Title: Mechanisms Driving the Lactate Switch in Chinese Hamster Ovary cells

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Running title: Mechanisms Driving the Lactate Switch

Abstract

The metabolism of Chinese Hamster Ovary (CHO) cells in a production environment has been extensively investigated. However, a key metabolic transition, the switch from lactate production to lactate consumption, remains enigmatic. Though commonly observed in CHO cultures, the mechanism(s) by which this metabolic shift is triggered is unknown. Despite this, efforts to control the switch have emerged due to the association of lactate consumption with improved cell growth and productivity. This review aims to consolidate current theories surrounding the lactate switch. The influence of pH, NAD^+/NADH, pyruvate availability and mitochondrial function on lactate consumption are explored. A hypothesis based on the cellular redox state is put forward to explain the onset of lactate consumption. Various techniques implemented to control the lactate switch, including manipulation of the culture environment, genetic engineering, and cell line selection are also discussed.

Keywords

Lactate, Metabolism, Lactate switch, Chinese Hamster Ovary, CHO, redox,

Introduction

The biopharmaceutical market is increasing 60% faster than the rest of the pharmaceutical industry (Aggarwal, 2012) and is estimated to be worth $100 billion (Templeton, Dean, Reddy, & Young, 2013). Since 1987, when the first mammalian-expressed therapeutic protein was produced by Chinese Hamster Ovary (CHO) cells (Jayapal, Wlaschin, Hu, & Yap, 2007), CHO cells have held a large share of that market.

Mammalian Cell Metabolism

The metabolism of CHO cells in a production environment has been an area of interest for several years due to its association with productivity. The metabolism of CHO cells appears to change throughout a production run and in response to nutrient availability. Typically, mammalian cells are thought of as being either glycolytic or oxidative. Some cells survive by primarily producing ATP through glycolysis. These cells consume glucose at a high rate and produce lactate to replenish the NAD^+ which is required for glycolysis to continue (Figure 1) (Zheng, 2012). Glycolysis produces only 2 ATP per glucose molecule, compared to the ~36 ATP generated through oxidative phosphorylation (OXPHOS) (Valvona, Fillmore, Nunn, & Pilkington, 2016). Some cells harness this and produce most of their ATP through OXPHOS in the mitochondria. ATP synthase is driven by the proton gradient across the inner mitochondrial membrane, which is itself generated by NADH and $FADH_2$ feeding electrons into the electron transport chain (ETC). The production of these reducing equivalents in mitochondria occurs largely due to flux through the tricarboxylic acid (TCA) cycle. Pyruvate, generated from glycolysis or amino acid catabolism, is commonly considered the default substrate for powering the TCA cycle. However, glutamine is also a major driver of the TCA cycle and can be catabolized as an energy source. In reality, most cells switch between oxidative and glycolytic metabolism in response to the extracellular environment. For

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example, cells grown in low glucose environments are able to upregulate mitochondrial function to maximize ATP synthesis (Potter, Newport, & Morten, 2016).

The Lactate Switch

Most CHO cells in a production system are glycolytic and will initially produce lactate from over 75% of the glucose supplied (Young, 2013). Concentrations of lactate below 20mM are well tolerated, but if the concentration rises above 40mM deleterious effects on growth and productivity occur (Fu et al., 2016). One of the key changes that can occur during a production run is the transition from a lactate producing to a lactate consuming culture, demonstrating a switch in metabolism. This metabolic switch is widely regarded as a desirable characteristic. The accumulation of lactate not only limits cell growth, but increases the osmolality of the media in a pH controlled environment due to base addition. Therefore, switching to lactate consumption prevents acidification of the medium and, as less base is added to control the pH, osmolality is maintained at a lower level and more nutrient feeds can be added (Gagnon et al., 2011). Analysis of over 200 production runs revealed a strong correlation between cultures which undergo the lactate switch and high productivity (Le et al., 2012). Numerous other studies (Charaniya et al., 2010; Luo et al., 2012; Mulukutla, Gramer, & Hu, 2012; Sun et al., 2013; Templeton et al., 2013) have also described lactate consumption as beneficial, and associate it with improved metabolic efficiency (Liste-Calleja et al., 2015; Luo et al., 2012).

Although the occurrence of the lactate switch is frequently reported in CHO cell literature, the exact mechanism by which it arises remains unclear (Table 1). This review aims to reconcile the literature surrounding this subject and proposes a reasonable hypothesis for the driver of the lactate switch at a molecular level.

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Part I: Molecular Mechanisms Behind the Lactate Switch

There are two key proteins involved in lactate consumption: the monocarboxylate transporter (MCT) through which lactate enters and exits the cell in co-transport with H^+ (Halestrap & Price, 1999; Liste-Calleja et al., 2015); and lactate dehydrogenase (LDH) which interconverts lactate into pyruvate allowing it to enter central metabolism. Control over either of these proteins has the potential to control the lactate switch.

The Monocarboxylate Transporter

MCT regulation takes place predominantly at the expressional level. Studies have shown that the expression of MCTs is altered in muscle in response to exercise or inactivity (Halestrap & Price, 1999; Halestrap & Wilson, 2012), demonstrating that MCT levels can change in response to stimuli. AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor gamma coactivator 1α (PGC1α) are thought to be involved in the transcriptional control of MCT1 (Halestrap & Wilson, 2012), the primary MCT found in CHO cells (Jeong et al., 2001). However, once expressed, the activity of MCTs is controlled entirely by the relative concentration of lactate and H^+ across the cell membrane (Cheeti, Warrier, & Lee, 2006; Halestrap & Price, 1999; Halestrap & Wilson, 2012; Wilkens, Altamirano, & Gerdtzen, 2011). This property of MCTs can explain fluxes in extracellular lactate concentration in a culture environment (Figure 2). Initially, the concentration of lactate outside the cell is low, so any lactate produced by the cell is exported. Cytosolic acidification, which occurs as a result of glycolysis, also drives lactate out of the cell by creating a pH gradient across the plasma membrane. This results in accumulation of lactate in the extracellular environment but benefits the cell as it works to de-acidify the cytosol. The pH buffering applied to cells in bioreactors could explain why lactate reaches such high concentrations in the culture media. Base addition to the medium increases the pH outside the

cell. This maintains the pH gradient across the plasma membrane, allowing the MCT to continually export lactate. For lactate consumption to occur, one of two things must happen. Either the concentration of H^+ inside the cell must drop, and the influx of protons down their concentration gradient will transport lactate alongside it (Figure 2D), or the concentration of intracellular lactate must drop lower than that of extracellular lactate to the point where it can overcome a pH gradient (Figure 2C). This could occur if protons are utilized in cellular reactions or their production is decreased (e.g. a reduction in glycolytic flux), or if lactate is oxidized by LDH. Oxidation of lactate would decrease its concentration within the cytosol and drive the influx of lactate into the cell. Thus, a reversal of the H^+ and lactate gradients across the plasma membrane is required for lactate consumption (Wilkens et al., 2011). This is supported by the work of Liste-Calleja et al. (2015). Their research on HEK293 cells showed that if pH control was in place, the switch to lactate consumption did not occur until glucose was depleted, but occurred when glucose was in excess (30mM) if the pH was not controlled. A lack of pH control means H^+ ions accumulate equally across the plasma membrane, resulting in an influx of lactate into the cell at an earlier stage. Further, direct addition of lactic acid at the beginning of the culture, which reduced the pH to <6.8, drove lactate consumption whereas addition of sodium lactate did not. This emphasizes the importance of balancing pH and lactate gradients between the intra and extracellular environments. However, this mechanism cannot be used to fully explain lactate consumption as there must be a change in LDH activity or in H^+ levels prior to altered influx/efflux patterns.

LDH Isozymes and Expression

For lactate to re-enter metabolism and be consumed, it must be oxidized to pyruvate via LDH. This means that regulation of LDH can control the flux between pyruvate and lactate. The activity of LDH has been shown to change throughout a fed-batch culture, with a change

in activity occurring around the time of the lactate switch (Ma et al., 2009).

There are three *LDH* genes; *LDHA*, *LDHB* and *LDHC*. *LDHC* is germ cell specific and forms homotetramers (Odet et al., 2008; Valvona et al., 2016) while *LDHA* and *LDHB,* if expressed in the same cell, can form heterotetramers. The *LDHA* gene encodes the LDH-M protein while *LDHB* encodes LDH-H. Since LDH is tetrameric, it can exist in five different isoforms depending on the ratio of LDH-M and LDH-H peptides which assemble. As the LDH-M and LDH-H proteins differ in their affinity for pyruvate and lactate, each of these isozymes has slightly different kinetic properties. LDH-M has a higher affinity for pyruvate, so preferentially catalyzes the forward reaction, while LDH-H has a higher affinity for lactate so is prone to catalyzing the reverse reaction (Figure 3).

The presence of LDH isozymes varies between tissues and is dependent on the metabolic status of the cell (Ross et al., 2010; Valvona et al., 2016). No consensus is available for the predominant form of LDH found in CHO cells. Some researchers claim *LDHA* is the sole *LDH* gene expressed by CHO cells (Dorai et al., 2010), while others report mixed *LDHA/B* (*LDHA3B* and *LDHA2B2*) isozymes (Jeong et al., 2001) or even claim the germ cell specific *LDHC* to be present (Choi et al., 2007; Szperalski, Jung, Shao, Kantardjieff, & Hu, 2011). *LDHA* transcripts have been reported by several research groups (Jeong et al., 2001; Qian et al., 2011; Szperalski et al., 2011; Yuk et al., 2014) so it is likely that this gene, at the very least, is expressed in CHO cells. Although seeming unusual, activation of the *LDHC* variant has been described in human cancers, so the presence of this isozyme in CHO cells is not implausible (Koslowski et al., 2002).

The different kinetic properties of each isozyme could allow the rate of lactate production and consumption to be controlled by the relative abundance of each isoform. In this sense, a switch to lactate consumption could be associated with increased *LDHB* expression.

Isozymes with more *LDHB* (LDH-H) subunits exhibit higher affinity for lactate so would preferentially catalyze the reverse reaction, regenerating pyruvate. The expression profiles of the LDH genes have been shown to change during aging (Ross et al., 2010), cell transformation (Koslowski et al., 2002), hypoxia (Semenza et al., 1996), and in one instance during CHO cell culture. LDH-C expression was reported to double after a temperature shift during which lactate consumption was initiated (Szperalski et al., 2011). This is interesting, as the LDH-C isoform is associated with an increase in the oxidative reaction, preferentially using lactate as a substrate (Fu et al., 2016; Koslowski et al., 2002). Indeed, CHO cells engineered to express LDH-C demonstrated increased uptake of supplemented lactate (Fu et al., 2016).

However, LDH is a stable, highly expressed enzyme with high specific activity, thus it is assumed a state close to dynamic equilibrium is reached within a cell (Halestrap & Wilson, 2012; S. H. Kim & Lee, 2007a; Quistorff & Grunnet, 2011a; Wahrheit, Niklas, & Heinzle, 2014). It has been argued that, as the kinetic properties of isozymes do not affect the equilibrium constant (K_{eq}), changes in LDH isoform ratios would not affect lactate accumulation (Quistorff & Grunnet, 2011a). This is because, if the K_{eq} is the same, the ratio of products to reactants also remains the same. However, the isozyme pattern within a cell would affect how quickly the reaction once again reaches equilibrium if a disturbance should occur (Quistorff & Grunnet, 2011b; Wang et al., 2016). Additionally, even if the reaction is considered close to dynamic equilibrium, the cellular environment is not a closed system. Lactate and pyruvate can enter and exit the cell, while pyruvate, NAD^+ and NADH can be consumed or produced in other metabolic reactions. In a bioreactor, the dynamic culture environment may mean the isozyme pattern adapts over time and exerts some control over the transition to lactate consumption. Further research is needed to confirm which *LDH* genes are expressed by CHO cells and to track their expression profiles during a production run to

determine the influence *LDH* gene expression has on the lactate switch.

LDH can be regulated at the level of expression, through transcription factors or epigenetic modifications; by post-translational modifications; or by degradation control. There are many reports of *LDH* transcription being up or downregulated. For example, BCL2 has been reported to dramatically reduce *LDHA* transcripts (Dorai et al., 2010), silencing of AMPK upregulated LDH expression (Faubert et al., 2013), while conflicting reports exist for the effect of copper on *LDHA* transcript levels (Qian et al., 2011; Yuk et al., 2014). Many cancer-related proteins such as c-Myc, KLF4, FOXM1, EGFR and HIF1 regulate *LDHA* expression along with other factors such as cAMP, estrogen and even lactate itself (Miao, Sheng, Sun, Liu, & Huang, 2013; Valvona et al., 2016). Stimulation of LDH expression by a high lactate concentration was also reported by Korke et al. (2004). This list is far from exhaustive, and with LDH playing a major role in redox homeostasis it is likely under complex but tight control. Additionally, methylation of LDH genes has been shown to control transcript levels in the cell, with *LDHB* expression being repressed in cancer cells due to hyper-methylation of the promoter (Alcivar et al., 1991; Maekawa et al., 2003).

LDH Post-Translational Modifications

Furthermore, the presence of post-translational modifications influences LDH activity. Phosphorylation increases tetrameric assembly of the protein and its affinity for NADH (J. Fan et al., 2011), while acetylation inhibits LDH activity and drives its degradation (Zhao et al., 2013). An increase in phosphorylation and a decrease in acetylation has been linked to cancer, a disease which typically causes excess lactate production by cells due to the Warburg effect (Potter et al., 2016; Warburg, Wind, & Negelein, 1927). Transient changes in the post-translational modification status of LDH in CHO cells therefore have the potential to alter the rate of lactate production, although whether this could drive complete reversal of

LDH activity is unknown.

Lactate:Pyruvate

Despite the ability of cells to exert control over LDH, its near equilibrium state means the primary driver of LDH flux is the relative concentration of its substrates (Quistorff & Grunnet, 2011a). Typically, the NAD^+/NADH ratio in the cytosol is ~700:1 while the lactate/pyruvate ratio is ~20:1 (Wilkens & Gerdtzen, 2015). If levels of pyruvate or NADH rise, the forward reaction is favored, whereas if lactate or NAD^+ levels rise, the reverse reaction is favored. Changes to the usual product/reactant ratio move the position of equilibrium can drive reversal of the LDH reaction and thus lactate consumption.

In this sense, lactate itself could be considered the driver of the switch. Indeed, Kyriakopoulos and Kontoravdi (2014) reported that all of their cultures reach a similar lactate concentration (~12.5mM) prior to the switch. However, this suggestion is difficult to reconcile across the field. There are many reports of lactate concentrations reaching significantly higher levels than this, in some cases >30mM (Y. Fan et al., 2015; Lu et al., 2013; Luo et al., 2012; Nolan & Lee, 2011; Wahrheit et al., 2014) and of lactate consumption continuing even when lactate is almost depleted (Le et al., 2012). Additionally, because lactate is limited to this one metabolic reaction, its own concentration relies almost entirely upon the relative concentrations of pyruvate, NAD^+ and NADH. Consequently, even if lactate concentration was the driver of the metabolic shift, it must first be preceded by a change in pyruvate, NAD^+ or NADH concentration.

Unlike lactate, pyruvate can partake in multiple metabolic pathways meaning its concentration can be influenced by several factors. When pyruvate is metabolized by enzymes other than LDH, the pyruvate pool is depleted. To replenish this, LDH catalyzes the reverse reaction and lactate consumption ensues, meaning control over other pyruvate

consuming reactions could trigger the lactate switch. For example, phosphorylation of pyruvate dehydrogenase (PDH) by pyruvate dehydrogenase kinase (PDK) results in its inactivation (J. W. Kim, Tchernyshyov, Semenza, & Dang, 2006). During the early stages of CHO cell cultures, PDH may be inactive due to phosphorylation, causing a buildup of pyruvate which drives lactate production. If pyruvate dehydrogenase phosphatase then dephosphorylated PDH, the pyruvate pool could be consumed by the mitochondria. The subsequent reduction in cytosolic pyruvate would then trigger the reversal of LDH to replenish pyruvate from the available lactate. Likewise, pyruvate carboxylase (PYC) and the malic enzyme (decarboxylating malate dehydrogenase) feed pyruvate into the TCA cycle and deplete the pyruvate pool. The theory that pyruvate is redirected into the mitochondria at the time of the lactate switch is supported by experimental data showing increased OXPHOS at a similar time point (Templeton et al., 2013; Zagari, Jordan, Stettler, Broly, & Wurm, 2013).

Glycolytic Flux Control

The intracellular pyruvate concentration is also affected by the rate of its production, typically through glycolysis. High glycolytic flux can result in an accumulation of pyruvate if the production rate exceeds its consumption. A high rate of pyruvate production causes the accumulation of lactate observed during the early stages of fed-batch cultures (Wilkens et al., 2011). This is supported by metabolic profiling data which show accumulation of alanine (Sheikholeslami, Jolicoeur, & Henry, 2014; L. Zhang, Shen, & Zhang, 2004) which sometimes undergoes its own metabolic shift similar to that of lactate (Ma et al., 2009). Alanine transaminase produces alanine from pyruvate, so this is indicative of excess pyruvate production. A drop in glycolytic flux leads to depletion of the pyruvate pool which lactate is used to replenish. Glycolytic flux is regulated at several well-established control points. The regulatory enzymes include hexokinase, phosphofructokinase (PFK), and pyruvate kinase (Berg, Tymoczko, & Stryer, 2002). Feedback inhibition is common; for example, PFK can be

inhibited by ATP, low pH and lactate while hexokinase is inhibited by glucose 6-phosphate (Berg et al., 2002; Halestrap & Price, 1999; Leite, Da Silva, Coelho, Zancan, & Sola-Penna, 2007). In this way, lactate consumption can be linked to glycolytic enzyme activity (Mulukutla et al., 2012).

Glycolytic channeling has also been suggested as a method to control the pyruvate available for conversion into lactate. Wahrheit et al. (2014) theorized that under conditions of high glucose, channeling through glycolytic enzymes is high and a distinct pool of pyruvate forms which is used to produce lactate. When glucose levels dropped, glycolytic enzymes were more diffuse through the cytosol and pyruvate was freely available to enter the mitochondria. In this way, glycolytic flux as well as the pyruvate pool available to LDH can be controlled, and therefore the potential to drive the lactate switch exists.

High glycolytic rates are linked to rapidly dividing cells (Zheng, 2012). This could explain the transition to lactate consumption being associated with entry into the stationary phase (Y. Fan et al., 2015; Ma et al., 2009; Mulukutla et al., 2012), as glycolytic flux decreases alongside cell growth. For example, Toussaint et al. (2016) reported that the switch to lactate consumption occurred consistently 24 hours prior to the culture reaching maximum cell density.

Glucose transporter 1 (GLUT1) transports glucose into cells. It is often overexpressed in cancers (Berg et al., 2002), which generally have high glycolytic flux due to the Warburg effect (Potter et al., 2016). Therefore, a reduction in GLUT1 expression could indirectly drive the lactate switch by restricting glucose uptake and therefore glycolytic flux. This is supported by experimental data in CHO systems which show lactate consumption occurs only when glucose consumption and glycolytic flux are low (Le et al., 2012).

The availability of nutrients to feed into glycolysis also affects its total flux. This explains the

recurrent observation that cells switch to consuming lactate upon depletion of glucose (Martinez et al., 2013; Sun et al., 2013; Tsao et al., 2005). The reduction in glycolytic flux caused by lack of input depletes pyruvate and NADH leading to lactate consumption. In this way, cells compensate for the carbon they can no longer obtain from the components of the culture medium with lactate. However, other studies show the lactate switch occurring even in the presence of excess glucose. In these instances, often another major carbon source, glutamine, is instead depleted (Ghorbaniaghdam, Chen, Henry, & Jolicoeur, 2014; Nolan & Lee, 2011; Wahrheit et al., 2014). Although unintuitive, depletion of glutamine could also drive the lactate switch by reducing the pyruvate pool. Glutaminolysis, a pathway often favored by cancer cells (Jin, Alesi, & Kang, 2016), can replenish pyruvate in the cell. Glutamine enters the TCA cycle (Figure 1) after conversion to α-ketoglutarate and exits as malate. Malate is then oxidized to pyruvate by the malic enzyme (decarboxylating malate dehydrogenase). It follows then that exhaustion of extracellular glutamine could drive the lactate switch if the contribution of glutaminolysis to the pyruvate pool had been significant. Despite this, nutrient deprivation alone cannot be used to explain the lactate switch as the phenomenon has been reported in fed batch cultures where nutrients are still present in excess (Carinhas et al., 2013; Kyriakopoulos & Kontoravdi, 2014).

Redox Balancing

As NAD^+ and NADH are also substrates of LDH (Figure 4), the redox state of the cytosol is an important factor in determining the direction of the reaction. If NADH levels rise, such as when glycolytic flux is high, LDH reduces pyruvate to lactate which regenerates NAD^+. As the reaction is reversible, LDH can be used to maintain redox balance. In this way, the redox state of the cell can be linked to the relative lactate and pyruvate concentrations (Halestrap & Wilson, 2012), meaning changes in redox potential could drive lactate consumption. This theory can be integrated with reduced glycolytic flux driving a lactate shift. Initially, high

glycolytic flux causes an overflow in NADH production and lactate is produced to replenish NAD^+. A subsequent reduction in glycolytic flux reduces the NADH pool, shifting the NAD^+/NADH ratio and lactate is consumed to restore the balance. It has been suggested that cytosolic NADH is consumed by mitochondria during the metabolic shift (Nolan & Lee, 2011). During lactate consumption, NADH is produced and the reducing power can be indirectly transported into the mitochondria via the malate-aspartate shuttle (Mulukutla et al., 2012). Again, this is supported by studies which demonstrate increased OXPHOS after the lactate switch (Templeton et al., 2013; Zagari et al., 2013). The idea that lactate consumption occurs with the aim of maintaining redox balance has been suggested by other researchers in the field (Nolan & Lee, 2011; Wilkens et al., 2011; Zalai et al., 2015).

Further support for the redox argument comes from the accumulation of glycerol in CHO cultures which has been reported by several groups (Blondeel et al., 2016; Carinhas et al., 2013; Dickson, 2014; Sellick et al., 2015). The pathway for producing glycerol involves another redox sensitive reaction, the conversion of dihydroxyacetone phosphate (DHAP) to glycerol 3-phosphate alongside the oxidation of NADH. As glycerol accumulates in CHO cultures, and the glycerol 3-phosphate dehydrogenase enzyme is reportedly upregulated upon expression of a recombinant protein (Blondeel et al., 2016), the cells may be using this reaction to restore the cytosolic NAD^+ pool and allow continuation of glycolysis in a similar manner to the LDH catalyzed reaction.

Redox potential is an important driver of biochemical reactions and a disturbance in its balance is associated with poor cell health and damage to macromolecules such as proteins and lipids (Gruning, Lehrach, & Ralser, 2010; Wilkens et al., 2011). If lactate consumers are better able to regulate their redox balance through LDH, this could explain the improved process performance. Conversely, cells unable to consume lactate have greater difficulty maintaining their redox state leading to reduced titer and cell growth rates.

Mitochondrial Activity

The redox state of a cell is strongly affected by the balance between glycolysis and TCA cycle activity (Zalai et al., 2015), implicating mitochondrial function in the lactate shift. NADH is produced during glycolysis and consumed by the mitochondrial ETC. If this is unbalanced, and glycolysis cannot meet the needs of the mitochondria, lactate can be consumed as a method of elevating NADH. Thus, either a decrease in glycolysis or an upregulation of mitochondrial activity could drive lactate consumption.

Copper deficiency has been shown to inhibit the lactate switch (Kang et al., 2014). Again, the balance between glycolysis and mitochondrial function can explain this phenomenon. Copper is a vital prosthetic site in complex IV of the ETC, meaning its deficiency compromises the ETC and the requirement for NADH is reduced. NADH levels remain high and lactate is not consumed. Further support for increased oxidative activity or decreased glycolysis driving the lactate switch comes from Templeton et al. (2014). Upregulation of the anti-apoptotic gene BCL2, which is known to interact with mitochondria, caused lactate consumption at a higher rate. This was accompanied by increased activity of some mitochondrial enzymes. By this mechanism, stimulation of mitochondrial function can be used to decrease the pyruvate and NADH pools (Dorai et al., 2009) and tip the balance between glycolysis and OXPHOS.

Summary

As described, a myriad of potential control mechanisms exist for the switch to lactate consumption. However, there are some which can be argued against based on the study conducted by Gagnon et al. (2011). In this study, a glucose feed system was established based on pH. Addition of glucose only occurred when the pH began to rise, as this was indicative of lactate consumption and therefore glucose depletion. This resulted in glucose addition being so tightly controlled that glucose was undetectable by their automated analyzer (<1.1mM)

and lactate fluctuations were so small they could not be accurately measured. To prevent any discernible changes in lactate concentration, the switch between lactate production and consumption must have occurred rapidly. For this reason, it is proposed that the switch to lactate consumption is not initially driven by transcriptional changes as this would not allow a rapid enough response. In this case, neither expression levels of the enzymes nor their relative isoforms could drive the switch. However, this does not rule out expressional changes occurring alongside the switch under normal conditions to achieve stable, long-term metabolic adaptation.

In conclusion, it is likely the trigger of the lactate switch is the redox state of the cell. This alters the flux through LDH due to the near equilibrium state of the reaction. It allows LDH to behave as a powerful redox sensor, with both the ability to replenish NAD^+ for the continuation of glycolysis and to regenerate pyruvate when glycolysis slows through sensing a reduction in NADH levels. A change in redox status would also happen quickly enough to explain the findings of Gagnon et al. (2011). It is possible that a rapid response to the metabolic/redox state exists, which is controlled by the relative ratio of pyruvate/lactate and NAD^+/NADH, then if the metabolic phenotype remains stable the cell will adapt its proteome to the new conditions.

Part II: Approaches to Controlling the Lactate Switch

Because the lactate switch is considered a desirable characteristic in fed-batch culture, there has been a significant amount of research into developing methods to ensure the switch occurs (Zagari et al., 2013). Often, the strategies used target the aforementioned molecular processes.

Metabolic engineering

A common method of improving metabolism in cultures is to genetically engineer metabolic

pathways in the cell. Advances such as the sequencing of the CHO cell genome (X. Xu et al., 2011) and the development of a genome scale metabolic model (Hefzi et al., 2016) mean new enzymes and pathways can be targeted rationally. With regards to lactate accumulation, the genes selected for engineering tend to surround pyruvate metabolism (Chen, Liu, Xie, Sharp, & Wang, 2001; Fogolin, Wagner, Etcheverrigaray, & Kratje, 2004; Jeong et al., 2006; Toussaint et al., 2016).

Since lactate is produced and consumed by a single reaction, the most obvious choice when engineering for reduced lactate formation is to knockout or downregulate LDH (Chen et al., 2001; Jeong et al., 2006; S. H. Kim & Lee, 2007a). Generally, similar results were obtained with this type of engineering, with knockdown of LDH being associated with decreased glucose consumption and decreased production of lactate without impairment of cell viability or productivity (Chen et al., 2001; S. H. Kim & Lee, 2007a). However, it should be considered that reducing lactate production also hinders regeneration of NAD^+ from NADH in the cytosol, so it is possible that glycolytic flux could be restricted.

Other methods of metabolic engineering aim to reduce lactate formation by diverting pyruvate into other pathways. For example, overexpression of yeast PYC, which localizes to the cytoplasm, generates a favorable metabolic phenotype. Pyruvate is converted into oxaloacetate which is then reduced into malate, forming NAD^+ in the process. Malate is transported into the mitochondria via the malate-aspartate shuttle (Wilkens & Gerdtzen, 2015) where it is oxidized back to oxaloacetate, forming NADH. This type of metabolism is advantageous for several reasons. Firstly, the cytosolic pyruvate pool is decreased, reducing the production of lactate through competition with LDH. Secondly, NAD^+ is regenerated in the cytoplasm without the need to produce lactate, allowing glycolysis to continue. Thirdly, the malate pool within the mitochondria is increased. This encourages flux through the TCA cycle, forming NADH in the mitochondria to fuel the ETC. Finally, the increased pool of

oxaloacetate formed from malate in the mitochondria will increase the rate of condensation of oxaloacetate and acetyl CoA. This increases flux through the TCA cycle and reduces the cytosolic pyruvate pool as pyruvate will be consumed to replenish acetyl CoA (Figure 1) (Wilkens & Gerdtzen, 2015).

Attempts at expressing recombinant PYC in production systems have been met with varying levels of success. The earliest research was carried out by Irani et al. (2002; 1999) in BHK-21 cells. Over two studies, they showed that clones expressing PYC had prolonged viability, reduced uptake of glucose and glutamine, reduced lactate production and a higher protein titer (Irani et al., 2002). Experiments in CHO cells produced largely comparable results with an increase in viability paired with reduced lactate consumption again being observed (Fogolin et al., 2004; S. H. Kim & Lee, 2007b; Toussaint et al., 2016). However, the effect on growth profiles and maximum cell density varied (Fogolin et al., 2004; Toussaint et al., 2016). Despite this, titer was increased in both studies (Fogolin et al., 2004; Toussaint et al., 2016). The overarching conclusion is that metabolic efficiency was improved, with less carbon lost to lactate. However, a more recent study (Wilkens & Gerdtzen, 2015) reported lower growth rates, volumetric and cell specific productivities in PYC CHO cells, although the glucose/lactate ratio was improved.

Diverting pyruvate away from LDH could also be achieved by upregulating PDH activity, thereby feeding pyruvate into the TCA cycle via acetyl CoA (Figure 1). A study conducted by Zhou et al. (2011) attempted to reduce lactate production through knockdown of both LDH and PDK. They achieved a more desirable metabolic state with reduced lactate accumulation. This change in metabolism was accompanied by increased volumetric and specific productivity.

Over-expression of malate dehydrogenase 2 (MDH2) is another approach to reducing the

pyruvate pool. The rationale behind this approach is to relieve the bottleneck in the TCA cycle caused by the MDH2-catalyzed reaction (Chong et al., 2010; Wilkens & Gerdtzen, 2015) and therefore reduce buildup of malate in the culture medium (Chong et al., 2010). This should result in increased oxaloacetate in the mitochondria, leading to greater citrate synthase activity and overall more pyruvate feeding into the TCA cycle. Additionally, this reaction produces NADH, so should encourage a more favorable redox state in the mitochondria. The two studies investigating over-expression of MDH2 in CHO cells had mostly contradictory results. Chong et al. (2010) demonstrated an increase in cell number whereas Wilkens and Gerdtzen (2015) showed a significant decrease. These results led to opposing results on titer. However, both studies did succeed in reducing the amount of lactate produced (Chong et al., 2010; Wilkens & Gerdtzen, 2015).

The utilization of alternative carbohydrates has been suggested as a method to reduce lactate accumulation. The GLUT5 fructose transporter has a lower affinity for fructose than the GLUT1 glucose transporter has for glucose. In this way, expression of GLUT5 and the replacement of glucose in the media with fructose could reduce lactate accumulation by reducing glycolytic flux (Wlaschin & Hu, 2007). CHO cells do not normally survive well in media containing fructose but not glucose; this is due to low, or potentially absent, expression of the GLUT5 transporter (Le et al., 2013; Wilkens & Gerdtzen, 2015; Wlaschin & Hu, 2007). Studies investigating the engineered expression of GLUT5 in the presence of fructose show favorable lactate profiles indicating more efficient metabolism (Le et al., 2013; Wilkens & Gerdtzen, 2015; Wlaschin & Hu, 2007). Le et al. (2013) used a time-sensitive promoter to drive GLUT5 expression in later culture stages resulting in a biphasic approach. This meant glucose could be used to stimulate cell growth in early culture, later followed by a switch to fructose feeding which favored balanced metabolism.

Engineering CHO cells to be resistant to apoptosis has been investigated to prolong cell

viability in bioreactors (Dorai et al., 2010; Dorai et al., 2009; Templeton et al., 2014). This type of engineering is largely successful with three studies reporting increased viable cell densities in engineered clones (Dorai et al., 2010; Dorai et al., 2009; Templeton et al., 2014). An interesting observation is the alteration in lactate metabolism seen in these apoptosis resistant clones. Dorai et al. (2010; 2009) showed that engineered clones underwent the lactate shift where control cell lines did not. This was supported by Templeton et al. (2014) whose BCL-2Δ overexpressing cells had a slower rate of lactate production and a greater uptake rate during the consumption phase compared to controls. Overall, they concluded that more pyruvate was directed into the mitochondria rather than to lactate and that apoptosis resistant cells did not rely as strongly on the production of lactate to maintain their redox balance.

The heterogeneity of the CHO cell population must be considered in the interpretation of studies surrounding metabolic engineering. Clonal variation can have a large impact on growth, titer and metabolic efficiency. Simply testing one, single-cell derived clone is not sufficient to draw conclusions. If attempts at identifying suitable proteins for engineering are to be continued, mixed clonally-derived cell lines or a panel of single-cell derived populations should be assessed.

Media and process control

Rather than engineering cells, their metabolism can be altered by changing their extracellular environment. This includes both components of culture media and other process controls such as pH and temperature.

Glucose is absorbed and metabolized by cells very quickly; this generally leads to inefficient metabolism and a buildup of lactate in the culture medium (Altamirano, Paredes, Cairo, & Godia, 2000). If another sugar that is imported or metabolized more slowly is used, this

would reduce glycolytic flux and therefore the accumulation of lactate. Galactose (Altamirano, Cairo, & Godia, 2001; Altamirano, Illanes, Becerra, Cairo, & Godia, 2006; Altamirano et al., 2000; Altamirano, Paredes, Illanes, Cairo, & Godia, 2004; Sun et al., 2013; S. Xu, Hoshan, & Chen, 2016), fructose (Altamirano et al., 2000; Le et al., 2013; Wlaschin & Hu, 2007; S. Xu et al., 2016), mannose (Altamirano et al., 2000; Berrios, Altamirano, Osses, & Gonzalez, 2011; S. Xu et al., 2016) and maltose (S. Xu et al., 2016) have all been investigated as alternative energy supplies.

Altamirano et al. extensively studied the use of galactose in CHO cell cultures. They initially found that galactose was consumed more slowly than glucose, as expected, and that this resulted in lower specific lactate production. However, cell density was drastically reduced (Altamirano et al., 2000). To remedy this, they attempted growing cells in low glucose with higher levels of galactose. This allowed a switch to lactate consumption upon glucose depletion and increased cell growth (Altamirano et al., 2006). This was supported by later studies (Sun et al., 2013) which demonstrated higher cell density, higher specific productivity and the lactate switch when combining glucose and galactose in feeds.

A reduction in glycolytic flux and the resulting overflow into waste metabolites can also be achieved by maintaining glucose at low levels in the culture environment without supplementing other sugars. Controlled feeding aims to maintain low glucose without allowing complete depletion. Historically, various methods have been used, including feeding based on models of stoichiometry and cell growth, and feeding based on direct metabolic measures such as oxygen consumption (Gambhir, Europa, & Hu, 1999; Xie & Wang, 1994; W. C. Zhou, Rehm, & Hu, 1995). Tight control over glucose levels offered by technological advances means glucose restriction is now a more plausible method of controlling lactate accumulation. The use of a traditional fed-batch process raises concerns with regards to glucose starvation as opposed to limitation. Although glucose starvation would still reduce

lactate buildup, this would have detrimental effects on product quality. Cells starved of glucose for even short periods of time produce different glycosylation patterns on the protein of interest (Toussaint et al., 2016).

Gagnon et al. (2011) developed a technique which used pH as a surrogate for remaining glucose in the media. A rise in pH is indicative of lactate consumption by the cells as protons are co-transported into cells by MCTs. Lactate consumption was presumed to be a result of low glucose, so the system was programmed to feed glucose to the bioreactor when the pH increased above a set-point. The addition of glucose triggers lactate production and a drop in pH occurs, preventing further glucose feeding. Because this method relies primarily on pH measurements, it is scalable in normal bioreactor systems and was shown to be successful up to 2500L.

In recent years, more sophisticated techniques have been introduced involving online auto-sampling giving real time monitoring of culture metabolites. Direct monitoring of glucose and lactate can be used to prevent overflow metabolism (Matthews et al., 2016; A. Zhang et al., 2015). Matthews et al. (2016) used Raman spectroscopy to measure glucose and lactate levels, and only initiated glucose feeding when both glucose and lactate concentrations fell below their set-points, preventing lactate accumulation.

Technological advances also allow larger scale omics studies to be conducted. For example, Mulukutla et al. (2017) recently used metabolomic profiling to identify metabolites which accumulated during a CHO fed batch culture. Of these, they identified several which resulted from overflow metabolism and were inhibitory to cell growth. Thus, studies such as these can allow rational design of culture media to prevent accumulation of inhibitory compounds.

Supplements can also be added to culture media in a bid to produce a favorable lactate profile. Copper is a common example of this (Luo et al., 2012; S. Xu et al., 2016; Yuk et al.,

2015; Yuk et al., 2014). The metabolism of copper deficient cells is pushed towards a glycolytic phenotype (Nargund, Qiu, & Goudar, 2015), potentially caused by perturbations in ETC function (Kang et al., 2014; S. Xu et al., 2016). In this sense, the composition of the culture medium can alter the balance between mitochondrial and glycolytic activity, with the potential to drive the lactate switch.

However, altering the components of culture media does not come without risks. For example, sugar composition can have a large effect on glycosylation of the final product (Berrios et al., 2011; Surve & Gadgil, 2015; S. Xu et al., 2016) while high copper concentrations were shown to increase basic variants (S. Xu et al., 2016; Yuk et al., 2015) and cause product aggregation (Qian et al., 2011). The accumulation of waste products can also affect the quality of the recombinant molecules (Toussaint et al., 2016). A balance must be obtained between striving for quality and productivity.

The pH of culture media can be altered with relative ease through base or acid addition. Decreasing the pH in culture systems has been shown to drive onset of the lactate switch in CHO and HEK293 cells expressing recombinants proteins (Liste-Calleja et al., 2015; Zalai et al., 2015). A pH shift may be paired with a temperature shift (Zalai et al., 2015), which is again easy to control. Hypothermia reduces global metabolic activity, thereby reducing glucose uptake and the production of lactate, although hypothermia alone is often not sufficient to drive a complete switch in lactate metabolism (Fogolin et al., 2004; Nolan & Lee, 2011; Tsai, Yoon, Chuppa, Konstantinov, & Naveh, 1996; Weidemann, Ludwig, & Kretzmer, 1994).

Selection of cells lines

In a number of studies, cells grown under the same conditions display differing phenotypes with regards to lactate production/consumption (Gagnon et al., 2011; Hinterkorner et al.,

2007; Luo et al., 2012; Zagari et al., 2013). This evidence suggests that the lactate metabolism of a cell is somewhat predetermined and therefore the initial cell selection process could be optimized to select clones with a favorable metabolic phenotype. Hinterkorner et al. (2007) used Rhodamine 123 to stain and sort cells based on their mitochondrial membrane potential. From a population of high lactate producers, sorting for cells with low membrane potential gave rise to sub-clones which had lower rates of lactate production alongside increased growth. Other studies have also linked mitochondrial function to the lactate profile and suggested mitochondrial oxidative capacity as a possible selection marker (Luo et al., 2012; Zagari et al., 2013). It is possible that cells with increased mitochondrial capacity have a propensity to switch to lactate consumption as the balance between glycolysis and the TCA cycle is more likely to favor the latter.

This leads to interesting possible developments in early clone selection. Enhancing the host population for cells with desirable characteristics could prove a useful method for ensuring a larger proportion of clones produced have favorable metabolic characteristics. In turn, this would reduce the number of clones which must be expanded and assessed in early clone selection, therefore reducing the cost and labor required. However, Hinterkorner et al. (2007) found that even cells from a clonal population had a large amount of variation in their specific glucose uptake and lactate production rates and went on to suggest that two rounds of metabolic selection may be required, one for the host cell population and once after clonal selection. This adds extra stages to the development process, but the long-term benefits have the potential to outweigh the initial cost and time investment.

Summary

As described, a number of methods have been investigated in an attempt to control the lactate switch, with varying levels of success. Some methods, such as supplementing the culture

medium, can be carried out with relative ease, whereas altering the cell line screening process or introducing metabolically engineered cell lines would raise more difficulties from technical and regulatory standpoints.

Variability in CHO cells is high due to the instability of their genome and changes in the transcriptome of the cell (Li et al., 2016). This means there will be variation in host cells used by individual research groups. Given that different results are sometimes obtained from CHO cell clones derived from the same host population (Hinterkorner et al., 2007; Zagari et al., 2013), results should be generalized with caution. Additionally, due to strong industry ties, investigations into controlling the lactate switch are often conducted using proprietary media and feeds. This means that the extent of differences in the initial culture environment is unknown and cannot be properly accounted for. Some experiments are also conducted in shake flasks rather than bioreactors, which lack the same control, again causing problems when attempting to reconcile results and extrapolate findings to large scale production systems (Gagnon et al., 2011).

It is worth considering that the desirable traits of a cell line vary during a production run. High growth rates early in culture allow high cell densities to be reached, generally improving volumetric titer. Later, balanced metabolism which generates high specific productivity is preferred. As a result, the biphasic approach will likely become more popular over the coming years. Feed design could be altered depending on the timing of its addition and the use of inducible gene switches will allow genetic engineering to be controlled to enhance growth or specific productivity depending on the culture phase.

Conclusion

Identifying the specific cause of the lactate switch would allow more focused strategies aimed at its control to be developed. A deeper understanding of the molecular basis by which

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2
3 the switch is triggered would also aid in generalizing results between research groups and cell
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5 types. The CHO cell genome was published in 2011 (X. Xu et al.) and hamster microarrays
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7 have since been developed (Qian et al., 2011); these tools will be invaluable in identifying
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9 potential markers of the lactate switch moving forward. Moving away from studying this
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11 phenomenon in a CHO cell system could also be of use. Lactate shuttling has been described
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13 in brain tissues where astrocytes produce lactate that fuels OXPHOS in neurons (Potter et al.,
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15 2016; Zheng, 2012). Similar shuttling patterns have also been described between cancer
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17 associated fibroblasts and cancer cells in the tumor microenvironment; this is termed the
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19 reverse Warburg effect (Potter et al., 2016; Zheng, 2012). Further investigation into these
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21 systems, (e.g. comparative proteomic/transcriptomic analysis of the lactate producing and
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23 lactate consuming cells) could shed light on the mechanism used by CHO cells to switch to
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25 this phenotype.
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29 Discovering why the lactate switch appears to be beneficial is also of importance. It stands to
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31 reason that the increased ATP yield from oxidative metabolism during lactate consumption
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33 allows cells to meet the energy requirements for protein synthesis. As stated in this review, it
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35 could also be the case that lactate consumption better equips cells to maintain their redox
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37 balnace, thus improving overall cell health and allowing increased rates of protein synthesis
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41 From this review, a hypothesis detailing the mechanism behind the lactate switch has been
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43 put forward. The nature of LDH catalyzed reaction means that the production and
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45 consumption of lactate can be used to maintain the redox state. Thus, it stands to reason that
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47 the purpose of lactate consumption is to restore the redox balance and therefore may be
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49 triggered by a disturbance in the NAD^+/NADH ratio. This ratio can be directly and indirectly
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51 influenced by several other suggested triggers of the lactate switch, meaning the redox state
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53 can be considered a universal effector in the switch to lactate consumption.
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3 **Tables**
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9 Summary of studies investigating the lactate switch.
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Reference	Suggested cause of the switch in lactate metabolism
(Altamirano et al., 2006)	Lactate consumption began when glucose was depleted.
(Altamirano et al., 2004)	Lactate consumption began when glucose levels decreased.
(Carinhas et al., 2013)	Lactate consumption occurred despite there being no nutrient depletion and prior to the culture reaching maximum cell density.
(Ghorbaniaghdam et al., 2014)	Lactate consumption began when glutamine was depleted.
(Kyriakopoulos & Kontoravdi, 2014)	The authors conclude that the lactate switch is triggered by the extracellular lactate concentration.
(Liste-Calleja et al., 2015)	The lactate switch was triggered by a pH shift.
(Luo et al., 2012)	The lactate switch occurred in cells with a greater oxidative capacity.
(Ma et al., 2009)	The lactate switch coincided with transition into stationary phase.
(Martinez et al., 2013)	Lactate consumption began when glucose was depleted.
(Mulukutla et al., 2012)	The authors suggest reduced glycolytic flux as the cause of lactate consumption, which is itself caused by a high lactate concentration and a reduced growth rate.
(Wahrheit et al., 2014)	Lactate consumption began when glutamine was depleted.
(Zagari et al., 2013)	Multiple experiments. In some instances, lactate consumption occurs when glutamine alone is depleted while some cultures did not consume lactate until both glutamine and glucose levels were depleted.
(Zalai et al., 2015)	The lactate switch can be controlled by a pH and temperature shifts.

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List of Figures

Figure 1

Metabolic map of glycolysis, the link reaction and the TCA cycle. Key enzymes are labelled. Pyruvate is a central metabolite which can be produced and consumed by a number of reactions. This diagram is non-exhaustive - pyruvate is also the product of various amino acid catabolism reactions.

Figure 2

The influence of lactate and pH on MCT flux. (A) When the concentrations of both lactate and H^+ are higher in the cytosol, there is a net efflux of both. (B) When pH is controlled, H^+ outside the cell are buffered. This means that extracellular H+ and lactate do not increase at the same rate, and net efflux of both occurs even when lactate concentrations outside the cell are high. (C) Lactate is consumed inside the cell due to reversal of the LDH catalyzed reaction or a reduction in glycolytic flux which decreases its production. There is a net influx of lactate and it is consumed from the culture medium. (D) The H^+ concentration inside the cell is reduced. This could be due to a decrease in glycolytic flux or its consumption in other cellular reactions. Lactate is transported alongside protons into the cell, despite a lactate gradient not being present. (E) Both lactate and H^+ concentrations are lower inside the cell. The net movement of lactate is an influx, meaning lactate is consumed from the culture medium.

Figure 3

The five isozymes of LDH. The LDHA gene which encodes the LDH-M protein has a greater affinity for pyruvate. Isozymes with a greater proportion of the LDH-M protein (i.e. LDH4 and LDH5) will therefore preferentially catalyze the forward reaction. The LDHB gene which encodes the LDH-H protein has a greater affinity for lactate. Isozymes with a greater

proportion of the LDH-H protein (i.e. LDH1 and LDH2) will preferentially catalyze the oxidation of lactate back to pyruvate.

Figure 4

The LDH catalyzed reaction. Pyruvate is reduced to lactate while NADH is concomitantly oxidized to NAD^+. This reaction is fully reversible.

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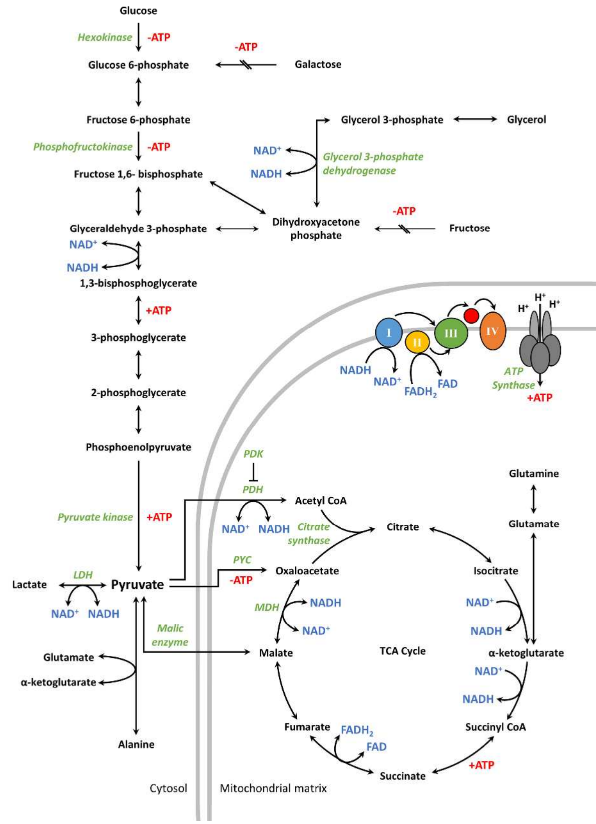

Metabolic map of glycolysis, the link reaction and the TCA cycle. Key enzymes are labelled. Pyruvate is a central metabolite which can be produced and consumed by a number of reactions. This diagram is non-exhaustive - pyruvate is also the product of various amino acid catabolism reactions.

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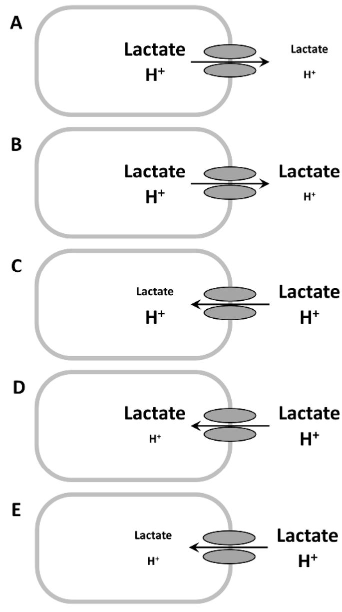

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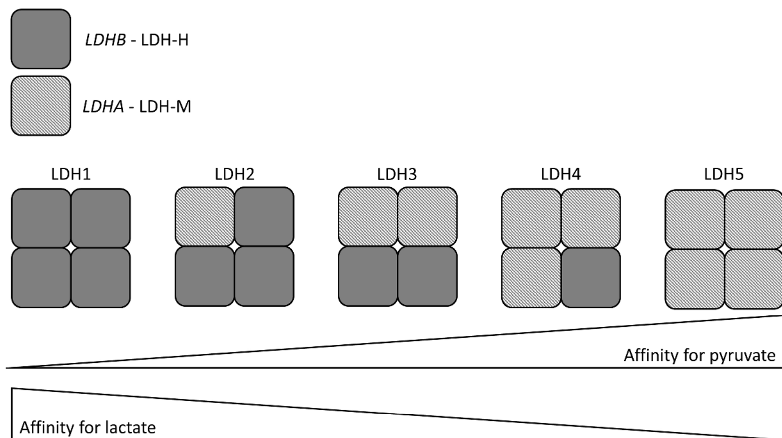

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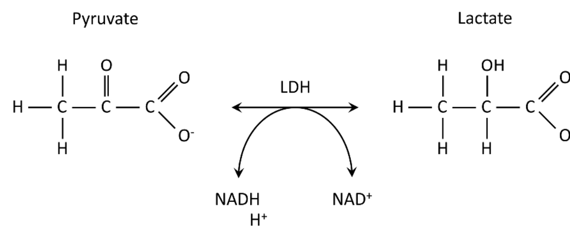

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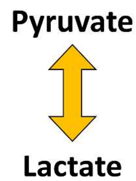

FOR GRAPHICAL TABLE OF CONTENTS: A switch in lactate metabolism is frequently observed in a CHO cell production environment. Cultures transition from a lactate producing to a lactate consuming state, a feature which is associated with enhanced process performance. However, the molecular mechanism through which this switch is conferred remains enigmatic. This review uses existing data to argue that redox imbalance is the primary driver of this metabolic switch.

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