevated lactate media was equivalent to the growth rate in ActiPro, 2) the viable cell density (VCD) on Day 3 was at least 4 x 10⁶ cells/mL, and 3) the cultures obtained criteria 1) and 2) for three passages. When cells were lactate-adapted, frozen stocks were made. Stock cultures were frozen in 10% dimethyl sulfoxide (DMSO) and stored in a liquid nitrogen tank. The LA cells were then cultured in media at the next lactate concentration increment. This procedure was repeated at 10 mM increments until stock cultures were obtained for 10 mM to 60 mM lactate.
3.4 LA Culture Characterization

Similar shake flasks and culture conditions to the lactate-adaptation protocol were used for the batch cultures. The two LA (30 mM and 50 mM) and the unadapted cells were used for the characterization studies. Frozen stocks were rapidly thawed into 30 mL of ActiPro with respective lactate concentration. After three days, cells were seeded at $5 \times 10^5$ cells/mL into the test media. Table 3.1 outlines media conditions used for each LA culture. All five conditions were conducted simultaneously. All conditions were examined in triplicate, which were conducted serially, from new frozen stock culture.

### Table 3.1: Experimental Conditions

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<td>Unadapted</td>
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<td>0 mM</td>
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<td>-</td>
<td>30 mM</td>
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3.5 VCD, Metabolites, and Amino Acid Quantification

Daily samples were collected from the batch culture for VCD, metabolite analysis, and amino acid quantification. VCD and cell viability were measured immediately using a Vi-CELL™ XR Cell Viability Analyzer (Beckman Coulter, Brea, CA). The cells were removed by centrifugation at 10,000 x g for 10 minutes. The culture supernatant was collected for metabolite and amino acid analysis and stored at -20°C. Measurements for glucose, lactate, glutamine, glutamate, IgG, and ammonia were obtained using the Cedex Bio Analyzer (Roche Diagnostics, Basel, Switzerland). Amino acid concentrations were obtained by capillary electrophoresis with high pressure mass spectrometry (CE-HPMS) using the REBEL (908 Devices, Boston, MA) except for glutamine and glutamate. Samples were diluted 1/100 in Rebel diluent for analysis on the Rebel.
3.6 Results and Discussion

3.6.1 Lactate Adaptation

To adapt CHO cells to higher lactate concentration, cells were cultured in increasing lactate concentrations using 10 mM increments (10 mM to 60 mM lactate). Figure 3.1 shows the growth profiles for the serial lactate-adaptation process versus time in days. During the lactate-adaptation process up to 20 mM lactate, the cells had fairly normal growth rates and final VCD on Day 3 despite the higher lactate concentration. The adaptation time increased for the 30 to 40 mM lactate cultures by an additional three-day passage. The total time to reach 40 mM LA cells was about 42 days. The total time to reach 60 mM LA cells was 76 days. The 30 mM and 50 mM LA cells were used for the characterization study, in parallel with unadapted cells. A previous strategy described by Freund et al. (2018) differed by placing CHO cells directly into 35 mM lactate media and waiting for the cells to return to normal growth rates. This lactate adaptation process took 40 days. Although the growth rates returned, it was not specified if there was a VCD criteria for adaptation. Since the growth rates and VCD were fairly constant during the serial lactate adaptation, one can assume this process might be less stressful than directly culturing cells in elevated lactate and waiting for normal growth rates to return.
Figure 3.1: Growth Profiles for the Lactate-Adaptation Process. The lactate concentration in ActiPro media was increased in increments of 10mM.

3.6.2 Cell Growth

Previous studies on LA cells showed that 35 mM LA cells had lower lactate production when cultured in media with an initial lactate concentration of 40 mM. This report did not directly compare the growth characteristics of the LA cells to unadapted cells such that metabolic changes beyond lactate and ammonia were not determined. In this study, two LA (30 mM and 50 mM lactate) and the unadapted cells were cultured in ActiPro and ActiPro with equivalently elevated lactate (see Table 3.1 for media lactate
concentration by LA cell type). Since Freund observed reduced lactate production with 35 mM LA cells, the 30 mM and 50 mM LA cells were expected to have reduced lactate production and consume lactate later in the cultures.

Growth profiles for the five conditions are shown in Figure 3.2A. All cultures in this work had growth rates of $0.031 \pm 0.02 \text{ h}^{-1}$ (SE) for the exponential phase. The growth rate between Days 4 and 5 decreased for all cultures as the glucose became limiting (Fig 3.2B). The final VCD for the unadapted cells and the 30 mM and 50 mM LA cells in the standard ActiPro media were similar. The 30 mM LA cells in 30 mM lactate ActiPro and 50 mM LA cells in 50 mM lactate ActiPro had a lower final VCD compared to unadapted cells (p-value=0.0123) (see Appendix B-1A for the growth profile on a linear scale). The cell viabilities for all the cultures were above 85% for the entire culture and were not significantly different (p-value=0.067) (Fig 3.2C). The LA cells maintained growth in the high lactate media, confirming lactate adaptation. The unadapted CHO cells were not cultured in the elevated lactate media, as previous data demonstrated poor growth and low titers under these conditions (Appendix C) (D. Chitwood, personal communication, October 31, 2021). Further, other researchers have observed that unadapted CHO cells in high lactate media have inhibited growth (Choi et al., 2007).
The protein production for the unadapted cells was higher than the 30 mM and 50 mM LA cells in either media (p-value<0.0001) (Fig 3.2D). The LA cells clustered by lactate adaptation and not the culture media. Previous research suggests that cells placed in higher lactate concentrations exhibit higher protein yields but similar to normal volumetric production rates (Choi et al., 2007; Lao & Toth, 1997). As it has been demonstrated previously, lactate production and consumption impact protein production (Freund & Croughan, 2018; Gagnon et al., 2011; Mulukutla et al., 2015). Despite the gradual lactate adaptation, the LA cells appear to have been potentially negatively impacted by the lactate adaptation process with respect to protein production. Fed-batch culture would be needed to confirm these results and determine overall effect of lactate adaptation on titer.
Figure 3.2: Growth, Glucose, and Titer Profiles for Lactate-Adapted and Unadapted Cells. A) VCD; B) Glucose. C) Cell Viability. D) Titer. “LA” = Lactate-Adapted. “/ 0” – ActiPro. “/ 30” – ActiPro with 30 mM Lactate initially. “/ 50” – ActiPro with 50 mM Lactate initially. Error bars represent standard error.
3.6.3 Metabolic Indicators and Products

To observe the effects of lactate adaptation on metabolism in standard and elevated lactate media, several metabolites were monitored during the five-day batch culture. Lactate and ammonia profiles are shown in Figures 3.3B and 3.4B, respectively. The lactate concentrations for the cultures in the elevated lactate media are expectantly higher than the cultures in standard media. For all cultures, the lactate profiles increased between Day 0 and Day 3, indicating lactate production. The difference in lactate levels during production between cultures were not significantly different (p-value=0.4300). After Day 3, the lactate profiles decreased for all cultures, indicating lactate consumption. There was a significant difference in the lactate consumption amounts with the elevated lactate media cultures consuming more (p-value=0.0133). For the elevated lactate media cultures, the amount of lactate consumed was greater than the amount of lactate produced. Contrary to previous reports that lactate consumption was not possible in the presence of glucose and excess lactate (Mulukutla et al., 2015), lactate consumption occurred in this thesis work. The shift from lactate production to lactate consumption aligns with the depletion of glutamine (Fig 3.3A). These results indicate that glutamine plays a significant role in lactate production and consumption. Since the glutamine consumption profile appears similar across the different cells and lactate level in the media, it does not appear that the lactate adaptation and lactate levels in the media affected glutamine metabolism.
Interestingly, the ammonia profiles also have two distinct phases for all five culture conditions. For all cultures, ammonia production occurred initially. For the 50 mM cells in 50 mM lactate, the ammonia production phase was one day longer than the other culture conditions. After Day 4, the cultures in elevated lactate had constant ammonia concentrations. In contrast, in standard media, both the LA and unadapted cultures produced ammonia after Day 4. There was a significant difference in the final ammonia values (p-value=0.0002). Decreased ammonia production has been previously observed for LA cells (Lao & Toth, 1997). Thus, it appears that lactate adaptation changed later-phase ammonia production when LA cells were cultured in elevated lactate media.
Figure 3.3: Glutamine, Lactate, and Glutamate Profiles for Lactate-Adapted and Unadapted Cells. A) Glutamine; B) Lactate. C) Glutamate. “LA” = Lactate-Adapted. “/ 0” – ActiPro. “/ 30” – ActiPro with 30 mM Lactate initially. “/ 50” – ActiPro with 50 mM Lactate initially.

Error bars represent standard error.
Figure 3.4: Alanine and Ammonia Profiles for Lactate-Adapted and Unadapted Cells. A) Alanine; B) Ammonia. “LA” = Lactate-Adapted. “/ 0” – ActiPro. “/ 30” – ActiPro with 30 mM Lactate initially. “/ 50” – ActiPro with 50 mM Lactate initially. Error bars represent standard error.
3.6.4 Amino Acids

Since lactate and ammonia metabolism are related through glycolysis and the TCA cycle, and protein synthesis requires adequate amino acids, the media amino acids were quantified. Most of the amino acid profiles were not different between the unadapted and LA cells, independent of the lactate concentration in the media. A majority of the amino acids were observed to have linear consumption profiles which were independent of LA status and media (glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine). Only glutamine was observed to be completely consumed prior to glucose depletion. Arginine, asparagine, aspartate, and glutamate were consumed after a delay of a couple days; however, the profiles were similar across the LA and unadapted cultures independent of culture media. All amino acids profiles except for cysteine have been included in Appendix A. Cysteine was not included because its concentration was below detection for the REBEL at the dilution used on all samples.

Alanine was observed to have a significantly different profile due to media and adaptation (p-value=0.0008) (Fig 3.4A and Appendix A-1A). The alanine profiles had two distinct phases. Specifically, alanine was produced initially in all cultures. Then, the alanine profiles after Day 4, either plateaued or indicated alanine consumption. The LA cells in elevated media reached plateaus, while the unadapted and LA cells in the standard ActiPro demonstrated consumption.
Figure 3.5 is a simplified metabolic pathway that shows glycolysis, the TCA cycle, and alanine and lactate reactions. In early cell growth with available glucose, glucose is primarily shuttled consumed to generate pyruvate and is shuttled to the TCA cycle (pink and red pathways in Fig 3.5). With this ready supply of pyruvate, there is a supply of pyruvate for conversion to alanine (blue pathway in Fig 3.5). This conversion explains the initial increase of alanine in the cultures. With lower glucose availability, a significant difference in alanine and ammonia profile is observed late in culture between cultures in standard ActiPro and those in elevated lactate media. A connection between ammonia and alanine metabolism has been observed previously in other studies with lactate-supplemented media (Lao & Toth, 1997; Li et al., 2012). However, a mechanism had not been proposed related to lactate metabolism directly and elevated lactate conditions. Alanine is derived from pyruvate as is lactate (blue and orange pathways in Fig 3.5). There are two mechanisms for alanine synthesis and degradation from pyruvate, the alanine aminotransferase 1 (GPT) (cytosolic) and alanine aminotransferase 2 (GPT2) (mitochondria) pathways (Romero et al., 2004). However, from RNAseq data, only the mitochondria version is expressed in CHO cells (Kondratova et al., 2015; Kremkow, Baik, MacDonald, & Lee, 2015). Alanine consumption to produce pyruvate coincides with the consumption of α-ketoglutarate to generate glutamate (blue pathway in Fig 3.5). Glutamate in the mitochondria can be converted to α-ketoglutarate, which also yields ammonia (green pathway in Fig 3.5) (Li et al., 2012). Therefore, alanine consumption can lead to ammonia production. The two LA cultures in elevated lactate media had excess lactate at the end of the cultures. In these cultures, lactate could be converted to pyruvate,
which eliminated alanine consumption. The observed alanine plateau for the LA cultures in lactate media is consistent with the plateau observed for ammonia.

Arginine and aspartate are common nitrogenous products of glutamine and glutamate; thus, depletion of glutamine caused these two amino acid concentrations to decrease after its consumption (Wu, 2010). The depletion of glutamine also leaves a need for a nitrogen source; asparagine, which was consumed when glutamine was depleted, is a nitrogen source used by CHO cells (Duarte et al., 2014). Arginine, asparagine, and aspartate have also been reported to act as nitrogen signaling molecules in nitrogen starvation meaning these amino acids could replace a role that depleted glutamine cannot fill (Reidman, Cohen, Kupiec, & Weisman, 2019). While glutamate synthesis and degradation occur as previously mentioned, the overall decrease of glutamate after three days may occur due to depletion of glutamine. Glutamine is produced from glutamate by glutamine synthetase (Zhang, Sun, Yi, & Zhang, 2006). Therefore, upon glutamine depletion, glutamate could be consumed to produce glutamine, which has been observed longer in fed-batch cultures (Elliott et al., 2020).
In contrast to the majority of the consumption profiles and similar to alanine, glycine was also produced but with a delay until Day 2. The glycine profiles were similar across the LA and unadapted cultures regardless of the media. The increase in glycine likely comes from serine consumption. The delayed production could be a result of glutathione synthesis, which is made up of cysteine, glutamate, and glycine (Chevallier, Schoof, Malphettes, Andersen, & Workman, 2020). However, this relationship is difficult to determine without cysteine profiles. While lactate in the media allowed for lower consumption by substitution of lactate for alanine, only cells adapted to high lactate would survive these conditions.
Figure 3.5: **Simplified Metabolic Pathways in CHO Cells.** The common three letter abbreviations are used for all metabolites.
CHAPTER FOUR

4 CONCLUSIONS AND FUTURE WORK

4.1 Summary and Conclusion

This work investigated the performance of cells adapted to varying levels of lactate. The LA cells were characterized in batch growth along with unadapted cells in standard media. Additionally, the LA cells were cultured in media at elevated lactate concentrations equal to the concentration used for adaptation. It was hypothesized that LA cells would have lower lactate accumulation. The unadapted and LA cells in the standard media had similar lactate profiles. However, the lactate consumption for LA cells in elevated media was significantly higher in the consumption phase compared to the cells in standard media.

Lactate supplement to media resulted in slightly different final VCD; however, the exponential growth rates are not different. The LA cells in elevated lactate exhibited slightly lower VCD. Alanine and ammonia profiles were significantly different depending on the lactate concentration in the media. For the LA cultures in elevated lactate, the lactate provided a supply of pyruvate, which replaced the cell metabolic need for alanine and associated production of ammonia. Labeling studies with alanine and lactate could confirm these findings.
4.2 Future Work

Future work should include fed-batch cultures to see if these effects extend into production conditions. Studies with alternate cell lines would also prove beneficial. Conducting work on DG44 CHO cells would allow for more direct comparison to the work by Freund et al. (2018) and show how translatable lactate adaptation is between cell lines. As the relationship between glycine and cysteine concentrations was not confirmed, profiles of cysteine should be obtained. Cysteine was present, just below detection on the Rebel with 100-fold dilution. Lower dilution would be needed to accurately quantify. With the LA cells, more work can be completed to explore lactate induced genome instability and whether genetic changes are maintained in culture or are random mutations occurring.
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APPENDICES
Appendix Introduction

The appendix A includes 19 amino acid profiles for LA and unadapted cells listed in alphabetical order. Cysteine is not included due to its concentration being below detection on the REBEL at the dilution used on all samples. For easier comparison, only three y-axis scales are used (3, 6, and 9 mM).

The appendix B includes the VCD profile for LA and unadapted cells on a linear scale. It also includes an alanine profile plotted over the IVCD. The IVCD was calculated by using the trapezoid approximation between viable cell density points.

The appendix C includes the results of a lactate stress experiment with a control culture and culture with gradual lactate addition.
Figure A-1: Alanine, arginine, asparagine, and aspartate profiles for Lactate-Adapted and Unadapted Cells. A) Alanine; B) Arginine; C) Asparagine; D) Aspartate. Error bars represent standard error.
Figure A-2: Glutamine, glutamate, glycine, and histidine profiles for Lactate-Adapted and Unadapted Cells. A) Glutamine; B) Glutamate; C) Glycine; D) Histidine. Error bars represent standard error.
Figure A-3: Isoleucine, leucine, lysine, and methionine profiles for Lactate-Adapted and Unadapted Cells. A) Isoleucine; B) Leucine; C) Lysine; D) Methionine. Error bars represent standard error.
Figure A-4: Phenylalanine, proline, serine, and threonine profiles for Lactate-Adapted and Unadapted Cells. A) Phenylalanine; B) Proline; C) Serine; D) Threonine. Error bars represent standard error.
Figure A-5: Tryptophan, tyrosine, and valine profiles for Lactate-Adapted and Unadapted Cells. A) Tryptophan; B) Tyrosine; C) Valine. Error bars represent standard error.
APPENDIX B: Supplemental Growth and Alanine Profiles

**Figure B-1:** Supplemental Growth and Alanine Profiles. A) VCD. B) Alanine Profile over the Integral Viable Cell Density. The slopes of the lines indicate alanine productivity. Positive slopes show alanine production, while negative slopes show alanine consumption. Error bars represent standard error.
Figure C-1: VCD and Lactate Profiles for Control and Lactate-Stressed CHO cells. A) VCD. B) Lactate. In a previous shake flask study on CHO cells producing VRC01, a gradual amount of lactate was added into culture and monitored. The results show inhibited growth for lactate-supplemented cultures. Control cultures are CHO cells in standard ActiPro.