ANALYSIS OF METHYLGLYOXAL TOXICITY IN HYBRIDOMA CELL CULTURE

By

Benjamin M. Roy

Thesis

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

in

Chemical Engineering

May, 2004

Nashville, Tennessee

Approved:

Professor R. Robert Balcarcel

Professor Robert D. Tanner

To Robert and Karen Roy for their support and patience.

ACKNOWLEDGMENTS

My sincerest thanks go to those who helped with the completion of this project. I would like to thank Dr. R. Robert Balcarcel for his guidance and advice throughout the process. I would like to thank Dr. Robert Tanner for his input and creativity. I am also indebted to all the members of my research group: Lindsey Clark, Sean Hartig, and Yuansheng Yang, who never hesitated to share time and insight with regard to this work. Finally, thanks to my parents for continuing to stand behind my decisions and supporting me along the way.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

SPECIFIC AIMS

In recent years pharmaceutical development has increasingly centered on biologically based products such as monoclonal antibodies and therapeutic proteins. Significant portions of these drugs are produced in bioreactors by mammalian cell culture. Research to improve the efficiency of these processes is widespread, investigating strategies that range from gene elimination to supplemental fed-batch design. Each strategy is aimed at a particular target which is thought to decrease efficiency. One commonly cited target is toxin accumulation in the culture. Lactate and ammonia have long been cited as toxins that build up during a batch and inhibit processes that are essential to cell growth and production of the therapeutic product. The current study compares lactate to methylglyoxal (MG), an endogenous glycolytic byproduct that has recently been suggested as a damaging agent.

The specific aim of this study is to quantify the toxicity of methylglyoxal in hybridoma cultures and compare it directly to the toxicity of lactate. Each toxin is examined to determine its mode of toxicity and levels that induce these toxic effects. These results will be compared to previous studies that have been performed on various cell lines. The current work also aims to reveal provide new insight regarding the mode of toxicity for each toxin. Lactate is studied at concentrations higher than previously considered to produce a more accurate value of median growth inhibitory concentration, GI50. The effect of osmolarity in lactate toxicity is also examined here.

Methylglyoxal has been shown to induce apoptosis in other cell cultures, and is studied here to determine the extent of apoptosis in hybridoma cultures and the rate at which it is induced. Based on these results, the relevance of each toxin is examined via comparison to observable batch levels. This requires an estimation of intracellular levels of both toxins over the course of the batch. Lactate levels are well documented in batch culture; methylglyoxal, on the other hand, is difficult to quantify. To this end, a model is presented that predicts the variation of intracellular MG over the course of the batch. The combination of estimation and toxicity screens allows for speculation on the role of each toxin in causation of cell death and growth inhibition in a bioreactor.

Based on the results of this toxicity analysis, strategies are proposed for preventing the accumulation of methylglyoxal in culture. These range from fed-batch strategies to genetic engineering of the cells.

CHAPTER II

BACKGROUND AND SIGNIFICANCE

Biopharmaceuticals

Biologically based products have become increasingly significant in the pharmaceutical industry. During the years 1980-2001, 40 recombinant proteins and 10 monoclonal antibodies were approved in the United States, and in 2002, approximately 40% of drugs approved by the FDA were biopharmaceuticals (Reichert 2003). Recombinant antibodies alone made up for an estimated four billion dollars worldwide sales in 2003 (Souriau et al. 2003), and approximately twenty more of these therapeutics are currently in clinical trials (Brekke et al. 2003).

Mammalian cells such as Chinese Hamster Ovary (CHO), hybridoma, and myeloma are commonly used to produce biopharmaceuticals. The genomes of the cells are transfected with a gene that is expressed to produce the target compound. The cells are grown in a bioreactor, after which the resulting product is harvested and purified.

Batch Production

Production of biopharmaceuticals is restricted by cell density limitations and cell death over the course of a batch. In a typical batch, hybridoma cells suffer growth inhibition after approximately 30 hours and onset of significant viability decreases at around 60 hours (Balcarcel et al. 2001). Cell death in the bioreactor generally occurs as

a result of apoptosis, or programmed cell death. While the cause of apoptosis in a bioreactor is undetermined, generally the process is caused by insults such as nutrient limitation and toxin buildup. These insults trigger an apoptotic cascade which leads to the condensation of chromatin and the breakup of afflicted cells. The process generally occurs over a period of hours. This mode of cell death is contrasted with necrosis, which is stimulated by a gross insult to culture, and typically results in cell swelling and breakup without significant change to cellular nuclei (Al-Rubeai 1998).

Limitations in biopharmaceutical production imposed by growth inhibition and apoptosis are cause for concern in industry. Inefficient production leads to larger equipment and increased cost in an industry frequently criticized for its product pricing. Moreover, the increasing number of biopharmaceuticals coming to market are projected to overwhelm industrial production capacity; suboptimal production methods will only exacerbate this problem (Walsh 2000).

Batch Production Improvements

Various assertions have been made as to why problems are occurring in batch cultures and how to avoid them. Among the suggested causes are nutrient limitation, oxidative stress, and growth factor withdrawal (Al-Rubeai 1998). Techniques employed to combat these problems have had varying levels of success. These include improved medium design, feeding strategies for nutrient supplementation, various additives to the culture, and genetic modification of the production cell lines. The strategies behind these techniques generally take one of two forms: 1) upstream prevention of a problem

or 2) inhibiting the downstream cascade induced by an insult. Just a few of the numerous examples of proposed batch efficiency increases are examined here.

Hosts of feeding strategies have been employed in upstream efforts to improve batch efficiency. Many have achieved significant improvements in monoclonal antibody productivity and cell viability extension (Bushell et al. 1994; Xie et al. 1994; Xie et al. 1996; Zhou et al. 1997; Zhou et al. 1997; Gambhir et al. 1999; Schwabe et al. 1999; Cruz et al. 2000; Dowd et al. 2000; Europa et al. 2000; Jang et al. 2000; Sauer et al. 2000; Altamirano et al. 2001; Godia et al. 2002; Lin et al. 2002; Frahm et al. 2003). Though the specifics of these strategies are wide-ranging, they are typically based on variations of three common goals. The first of these goals is to improve the efficiency with which nutrients are consumed. When cells are provided excess nutrients, they tend to consume these nutrients in amounts exceeding demand for growth and production (Europa et al. 2000). Smaller nutrient doses can improve this efficiency and extend the life of the culture. The second goal seen among these techniques is the minimization of toxic byproducts. Lactate, ammonium, and alanine have been shown to accumulate to high levels in batch cultures (Zhou et al. 1997). The toxicity of these products has been examined on many occasions, and is thought to impact batch cultures (Miller et al. 1988; Hassell et al. 1991; Duval et al. 1992; Omasa et al. 1992; Ozturk et al. 1992; Kromenaker et al. 1994; Ludemann et al. 1994; Newland et al. 1994; Lao et al. 1997; Cruz et al. 2000; Patel et al. 2000). The third goal often cited in the design of fed-batch systems is the prevention of nutrient limitation (Jang et al. 2000). To this end, amino acids are analyzed to determine uptake rates and derive strategies to prevent their complete consumption.

Various genetic engineering strategies have also been adopted as upstream prevention methods. As in feeding strategies, the goal of many of these efforts has been to minimize toxin buildup and optimize nutrient usage. One target has been flux through the TCA cycle. The TCA, or citric acid, cycle breaks down acetyl carbons (in the form of acetyl-CoA) to carbon dioxide. In glycolysis, glucose is broken down to pyruvate; the energy released is stored and used in anabolic processes in the cell. In batch mammalian cell cultures, the product pyruvate is typically converted to lactic acid and excreted from the cells. Alternately, pyruvate can be converted to acetyl-CoA by pyruvate carboxylase and enter the TCA cycle. This process is more energy efficient, however, it has been shown to occur negligibly under normal conditions (Bonarius et al. 2001). Pyruvate carboxylase has thus been upregulated in baby hamster kidney (BHK) cells in attempt to increase efficiency (Irani et al. 1999). This resulted in an increase in viability and antibody production in the batch. In a separate work, a hybridoma cell line was engineered without the gene for the production of lactate dehydrogenase (Chen et al. 2001). In removing this enzyme, which catalyzes the production of lactate from pyruvate, growth, viability, and antibody production were improved in the batch.

Other upstream techniques have cited cell cycle control as a potential means of improving batch cultures (Jang et al. 2000; Altamirano et al. 2001; Godia et al. 2002). Rapamycin, a compound that slows progression through the cell cycle, has been shown to extend the viability of hybridoma cultures and increase maximum attainable cell density (Balcarcel et al. 2001).

Other efforts to improve batch production have focused on blocking negative effects such as apoptosis and growth inhibition downstream. To maintain growth that is typically limited in a batch, several have attempted to supplement culture with insulin and serum (Doverskog et al. 1997; Sanfeliu et al. 2000). This resulted in greater cell densities, but also seemed to stimulate cell death (Sanfeliu et al. 2000). Various genetic alterations have been made to cell cultures to block events in the apoptotic cascade (Simpson et al. 1997; Fassnacht et al. 1998; Mastrangelo et al. 1999; Terada et al. 1999; Mastrangelo et al. 2000; Sauerwald et al. 2002). These focused specifically on the upregulation of the anti-apoptotic gene bcl-2, transfection of caspase inhibitor XIAP, and the addition of various chemical reagents shown to inhibit apoptotic events.

The current work examines methylglyoxal (MG), a potential causal agent of apoptosis and growth inhibition in batch cultures, and lactate, a suspected growth inhibitory agent. Based on an analysis of the two toxins and their relevance to batch hybridoma production, strategies will be recommended for minimizing their accumulation.

Lactate

Lactate has long been suggested as a problem in batch cultures. Many studies have targeted the compound (Miller et al. 1988; Hassell et al. 1991; Duval et al. 1992; Omasa et al. 1992; Ozturk et al. 1992; Kromenaker et al. 1994; Lao et al. 1997; Cruz et al. 2000; Patel et al. 2000). Some cite it as simply a marginally relevant byproduct of the inefficient glycolytic pathway (vs. the TCA cycle). Many, however, have cited the compound itself as inducing toxic effects on growth in batch cultures, even in pH

controlled settings. A proposed mechanism for the toxicity of lactate is its inhibition of glycolysis (Omasa et al. 1992). Pyruvate is converted to lactate by lactate dehydrogenase (LDH), also regenerating NAD^+ from NADH cofactor. An intracellular buildup of lactate may inhibit LDH, which would, in turn, cause a buildup of NADH, and inhibit glycolysis (Lao et al. 1997). The current study examines lactate toxicity at greater concentrations than have been previously tested to more accurately describe its growth and metabolic inhibition. In addition, sodium chloride studies are presented to determine the extent to which lactate's toxic mechansim is influenced by osmolarity changes.

Methylglyoxal (MG)

In considering toxin accumulation in a bioreactor, byproducts that accumulate to millimolar levels in surrounding growth medium such as lactate and ammonium are typically focused on. The significant presence and toxic characteristics of these compounds makes them likely candidates to cause problems in a batch. However, compounds occurring at far lower concentrations may be extreme more relevant than these, based on a higher potency. It, thus, is useful to examine known toxins that accumulate to levels only a fraction of lactate and ammonium. Recent research suggests that methylglyoxal (MG) is such a compound (Chaplen 1998). This compound is potently toxic. That the compound is more toxic than lactate is demonstrated by naturally occurring "detoxification" mechanisms, which convert it to D-lactate. An increasingly significant body of evidence suggests that MG should be studied as a potentially harmful compound to production cell lines.

Methylglyoxal ($C_3H_4O_2$) is a small compound that contains two carbonyl groups and is extremely reactive (Figure 2.1). Excellent reviews are available regarding its toxicity and detrimental effects in many different settings (Thornalley 1996; Chaplen 1998; Kalapos 1999).

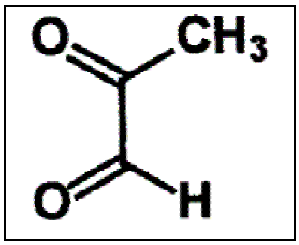

Figure 2.1: Methylglyoxal

Formation and Detoxification

Methylglyoxal is formed by several mechanisms intracellularly. Chief among these is the non-enzymatic decomposition of the glycolitic intermediates dihydroxy-acetone phosphate (DHAP) and glyceraldehyde-3-phophate (GAP). Under normal conditions, methylglyoxal is produced at a rate which is approximately 0.1-0.4% of glycolytic flux (Thornalley 1988). Methylglyoxal is also a natural intermediate of the Maillard reaction, which may occur to a significant extent if large pools of glucose accumulate intracellularly (Padayatti et al. 2001). Other potential sources of methylglyoxal include the catalyzed degradation of acetone, and the catabolism of threonine (VanderJagt 2003).

Detoxification of methylglyoxal also occurs through several pathways. The glyoxalase pathway involves two enzymes, glyoxalase I (GLO I) and glyoxalase II (GLO II), which transform methylglyoxal to D-lactate. In this pathway, glutathione, an

important antioxidant, initially reacts with free methylglyoxal. The product is then converted to S-D-lactoyl-glutathione ($CH_3CH(OH)CO$-SG) by glyoxalase I, which is further converted to D-Lactate by glyoxalase II. The latter reaction also regenerates glutathione. Methylglyoxal can also be detoxified through conversion to pyruvate via betaine aldehyde dehydrogenase or conversion to aminoacetone via aldose reductase. The major pathway for detoxification is reported to be the glyoxalase pathway, which detoxifies 10-40 fold greater amounts than aldose reductase (Vander Jagt et al. 2003). Dominant pathways are depicted in Figure 2.2.

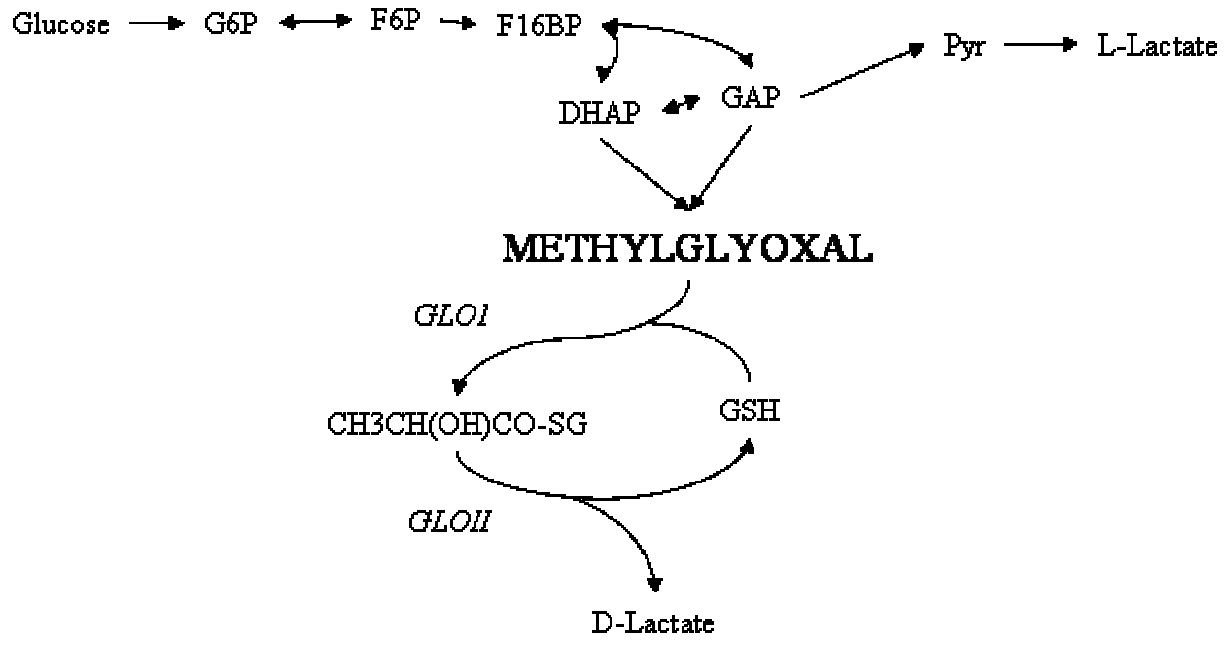

Figure 2. 2 Methylglyoxal is formed predominantly through the non-enzymatic decomposition of dihydroxy-acetone phosphate (DHAP) and glyceraldehyde-3-phophate (GAP). The toxin is detoxified through the glyoxalase system, with glutathione (GSH) as a cofactor.

The formation of MG occurs non-enzymatically, indicating that the compound occurs in all cells and organisms (Thornalley 1996). The glyoxalase system has been deemed ubiquitous, having been detected in bacteria, yeast, and mammalian cells (Thornalley 1993).

Reactivity

The major difficulty in studying MG stems from its reactivity with macromolecules. The compound is extremely reactive with amino acid residues of arginine, lysine, and cysteine. Under normal conditions, a majority of the compound is bound to proteins (Lo et al. 1994; Chaplen 1998; Degenhardt et al. 1998; Kilhovd et al. 2003). Assays of the compound vary from enzymatic conversion using glyoxalase enzymes to advanced chromatographic methods (McLellan et al. 1992; Chaplen et al. 1996; Chaplen et al. 1996; Chaplen et al. 1998; Martins et al. 2001; Lapolla et al. 2003). The accuracy of these assays is limited by MG bound reversibly and irreversibly to macromolecules. Recently, Chaplen et al. have described a more advanced method to trap bound MG which may have otherwise gone undetected (Chaplen et al. 1998). The accuracy of even this assay, however, is in question based on the effectiveness in quantifying bound MG.

The reactivity of MG not only makes it difficult to measure, but also makes it a dangerous presence in cellular systems. The compound has the potential to deactivate metabolic enzymes (Yan et al. 1999). Chaplen reports that many proteins instrumental in glycolysis and signaling pathways contain active site arginine and lysine residues, making them susceptible to MG-mediated modification (Chaplen 1998).

Presence in Disease

Methylglyoxal has been studied extensively because of its proposed impact in the pathology of diabetes. Blood levels of free methylglyoxal are significantly higher in diabetics (Thornalley et al. 1989). Reactive dicarbonyl-containing compounds such as MG have been linked to the formation of advanced glycation endproducts (AGEs), which are macromolecules whose structure and function has been altered by reaction with reducing sugars such as glucose and its derivatives (Singh et al. 2001). Methylglyoxal in particular is known to be a precursor to several AGEs which have been detected in diabetic patients (Vander Jagt et al. 2003). These AGEs are considered to be significant in the development of numerous diabetic complications including nephropathy, neuropathy, retinopathy, and vasculopathy (Singh et al. 2001). Advanced complications in diabetic nephropathy have been linked to elevated MG concentrations (Beisswenger et al. 2003). Many have thus assigned a significant role to MG in complications associated with diabetes, though its mechanism is not fully elucidated. The carbonyl-stress hypothesis proposes that hyperglycemia results in an increase in reactive carbonyl-containing species (carbonyl stress) that leads to an increase in modification of proteins, oxidative stress, and tissue damage (Baynes et al. 1999).

Methylglyoxal has also been studied in the context of cancer. Tumor cells display high rates of glycolysis, and thus would be expected to produce significantly higher amounts of MG than normal cells. The ability to deal with these increased levels of MG may thus be an important characteristic of “successful” tumor cells. Supporting this reasoning are studies which show that malignant cells display higher activity and expression of glyoxalase I than nonmalignant proliferating and quiescent counterparts

(Thornalley 1995; Davidson et al. 2002). Moreover, high levels of glyoxalase I have been linked to apoptosis resistance in tumor cells (Tsuruo et al. 2003). Methylglyoxal itself has been studied as a potential anti-tumor agent, with varying degrees of success (Thornalley 1993). In attempts to circumvent this inconsistency, glyoxalase I inhibitors have been investigated as potential anti-tumor therapeutics (Thornalley et al. 1996; Tsuruo et al. 2003).

Presence and Implications in Batch Production

Several aspects of MG formation and reactivity make it prudent to consider the compound as a potentially detrimental to batch biopharmaceutical production by mammalian cells. First of these is hyperglycemic conditions in the batch. Culture medium is routinely supplemented with glucose levels of 20 mM and greater to support rapid proliferation. However, formation of methylglyoxal in diabetes has been linked to hyperglycemic conditions such as these (Vander Jagt et al. 2003). In addition, hyperglycemic conditions physiologically are cited as the main risk factors in the development of methylglyoxal-related diabetic complications (Baynes et al. 1999).

Characteristic metabolism of production cell lines may increase the probability of high level MG accumulation. Increased cellular metabolism is a defining factor of a transformed cell lines (Plas et al. 2002). Since methylglyoxal production is closely linked to glycolytic metabolism, transformed cell lines may be consistently exposed to consistently higher levels of MG than their physiological counterparts. Recent studies based on the aforementioned advanced analytical techniques have found methylglyoxal levels in CHO cultures orders of magnitude higher than previously detected in humans

or culture (Chaplen et al. 1998). The combination of hyperglycemic conditions, increased metabolism, and measurements indicate that MG may be accumulating to high levels in batch cultures of transformed cell lines.

High levels of MG could induce a variety of problems in batch cultures of production cell lines. High levels of MG have been shown to induce apoptosis, and could hinder survival in cell culture (Kang et al. 1996; Godbout et al. 2002; Liu et al. 2003). Chaplen has also shown that CHO cultures transfected with glyoxalase I detoxifying enzyme display greater colony-forming ability than wild-type cells. High levels of MG could indirectly produce oxidative stress. The detoxification of MG is dependent on glutathione, an essential component in detoxifying reactive oxygen species (ROS). Increased levels of MG could reduce the amount of glutathione available to maintain low ROS levels. In addition, MG may be particularly toxic to transformed cell lines. These lines are characterized by their ability to rapidly proliferate, to which MG has demonstrated a selective toxicity (Kang et al. 1996; Thornalley et al. 1996).

Documented MG Toxicity

The current work seeks to quantify the toxicity of methylglyoxal in hybridoma cultures. Several efforts have been made in other cell lines.
Exogenous methylglyoxal was shown to inhibit growth and induce toxicity in human leukemia (HL60) cells at median inhibitory concentrations of 238 μM and 363 μM, respectively (Ayoub et al. 1993). Further studies on this cell line indicate growth inhibition to be in the G1 phase and death to be from apoptosis (Kang et al. 1996).

Kang et al. also showed that the compound inhibited DNA synthesis, and that MG was selectively toxic in rapidly proliferating cells.

Other studies have attempted to clarify the pathway by which MG induces apoptosis. Liu et al. found that MG induced dose-dependent apoptosis and p38 mitogen activated protein kinase activation in rat mesangial cells. Apoptosis was found to be significant at MG concentrations greater than 100 μM for 16 hour incubation time (Liu et al. 2003). The kinase implicated in this study is commonly associated with various cell stresses and has been linked to hyperglycemia-induced apoptosis.

A study conducted on a fibrosarcoma cell line (Van Herreweghe et al. 2002) sought to further clarify MG's toxic mechanism by experimenting with tumor-necrosis factor (TNF), a pleiotropic cytokine. One effect of TNF addition to the cell line was accumulation of MG. This study showed that MG modification of proteins during this TNF-induced cell death is not random, but involves specific target molecules for MG. One advanced glycation endproduct was characterized as being produced from reaction of protein with MG. This endproduct was shown to be important to the TNF induced cell death.

MG has thus been established as an inducer of apoptosis in what may be a very specific pathway. The toxicity of MG in production cell lines is of interest as a potential cause of cellular growth inhibition and apoptosis in a bioreactor, but has only been minimally investigated in the context of CHO cells (Chaplen et al. 1998). There are reasons to believe, however, that MG may be a more significant factor in hybridoma cell lines. Both lines have similar metabolic characteristics, however, their growth and death profiles are much different. Cell death as a result of apoptosis in hybridoma culture has

been reported at approximately 90%, whereas necrotic cell death has been reported as the dominant mode in CHO cells (Singh et al. 1994). As shown previously and in the current work, methylglyoxal-induced death displays predominantly apoptotic characteristics, and thus is less likely to be a primary causal agent of cell death in CHO cells. Based on this, the current study will seek to quantify MG toxicity in hybridoma cells and compare these results to the toxicity of lactate, a more extensively studied batch toxin.

CHAPTER III

MATERIALS AND METHODS

Cell Culture

Non-adherent, murine hybridoma cells (ATCC CRL1606) that secrete an immunoglobulin (IgG) against human fibronectin were used in all of the described experiments. Cells were cultivated using a serum-free, hydrolysate-free Iscove's Modified Dulbecco's Medium (IMDM) formulation. The glutamine-free basal medium was supplemented with 4.0 mM glutamine, 10 mg/L insulin, 5 mg/L holo-transferrin, 2.44 μL/L 2-aminoethanol, 3.5μL/L 2-mercaptoethanol, and 10 U/mL penicillin-10 μg/mL streptomycin. For metabolic screens, the cells were cultivated in RPMI 1640 (Sigma) supplemented with 2 g/L sodium bicarbonate, 4.0 mM D-glucose, 10 mg/L insulin, 5 mg/L holo-transferrin, 2.44 mL/L 2-aminoethanol, 3.5 mL/L 2-mercaptoethanol, and 10 U/mL 10 U/mL penicillin-10 μg/mL streptomycin. During routine maintenance, cells were seeded at 2E5 viable cells/mL in T-Flasks in an incubator controlled at 37° C, 95% humidity, and 10% CO_2.

Test Chemicals

Methylglyoxal was obtained from Aldrich (#17,733-4) as 40% aqueous solution. Sodium pyruvate was obtained from Mediatech as 100mM aqueous solution (25-000-CI). L-Lactic Acid (#L6402) and Sodium Chloride (S-9625) were purchased from Sigma; one molar stock solutions of these compounds were mixed in water. All lactic acid

solutions were neutralized with sodium hydroxide before culture exposure to determine the effect of lactate apart from the pH change it imparts.

Six-Well Plate Exposures

Six-well plates (VWR #140685) were used to screen hybridoma cells to exogenous methylglyoxal, lactate, and sodium chloride exposure. Cells were grown in a T-175 flask to approximately 1E6 viable cells/mL. Cultures were centrifuged at 200g for ten minutes and resuspended in IMDM at 1E6 vcells/mL. Culture was seeded in well plates by diluting 1:2 (to ~5E5 vcells/mL) with exposure medium pre-heated to 37° C. Exposure medium was created directly before use through mixing of appropriate volumes of aqueous stock solutions in IMDM such that the exposure medium contained twice the exogenous concentration exposed to cells. Plates were seeded such that two wells were exposed to each toxin concentration. All lactate and sodium chloride exposures were seeded from a single culture, while methylglyoxal exposures were performed at a separate time. Well plates were incubated in 10% CO2 at 37° C for a given period of time and counted by trypan blue exclusion.

Endpoint Growth and Death

In order to determine the effect of each toxin on growth and induction of cell death, exposure period was set at 24 hours. Directly following the exposure period, density in each well was determined by trypan blue exclusion cell counts using a hemacytometer. These screens were executed for methylglyoxal, lactate, and sodium chloride toxin solutions.

Kinetic Cell Death

Separate exposures were performed to determine the kinetics of methylglyoxal-induced cell death. In this assay, exposure period was varied for each well. Two wells of each MG exposure concentration were counted at 2.67, 5.33, and 8 hours.

T-Flask Exposures

T-25 Flasks were used to screen for mode of cell death and the effect of gradual toxin addition.

Mode of Cell Death

Cultures were prepared and exposed to toxin-containing medium as in well-plate assays. Three flasks were prepared, one per toxin (methylglyoxal, lactate, sodium chloride). Selected toxin concentrations were known to cause significant cell death based on previously conducted well plate assays. Cultures were analyzed after 24 hours using an acridine orange- ethidium bromide dye mixture to determine relative amounts of apoptosis and necrosis (Mercille et al. 1994). Cells with three or more evident dots of condensed chromatin in the cell were deemed apoptotic.

Gradual Toxin Addition

In order to better understand the effect of acute dosage of methylglyoxal, MG was added gradually to culture in a T-Flask. Cultures were seeded in three T-25 Flasks at 5E5 vcells/mL. The first flask received a single, acute dose of methylglyoxal stock solution (final concentration 600 μM). The second received equal doses of

methylglyoxal every hour for the first six hours such that the final concentration of Methylglyoxal in the flask was 600 μM. The control flask received equivalent doses of water for the first six hours.

Metabolic Screens

Lactate and sodium chloride exposures were performed to determine their effect on glycolytic metabolism. Six-hour exposure and subsequent glucose assays were performed as described previously (Balcarcel et al. 2003). Cultures were centrifuged at 200g for 10 minutes and resuspended in RPMI test medium at approximately 3.5E6 viable cells/mL. Culture was seeded in 24-well plates by diluting 1:2 (to ~1.75E6 vcells/mL) with exposure medium pre-heated to 37° C. Two concurrent screens were used to test control and five toxin concentrations of sodium chloride and lactate, four wells were used for each concentration. Glucose concentration in each well was analyzed by enzymatic assay in 96-well plates. Initial concentrations were determined by medium samples taken prior to the screen containing appropriate toxin solutions but no culture.

Replicates

Ten total exposure assays were performed: 24-hour growth inhibition/induction of cell death for each toxin (3), kinetics of cell death determination for methylglyoxal (1), mode of cell death for each toxin (3), gradual addition of methylglyoxal (1), and metabolic screening for lactate and sodium chloride (2). Three replicates were produced for each assay except the metabolic screens, for which two replicates were

produced. Replicates were performed to minimize the effects of inoculum variation in the results and improve statistical significance.

Data Analysis

Calculations

Cell growth can be modeled by the following exponential function:

$$n_{T2} = n_{T1} e^{\mu \cdot \overline{f_v}(t_2 - t_1)} \quad (1)$$

In this equation n_{TX} represents total cell density at X = 1 (initial) and X=2 (final) time points. Average fraction viable, $\overline{f_V}$, is estimated by averaging initial and final viability. Viability is calculated from hemacytometer trypan blue exclusion counts:

$$f_v = \frac{n_V}{n_T} \quad (2)$$

where n_V and n_T represent viable and total cell density, respectively.

Based on equation 1, average growth rate over a given time interval can be calculated from the following:

$$\mu = \frac{\ln(n_{T1} / n_{T2})}{\overline{f_V}(t_2 - t_1)} \quad (3)$$

after n_{TX} and $\overline{f}_{V,}$ are determined by trypan blue exclusion. This calculation of growth rate assumes that only cells deemed viable by trypan blue exclusion can replicate.

Glucose consumption rate was calculated for metabolic screen data of lactate and sodium chloride. This rate is defined as the change in glucose concentration over the six hour screening period, $(t_2 - t_1)$.

$$q_{glc} = \frac{C_{glc} - C_{glc0}}{n_v(t_2 - t_1)} \quad (4)$$

In this equation, C_{glc} represents the concentration of glucose in the well plate.

Curve Fitting

Data from Cell Death/Growth Inhibition twenty-four hour assays were fitted to a sigmoidal model using Polymath software.

$$Y = \frac{X50^n}{X50^n + C_{ex}{}^n} \quad (5)$$

This fit was applied to viable cell density, growth, and viability data. In this equation, Y represents one of the aforementioned data sets given as a percentage of control, and is plotted against C_{ex}, toxin exposure concentration. The parameters in the equation, n (an empirical constant) and $X50$ (median effective dose) are fitted by Polymath. The labels IC50, LC50, and GI50 represent the median effective dose for viable density, viability, and growth response curves, respectively.

Statistical Analysis

Data from each experiment were analyzed by JMPIN software version 4.0.2. In all cases, mean data from each replicate was combined and analyzed as a conglomerate. Dunnett's method was used to compare sets of test points to control;

Tukey-Kramer HSD (Hamilton Standard Division) was used to compare individual pairs of data. All statistical significance was determined at the 95% confidence level.

CHAPTER IV

RESULTS

Exposure Endpoint Growth and Death

Three toxins (Methylglyoxal, Lactate, Sodium Chloride) were screened in twenty four hour exogenous exposures to hybridoma cells. Trypan blue exclusion cell counts following the twenty-four hour exposure allow for the construction of dose response curves of viability, growth, and cell density. Viable cell density is the preferred means of analysis since it is measured directly and holds no implicit assumptions.

Viable cell density profiles for lactate and sodium chloride appear very similar (Figures 4.1,2,3). Significant reduction in viable and total (not shown) density from control is observed at the 40 mM dose for lactate and the 60 mM dose for sodium chloride. Statistical differences in viable density for equivalent doses of lactate and sodium chloride is only seen at the 40 mM dose. Trypan blue viability profiles were also similar for lactate and sodium chloride (Figures 4.4,5,6). Significant reduction in viability at twenty four hours is seen for lactate doses greater than 80 mM and at the sodium chloride 100 mM dose. No significant difference in endpoint viabilities for the two toxins was observed at any dose at the 95% confidence level.

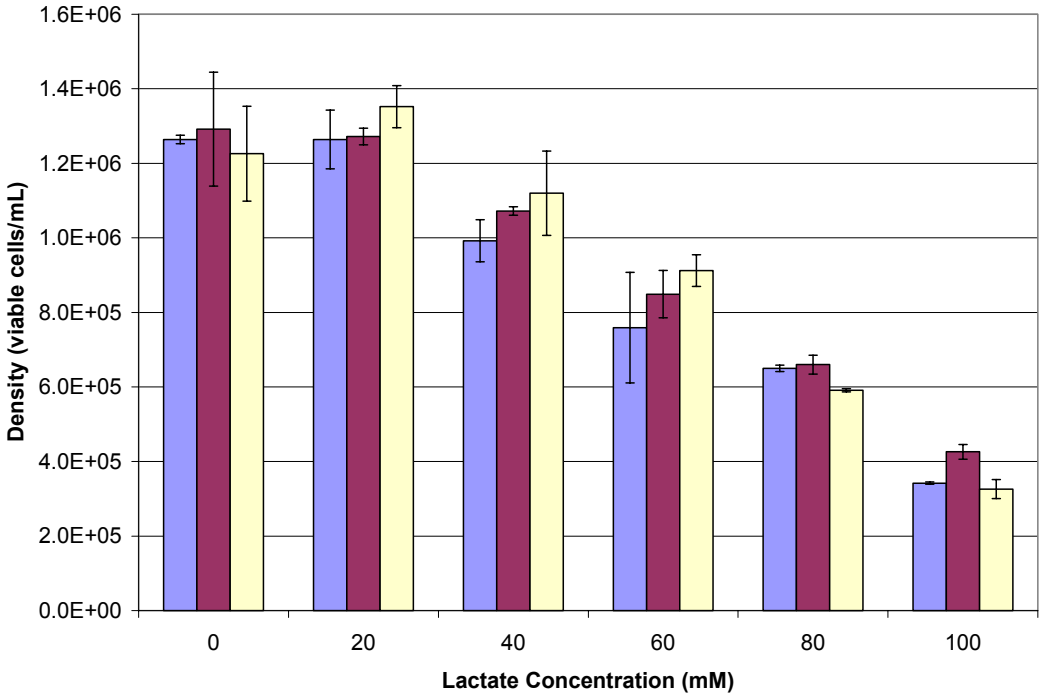

Figure 4.1 Viable cell density profile after twenty-four hour lactate exposure, three replicates.

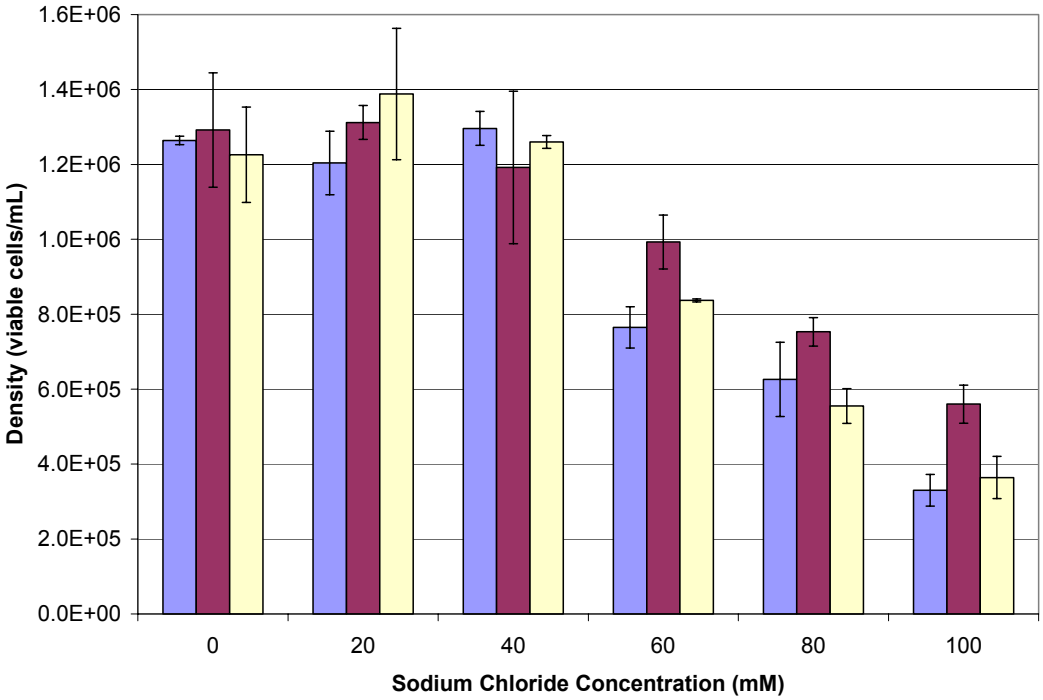

Figure 4.2 Viable cell density profile after twenty-four hour sodium chloride exposure, three replicates.

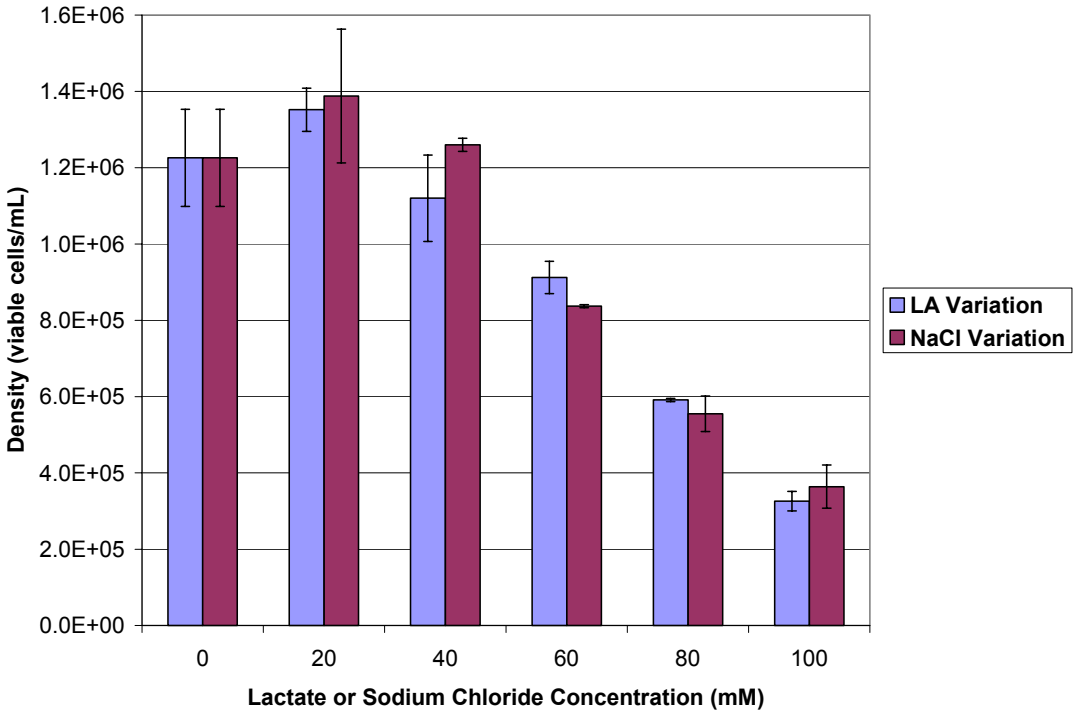

Figure 4.3 Viable cell density profile after twenty-four hour lactate and sodium chloride exposure, one replicate.

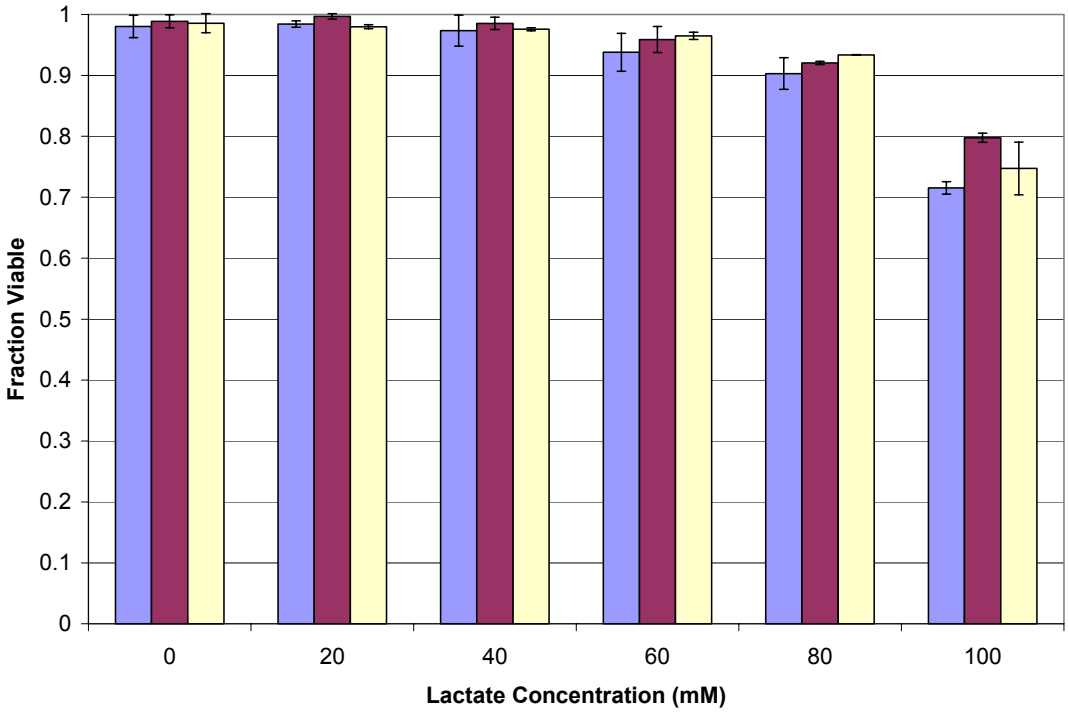

Figure 4.4 Viability profile after twenty-four hour lactate exposure, three replicates.

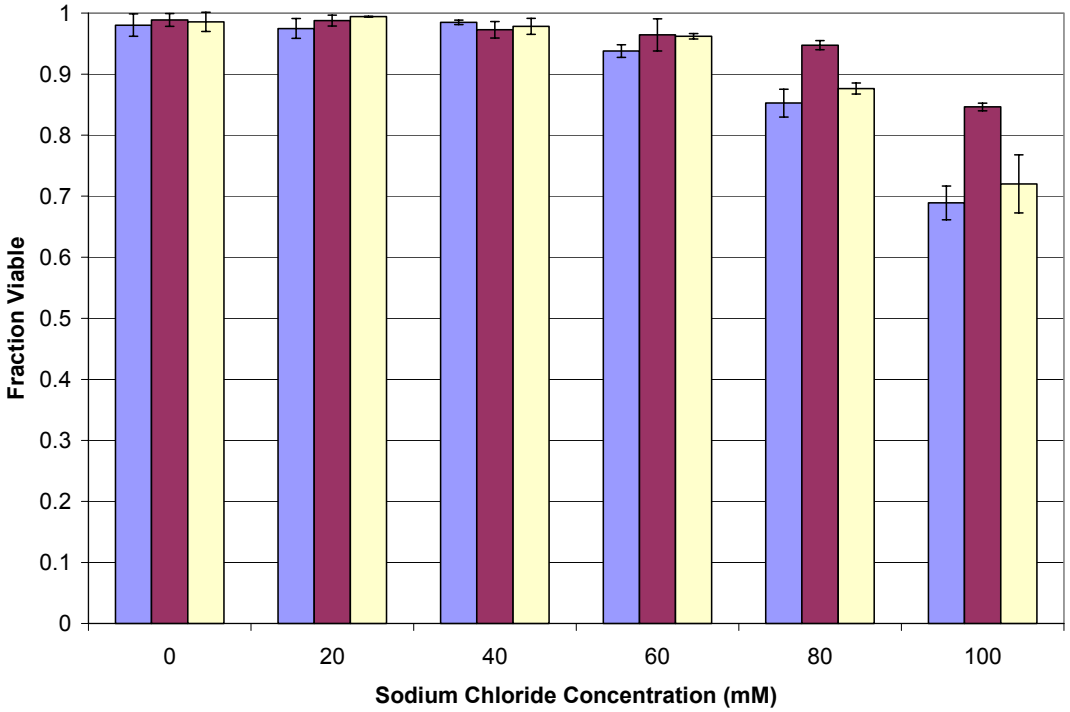

Figure 4.5 Viability profile after twenty-four hour sodium chloride exposure, three replicates.

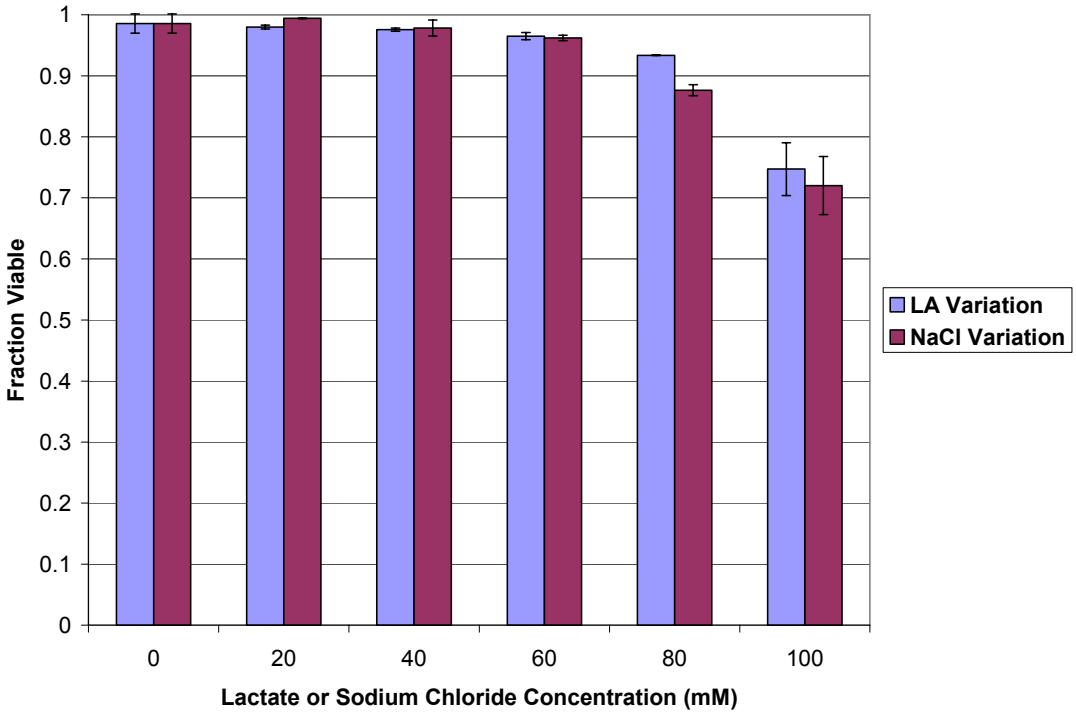

Figure 4.6 Viability profile after twenty-four hour lactate and sodium chloride exposure, one replicate.

Methylglyoxal screens produced large amounts of non-viable cells (Figures 4.7,8,9). Viable cell density was significantly reduced at a dose of 300 μM, and was below detection at doses of 800 μM and greater.

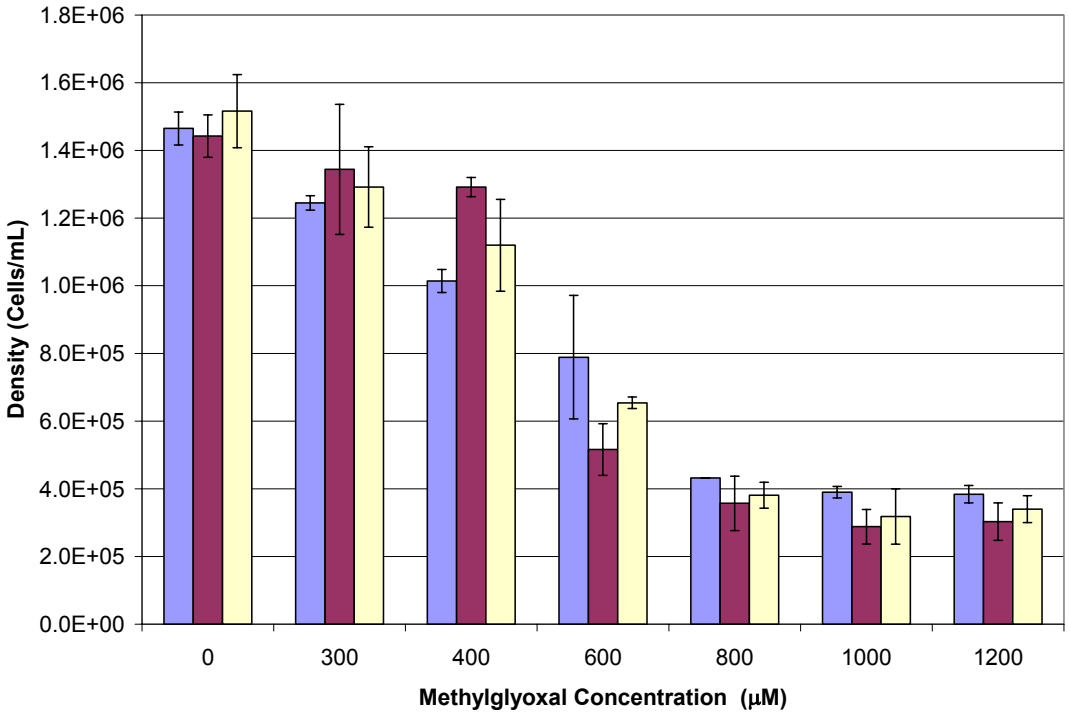

Figure 4.7 Total cell density profile after twenty-four hour methylglyoxal exposure, three replicates.

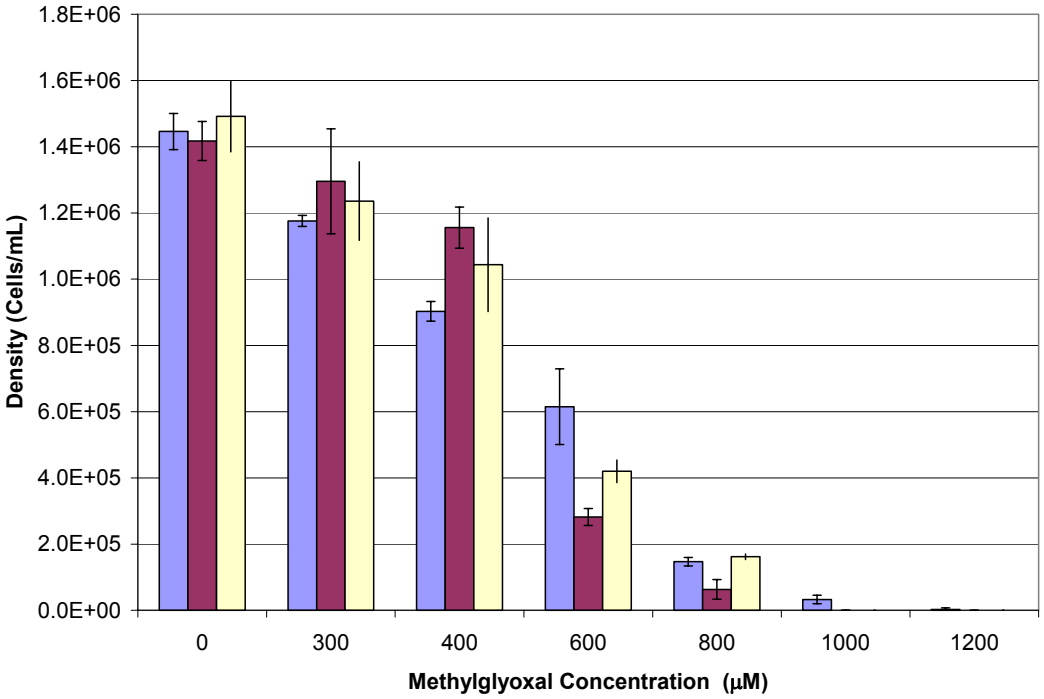

Figure 4.8 Viable cell density profile after twenty-four hour methylglyoxal exposure, three replicates.

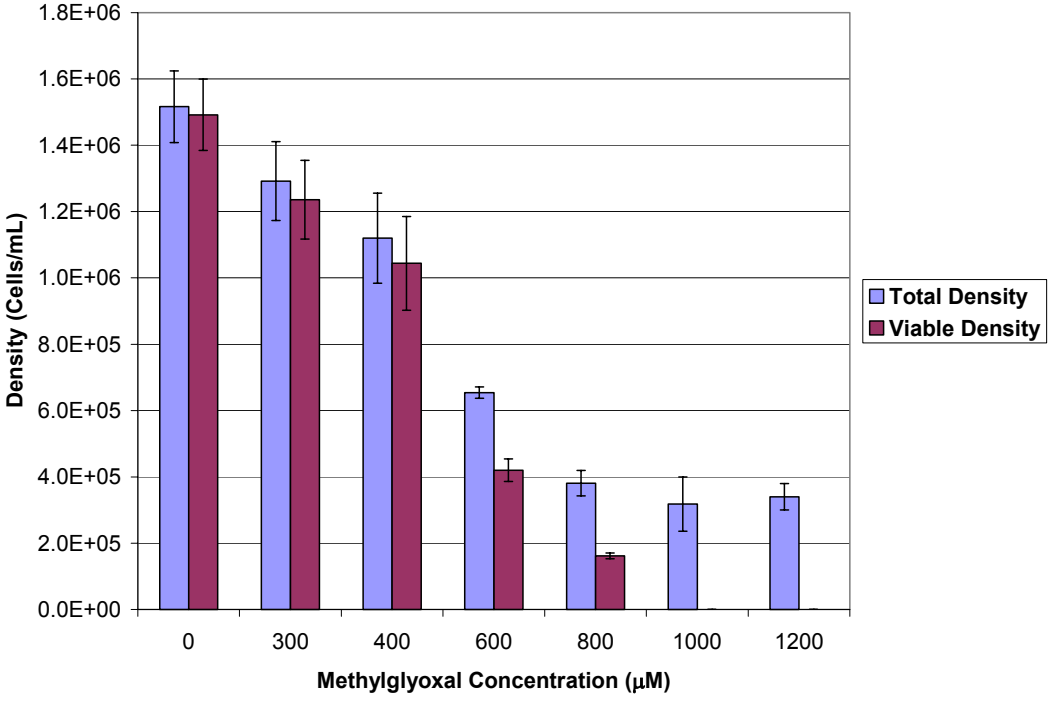

Figure 4.9 Total and viable cell density profile after twenty-four hour methylglyoxal exposure, one replicate.

Based on these dose response curves, sigmoidal curve fits were applied (Equation 5). IC50, LC50, and GI50, were determined as mean inhibitory concentrations of viable cell density, viability, and growth, respectively (Table 4.1; Figures 4.10,11). As is evident from smaller confidence intervals, viable cell density provides a more precise description of toxicity for this assay. Viable density profiles also allow for direct comparison of toxicity ranges between toxins (Figure 4.12).

Table 4.1. Median inhibitory concentrations (mM) for twenty four hour methylglyoxal, lactate, and sodium chloride exposures.

Toxin	IC50	LC50	GI50
Methylglyoxal	0.483 ± 0.016	0.674 ± 0.119	-
MG (Ayoub et al.)	0.238 ± 0.002	0.363 ± 0.008	-
Lactate	77.6 ± 5.3	-	70.6 ± 5.6
Lactate (Cruz et al.)	-	-	37
Sodium Chloride	81.2 ± 10.7	-	75.8 ± 12.3

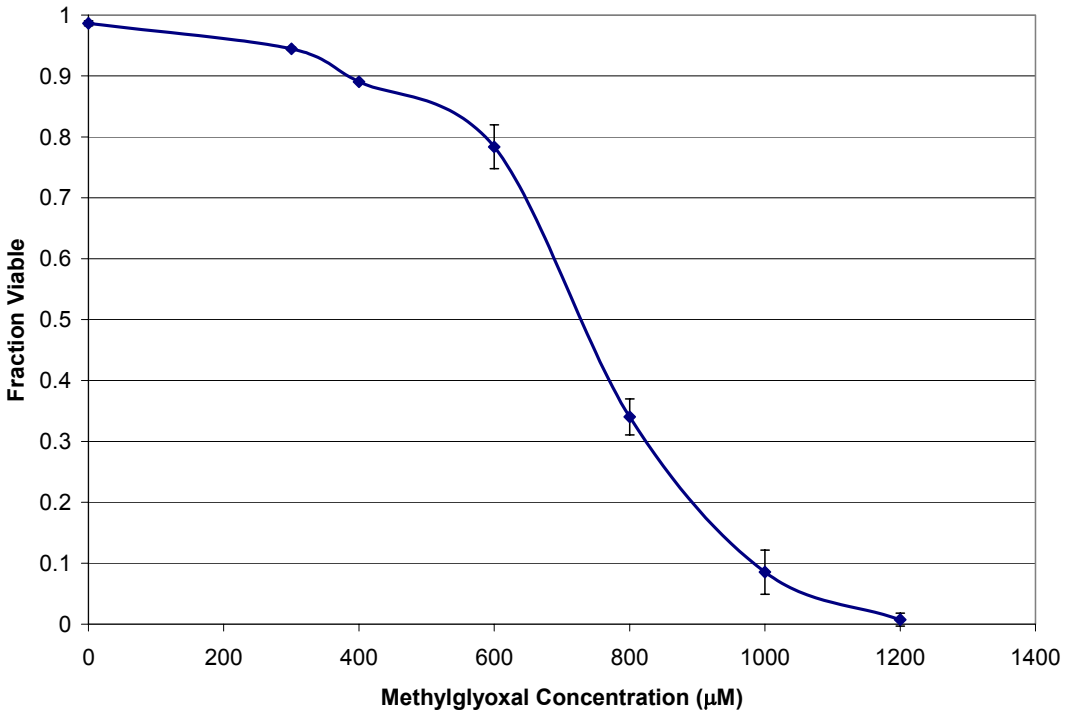

Figure 4.10 Viability profile after twenty-four hour methylglyoxal exposure, one replicate.

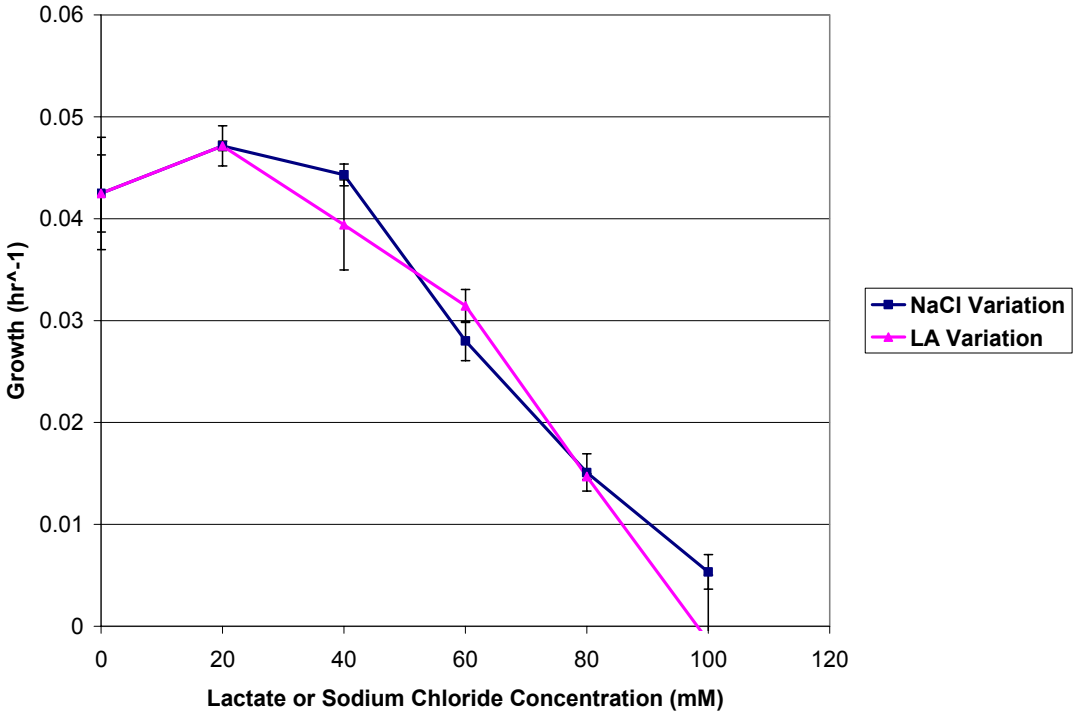

Figure 4.11 Growth profile after twenty-four hour lactate and sodium chloride exposure, one replicate.

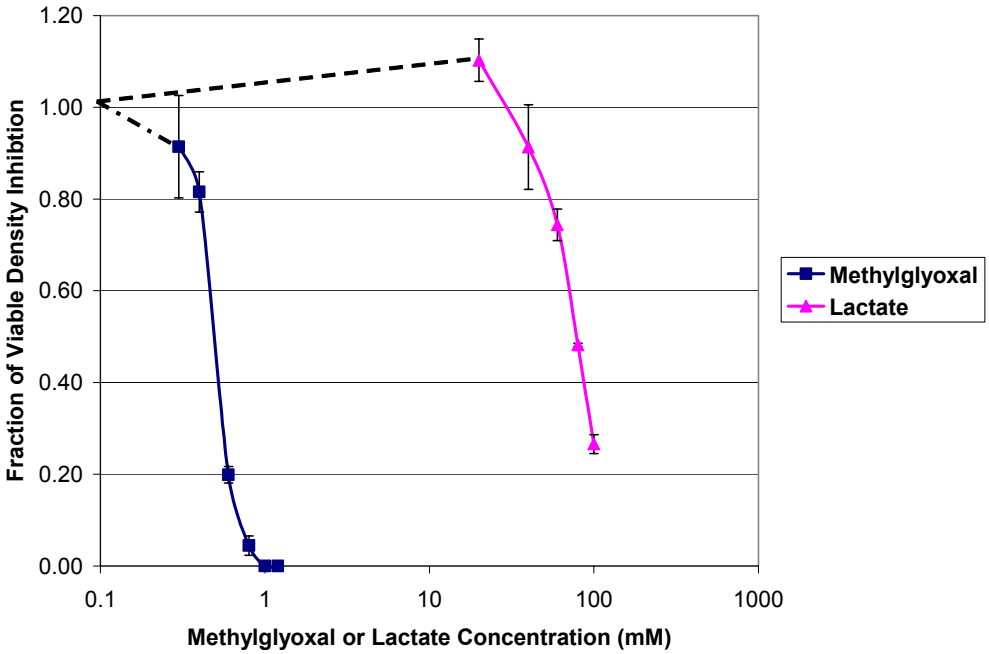

Figure 4.12 Viable density inhibition after twenty four hour lactate and methylglyoxal exposure, one replicate.

Mode of Cell Death

To determine the mode of death induced by each toxin, twenty four hour exposures were conducted in T-Flasks at concentrations shown to cause significant death. Results (Table 4.2), indicate that methylglyoxal induced a higher percentage of apoptosis than did the other toxins. The difference between lactate and sodium chloride was not significantly different at the 95% confidence level.

Table 4.2. Percent apoptotic among non-viable cells after twenty-four hour methylglyoxal, lactate, and sodium chloride exposures.

Toxin	Concentration	% Apoptotic
Methylglyoxal	600 μM	81 ± 7
Lactate	80 mM	37 ± 11
Sodium Chloride	80 mM	49 ± 7

Kinetics of Methylglyoxal-Induced Death

Well plate screens were used to determine the rate at which death was occurring after exogenous exposure for methylglyoxal. Viable density profiles versus time analyzed by Dunnett's method show that for each test concentration, significant death had occurred by 5.33 hours after exposure (Figure 4.13). Also noteworthy, significant amounts of debris were observed in the culture during later counts, most likely due to significant cell lysis, which would have affected the calculation of growth rate in 24 hour endpoint screens.

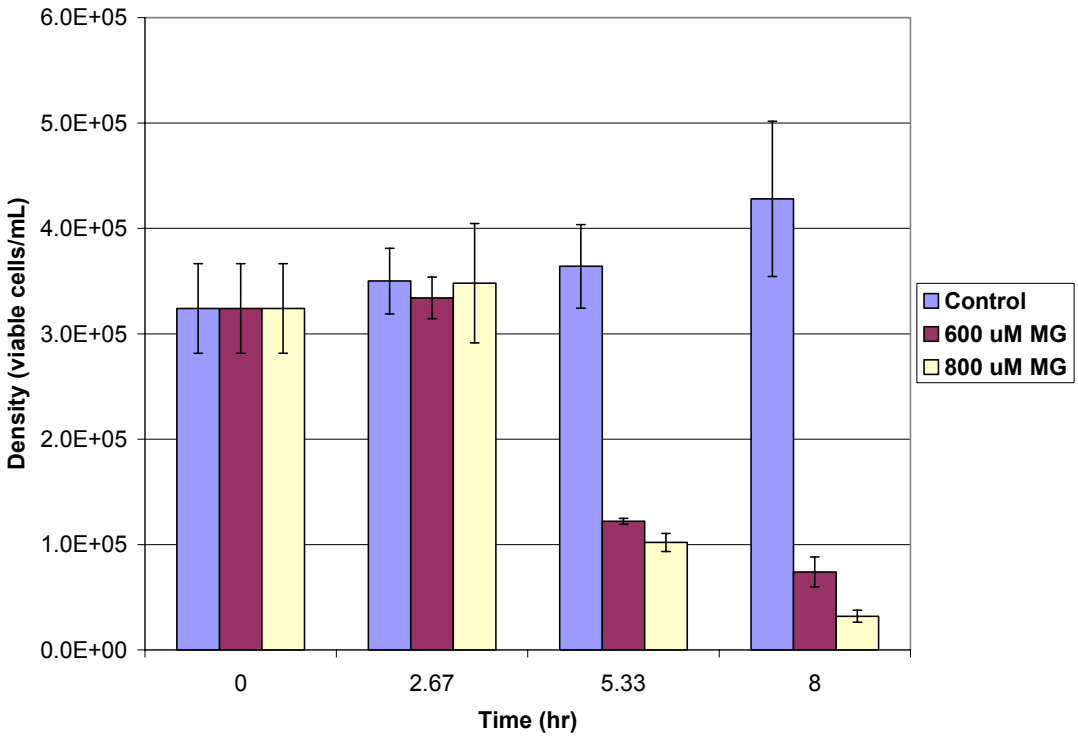

Figure 4.13 Kinetic viable cell density profile for methylglyoxal exposure, one replicate.

Gradual Addition of Methylglyoxal

Methylglyoxal was added gradually during the first six hours of culture to determine the sensitivity of the culture to the rate of accumulation of the toxin. Viability results from gradual addition were significantly different from acute dosage and control (Figure 4.14). In addition, the reduction in density from control was much more pronounced than that of viability for the gradual dosage case.

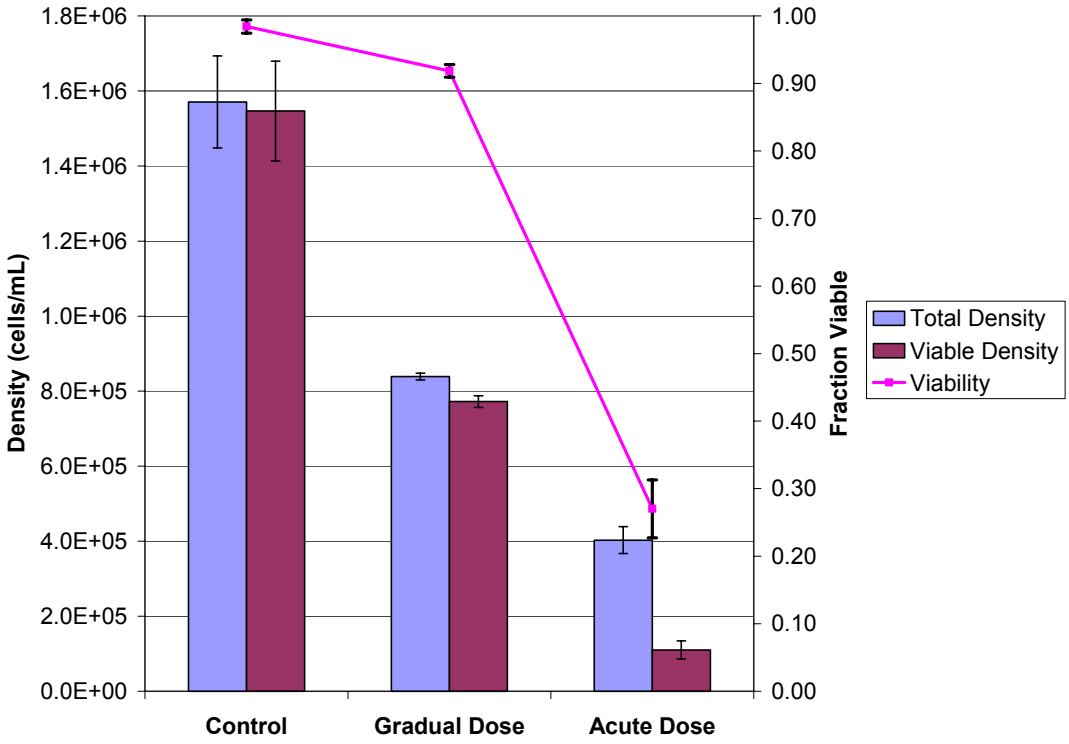

Figure 4.14 Density and viability profile after twenty-four hour exposure to methylglyoxal with varying dosage types.

Metabolic Screens

To analyze the toxicity profiles of lactate and sodium chloride, six hour exposures were conducted and glucose uptake rates measured. Both lactate and sodium chloride inhibited glycolysis (Figures 4.15,16,17). Inhibition was significant for concentrations of

lactate 40 mM and above, 80 mM and above for sodium chloride. In comparing equivalent doses, all rates for lactate doses were significantly lower than for corresponding sodium chloride doses.

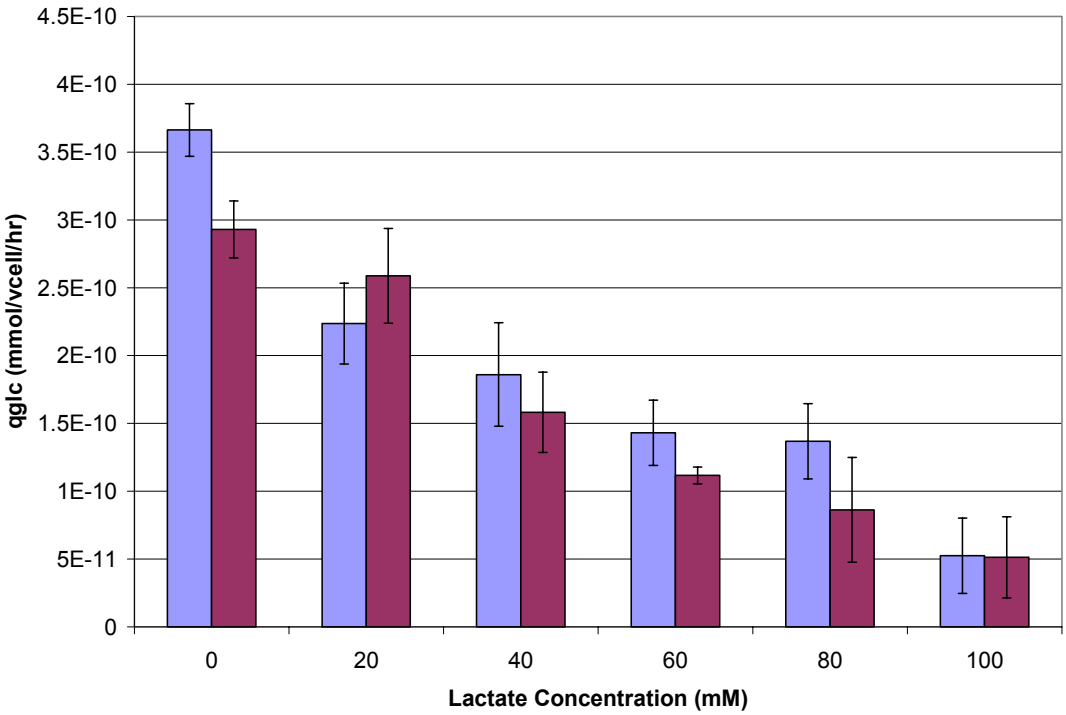

Figure 4.15 Glucose uptake rate during six-hour lactate exposures, two replicates.

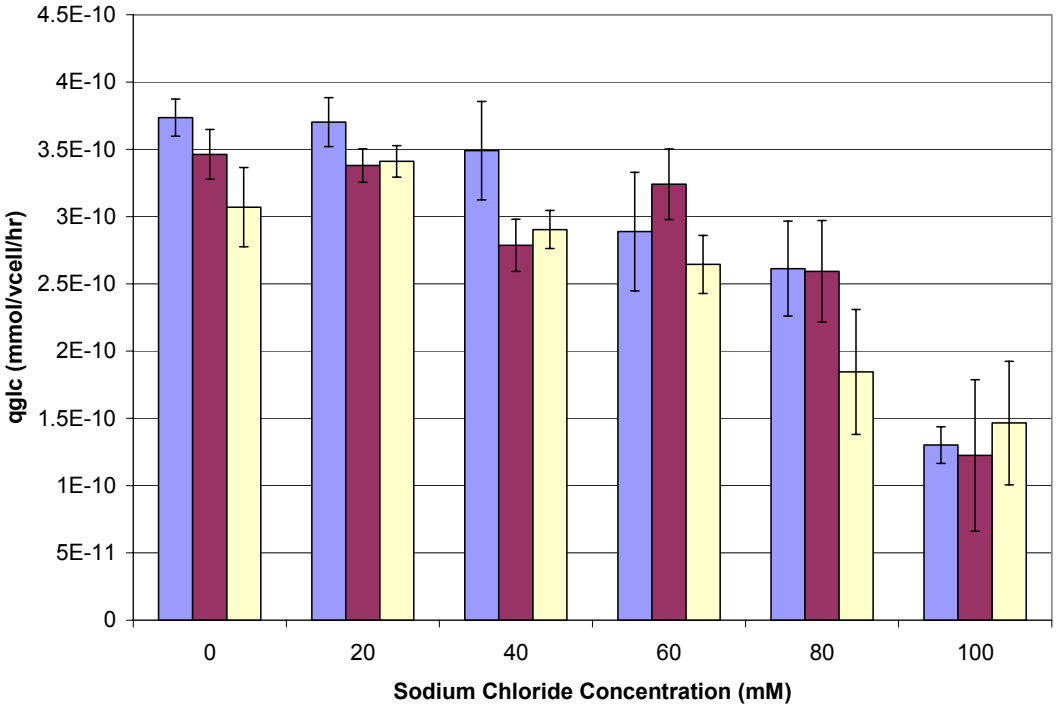

Figure 4.16 Glucose uptake rate during six-hour sodium chloride exposures, three replicates.

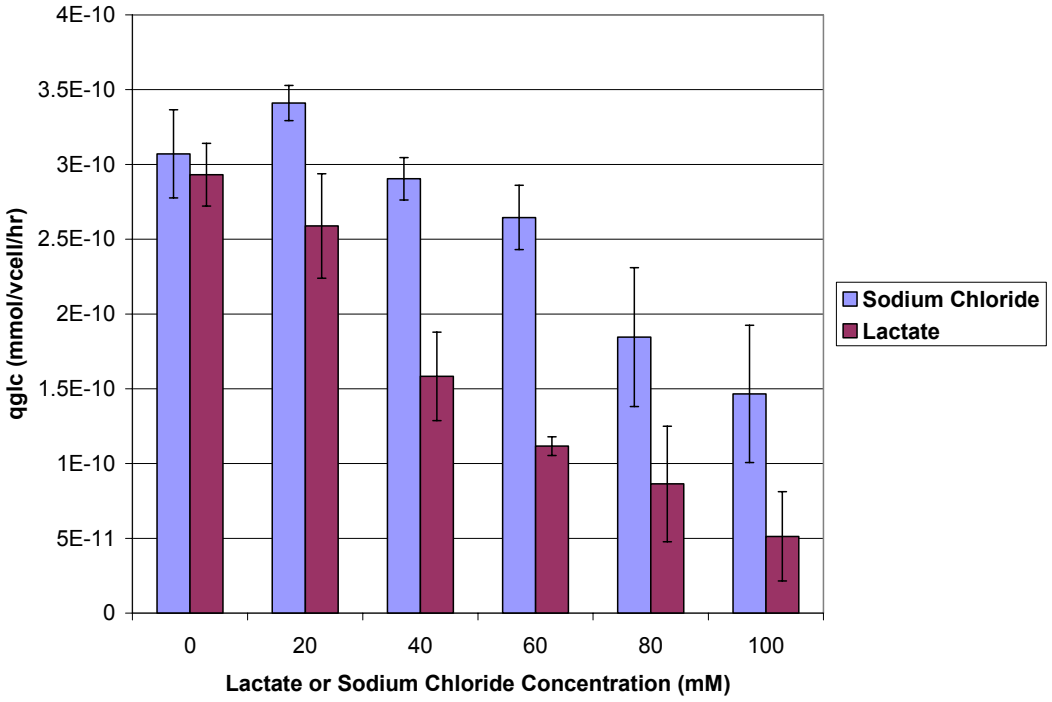

Figure 4.17 Glucose uptake rate during six-hour lactate and sodium chloride exposures, one replicate.

CHAPTER V

DISCUSSION

Experimental Design Shortcomings

The object of the 24 hour growth and viability screens was to quantify methylglyoxal and lactate levels that are detrimental to hybridoma cells. These screens are not without shortcomings in this regard. Toxic mechanisms are likely to depend on intracellular concentrations, whereas the toxicity here is quantified based on exogenous concentration. In the case of lactate, these concentrations have been shown to differ (Schmid et al. 1992). Although MG has been reported as freely permeable, some gradients are likely to exist for that compound as well. Attempts will be made in the analyzing the relevance of these toxins to account for these discrepancies.

Another shortcoming of these screens is the single, large dosing mechanism of toxin exposure. In a batch setting, these metabolic toxins are likely to undergo a more gradual increase. Miller has demonstrated that gradual doses of lactate are less toxic than large, acute doses (Miller et al. 1988). The current work demonstrates that the same is true for methylglyoxal (Figure 4.14); a single, acute dose is more toxic than the same dose applied gradually over a six-hour time span. In the case of methylglyoxal, this implies that cells are better able to detoxify the toxin in small doses or can amplify detoxification pathways after the initial dosing.

The conducted screens also assume that the concentration of toxin in the medium remains constant over the exposure period. At an average density of 5E5

viable cells/ mL and average cell size of 1800 cubic microns (spherical cells at approximately 15 μM in diameter), the volume of cells in culture is only about one thousandth of the total volume. Thus, even if cells "consume" MG via detoxification several times over, the bulk concentration is unlikely to be significantly affected. However, MG has shown itself to be very reactive with various macromolecules, including amino acids, proteins, and DNA. The reaction of methylglyoxal with bovine serum albumin, specifically, has been well documented (Lo et al. 1994). In addition, methylglyoxal has shown significant reactivity with the free amino acids arginine, lysine, and cysteine. Exposure medium for the screens (IMDM) contains the proteins albumin and insulin, as welll as large amounts of free amino acids. The concentration of free methylglyoxal in the medium can thus be expected to decrease as methylglyoxal becomes increasingly associated with these molecules as well as any secreted by the cells. Although gradients, dosage type, and reactivity effects are difficult to estimate they are likely to change the toxicity results presented herein.

Comparison to Previous Work

The results described here show consistency with some previous studies, and inconsistency with others. Liu et al. found that MG induces apoptosis at concentrations greater than 0.100 mM (Liu et al. 2003). Ayoub et al. demonstrated an IC50 of 0.238 mM (Ayoub et al. 1993). These values are significantly lower than demonstrated here, albeit for different cell lines. The toxicity of lactate here is similar to that shown by Lao, although sodium chloride results seem to differ (Lao et al. 1997). Lactate results are in contradiction with those of BHK cells, where GI50 was shown to be 37 mM, vs. the 70.6

mM shown here (Cruz et al. 2000). In addition, clear inhibition of glycolysis is shown here for both lactate and sodium chloride, which was not seen in the work of Cruz et al.

Relative Toxicity of Methylglyoxal and Lactate

Exposure screens of twenty four hours were conducted to compare the toxicity of methylglyoxal, a molecule that has recently been suggested as a dangerous toxin in production cell lines (Chaplen 1998), with lactate, a toxin that has been researched extensively and targeted as a waste product to be minimized in industrial production. It should be noted that lactate was screened in neutral form. Lactate buildup in a bioreactor which is not pH controlled is likely to be far more toxic. The toxins can best be compared by IC50, the median inhibitory concentration of viable cell density over the twenty-four hour period. The IC50 of methylglyoxal (0.483 $\pm$ 16 mM) was approximately two orders of magnitude lower than that of lactate (77.6 $\pm$ 5.3 mM) (Figure 4.12).

An alternative means of comparison, LC50 of methylglyoxal and GI50 of lactate, are based on viability and growth rate calculations that carry implicit assumptions, which may be incorrect to varying degrees. Growth rate (Equation 3), is calculated using an average viability over the period in question, and viability makes the assumption of no lysis during the exposure period. During methylglyoxal screens, significant debris was observed in exposed cultures, casting doubt on the validity of this “no lysis” assumption. Despite their shortcomings, however, apparent LC50 and GI50 results can provide insight into the respective mode of toxicity for each toxin, and are commonly used parameters (Figures 4.10, 11). A comparison of these parameters also reveals

approximately two orders of magnitude difference (0.674 ± .119 mM LC50 vs, 70.6 ± 5.6 mM GI50).

Batch Levels of Toxins

In order to evaluate the relevance of these toxins in a batch, batch levels of the toxins must be approximated. Although methylglyoxal has been shown to be orders of magnitude more toxic than lactate, a more complete analysis requires comparing actual batch levels of the toxins to toxicity results produced here.

Lactate

Lactate production has been well-characterized in batch cultures of production cell lines (Ozturk et al. 1991). Commonly, this is quantified by measuring exogenous levels during the batch. Lindsey Clark, also of the Balcarcel lab at Vanderbilt monitored exogenous levels of lactate over the course of 80 hours during batch culture of CRL1606 in a T175 flask (Figure 5.1). This data indicates that batch levels can reach approximately 18 mM during the course of the batch, and are greater than 12 mM after approximately 35 hours.

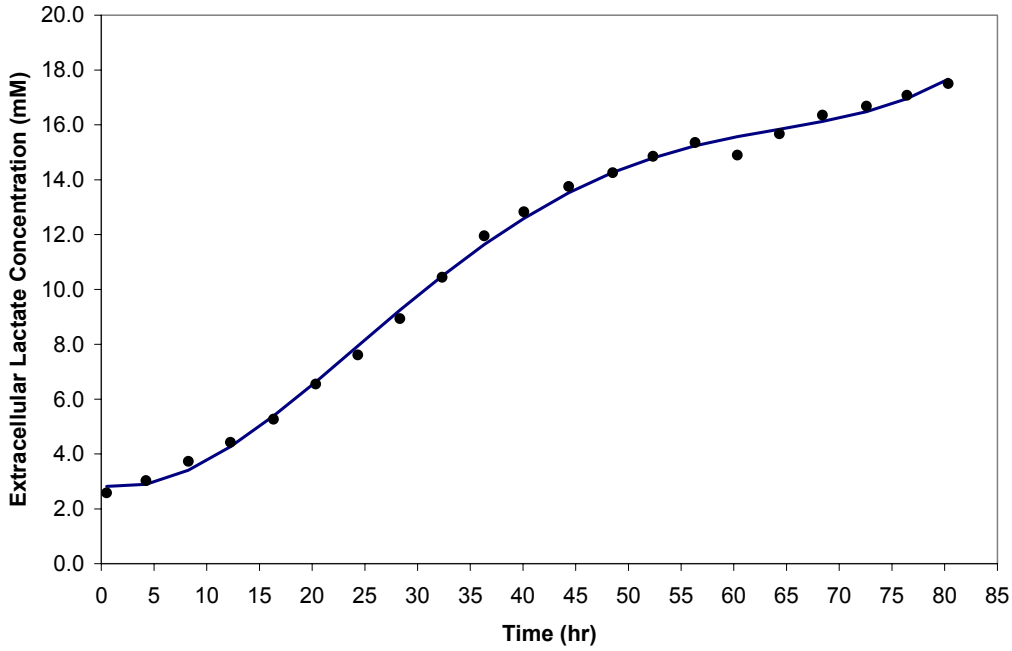

Figure 5.1 Lactate concentration in extracellular medium during batch hybridoma culture in T-175 flask (source: Lindsey Clark, personal communication).

These numbers are significantly below the threshold of lactate toxicity demonstrated here. However, toxicity of the compound more than likely depends on intracellular rather than the extracellular concentrations reported here. These intracellular concentrations are likely to vary depending on direction of toxin flux. In normal batch production, intracellular concentration can be expected to be greater, as the concentration driving force causes lactate to be exported and accumulate extracellularly. In the case of an extracellular dose of lactate, however, one can expect the opposite to be true. Schmid et al. has described this relationship between intracellular and extracellular lactate in hybridoma cells (Schmid et al. 1992).

Lactate in the current study caused significant growth inhibition at exogenous concentration of 40 mM, but not at 20 mM. Shmid et al. demonstrated intracellular

lactate concentrations of approximately 20% less than exogenous exposure concentrations. Applying the same relationship here, it is reasonable to assume that intracellular concentrations of below approximately 25-30 mM are not significant enough to induce growth inhibition. Shmid et al. similarly demonstrated an intracellular lactate concentration approximately 60% higher than extracellularly. Applying this relationship, it is not likely that intracellular lactate would reach 25 mM until at least 40 hours into a batch. According to a batch profile, growth appears to have significantly decreased from its peak by 40 hours (Figure 5.2). Lactate may reach levels significant enough to inhibit growth, is not the primary culprit in batch growth inhibition.

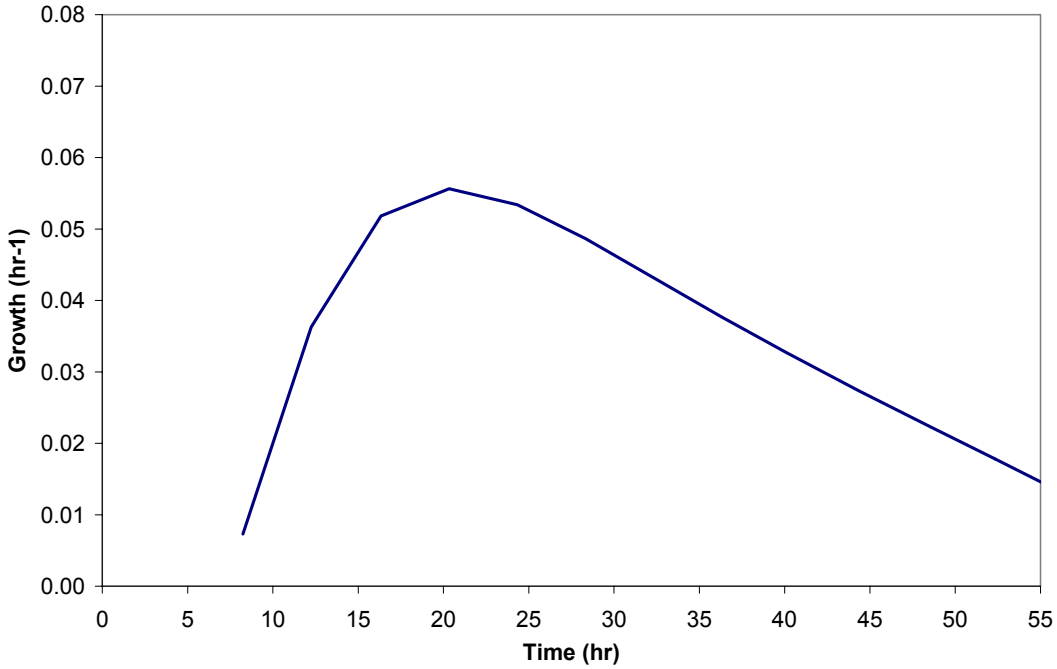

Figure 5. 2 Growth rate of hybridoma batch culture in T-175 flask (Source: Lindsey Clark, personal communication).

Methylglyoxal

Methylglyoxal levels in batch culture are more difficult to quantify, which makes study of the toxin extremely difficult. Current assays have detected levels as high as 300 μM in CHO cells (Chaplen et al. 1998), two to three orders of magnitude greater than previously detected. Our knowledge of MG metabolism makes these measurements easier to accept. The glycolysis-based formation of the compound makes glycolytically driven transformed cell lines susceptible to high levels of this toxin. Hyperglycemic conditions, as found in typical batch medium, may also drive increased formation of MG.

Mathematical Model

Even sophisticated measurement techniques may fall short quantifying intracellular MG. Thus, a model of intracellular concentrations based on batch hybridoma data and reported information about MG production may be useful in understanding the toxin. Hybridoma data in the model is taken from 80-hour batch analysis of growth and metabolism courtesy of a personal communication from Lindsey Clark. This data can be used to construct a model of methylglyoxal based on its material balance.

$$\frac{dMG}{dt} = \nu_F - \nu_D - q_{MG} - MG(\mu + \frac{1}{\overline{V_c}}\frac{d\overline{V_c}}{dt}) \qquad (5)$$

Toxicity of the compound is likely dependent on the intracellular concentration of the compound, thus concentration terms are defined in moles of methylglyoxal per liter of cytosol (M). Terms in the equation include rate of formation, ν_F (M/hr), rate of detoxification, v_D (M/hr), flux out of the cell q_{MG}, (M/hr), growth, μ (hr^{-1}), and average

cell volume, V_c (L/viable cell). In this model, production of the methylglyoxal is represented solely by ν_F. However, the compound levels are lowered by several means. These include detoxification pathways, flux into the surrounding medium, dilution by cell growth, and dilution by changing cell size. The latter two terms acknowledge the highly variable nature of growth and cell size during the batch and attempt to account for changes in intracellular concentration metabolites that may result from these phenomena.

Rate of Formation

Glycolytic flux is likely to be the major source of methylglyoxal, and thus will be the only one considered in this model. The toxin has been reported to form at a rate proportional (approximately 0.1-0.4%) to that of glycolysis; the current model will adopt the more conservative 0.1% estimate. Glycolytic flux is approximated by per cell glucose uptake rates in batch culture. These rates are fitted to a second degree polynomial as a function of time.

Detoxification

The primary detoxification mechanism of MG is the glyoxalase system, which consists of two enzymes, glyoxalase I and II, and glutathione as a cofactor. In the current model, only detoxification by glyoxalase I will be considered, i.e. the products of the GLOI-catalyzed reaction will be considered non-toxic. IN addition, the reaction of glutathione with MG will be considered to be rapid, and not limited by the concentration of glutathione. Thus, detoxification will be approximated by Michaelis- Menten kinetics (Equation 6).

$$v_D = \frac{v_{\max} * MG}{k_m + MG} \quad (6)$$

In this equation, parameter k_m will be approximated as 125 μM, the reported value for human red blood cells (Thornalley 1993). The other parameter $v_{\max}$ is an unknown that is related to the amount and activity of glyoxalase I enzyme present in the cells by the following:

$$v_{\max} = k_{cat}[E_T] \quad (7)$$

where $[E_T]$ is the total concentration of enzyme in the cell and k_{cat} is the catalytic constant of the enzyme. It is difficult to estimate glyoxalase amount, and as such, several cases for $v_{\max}$ will be considered over an appropriate range to fit existing data. Over the course of a single simulation, $v_{\max}$ will be assumed to be constant (that is to say the activity and amount of the enzyme do not change over the course of the batch).

Flux

Flux out of the cell, q_{MG}, is another questionable parameter, which must be approximated. Methylglyoxal has been traditionally reported as being completely permeable as a small polar compound (Thornalley 1996), but several factors complicate this approximation. That MG is produced intracellularly makes it likely that intracellular concentration would be higher. In addition, the reactivity of the compound increases the probability that it will bind to a compound before leaving the cell. Because of MG's tendency to associate with macromolecules (Lo 1994), this model will assume that only MG that has recently been formed is likely to exit the cell. Thus, flux will be defined as a fraction of the Methylglyoxal formation rate. The value of this fraction largely

determines the mean Methylglyoxal concentration in the batch, and will be set at 0.85 in the base case.

$$q_{MG} = .85 * v_F \tag{8}$$

Growth

Growth Rate μ for the model is defined according to equation 9.

$$\mu = \frac{1}{n_v}\frac{dn_v}{dt} \tag{9}$$

Viable cell density is fitted to a polynomial based on batch data. This is a more accurate definition of growth than is presented in equation 3, which is based on multiple measurements of viable cell density instead of simply final and initial measurements during a 24-hour screen. Viable cell density data was fitted to a 3rd order, time dependent polynomial.

Cell Volume

Specific cell volume will be approximated as a time-dependent polynomial. Mean viable cell volume was extracted from dynamic AFP-27 hybridoma flow cytometry data and fitted to a 5th degree polynomial (Hu 2003).

Simulation

Base-case simulation results can be seen in Figure 5.3. A few aspects of the plot are noteworthy. First, the profile of MG shows an exponential increase in concentration at approximately 60 hours in the batch. Based on the results of kinetic death screens (Figure 4-13), MG can be expected to induce death shortly (~4-5 hours) after an acute dose. The onset of significant cell death typically occurs around 60 hours in batch hybridoma cultures. A marked increase of MG at approximately 60 hours is

thus a potential cause of cell death in batch cultures. The cause of the MG spike at this time is related to growth inhibition and shrinking cell size. In the model, dilution by growth is a major factor in keeping MG concentration constant.

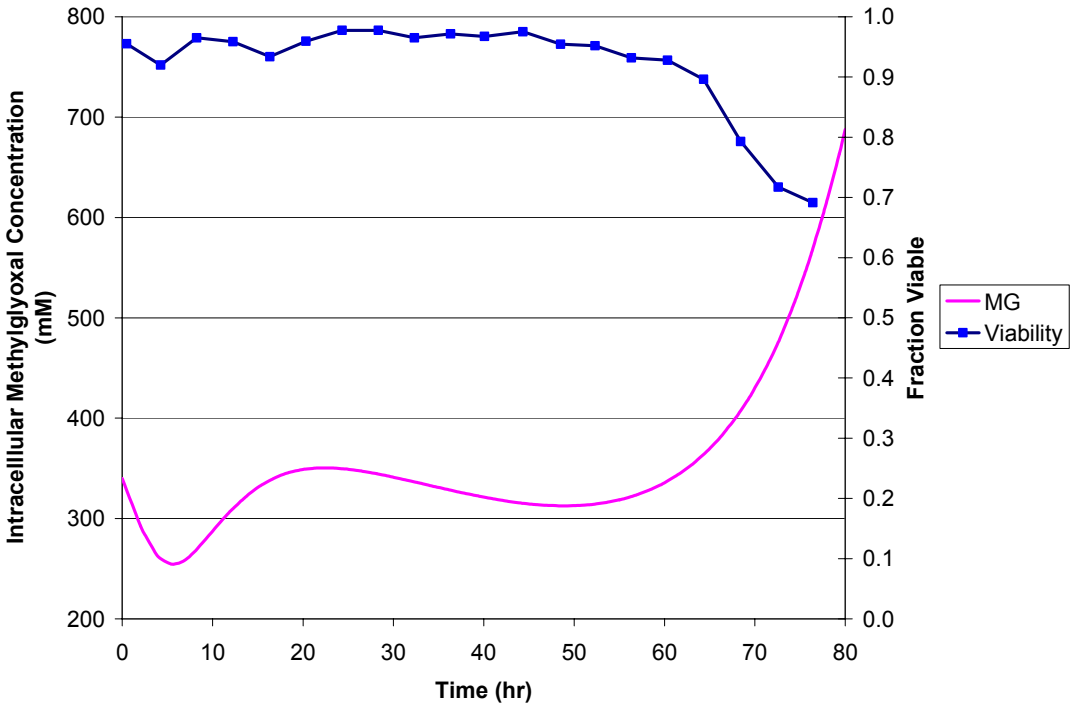

Figure 5.3 Hybridoma viability data (Source: Lindsey Clark, personal communication) and simulated intracellular methylglyoxal concentration during batch hybridoma culture.

The simulation case shown in Figure 5.3 is also cyclic. The regular maintenance of cells, or passaging, consists of reseeding the culture in fresh medium. Hybridoma cells are typically passaged at 24 or 48 hours; this cycle can be ongoing for months, indicating that minimal toxic effects are experienced. The model predicts a relatively constant intracellular MG concentration from approximately 20 to 50 hours at values which, according to the present study, are only marginally toxic. The cylic nature of the profile is dependent on a slight decrease in intracellular MG during the initial hours of

the culture, followed by a gradual rise back to the “steady-state” value by approximately 20 hours. This early recovery is due glycolytic rates which start relatively low, such that more MG is detoxified than is produced.

Implications of the Analysis

The base case model implies that MG accumulation could be a primary factor in the onset of cell death in a batch reactor. Validation of the model through more accurate predictions of $v_{\max}$ and q_{MG} in conjunction with time-dependent MG measurements would result in MG being considered a significant target in the effort to improve batch efficiency. Less significantly, the analysis implies that cells in culture are continually exposed to significant levels of MG. Bound MG, though not acutely toxic, may cause a gradual deterioration of function as proteins and other significant molecules alters these levels.

Sensitivity Analysis

Several parameters were chosen for the base case scenario to demonstrate the possibility of MG accumulating and inducing apoptosis late in a batch. Values chosen were $v_{\max}$ = 14 M/hr, and q_{MG} = 0.85 * vf. The value of q_{MG} largely determines the mean, or “steady-state” methylglyoxal concentration in the batch. This parameter was chosen to fit the simulation to intracellular CHO data. A lower fraction of synthesized MG that leaves the cell corresponds to a greater amount of MG to accumulate or be detoxified by the cell. A change in q_{MG} (and subsequently $v_{\max}$), thus shifts the base-case profile along the y-axis. The dynamic profile of MG is largely dependent on this $v_{\max}$ (Figure 5.4). This parameter is dependent on the amount of enzyme present in the cells.

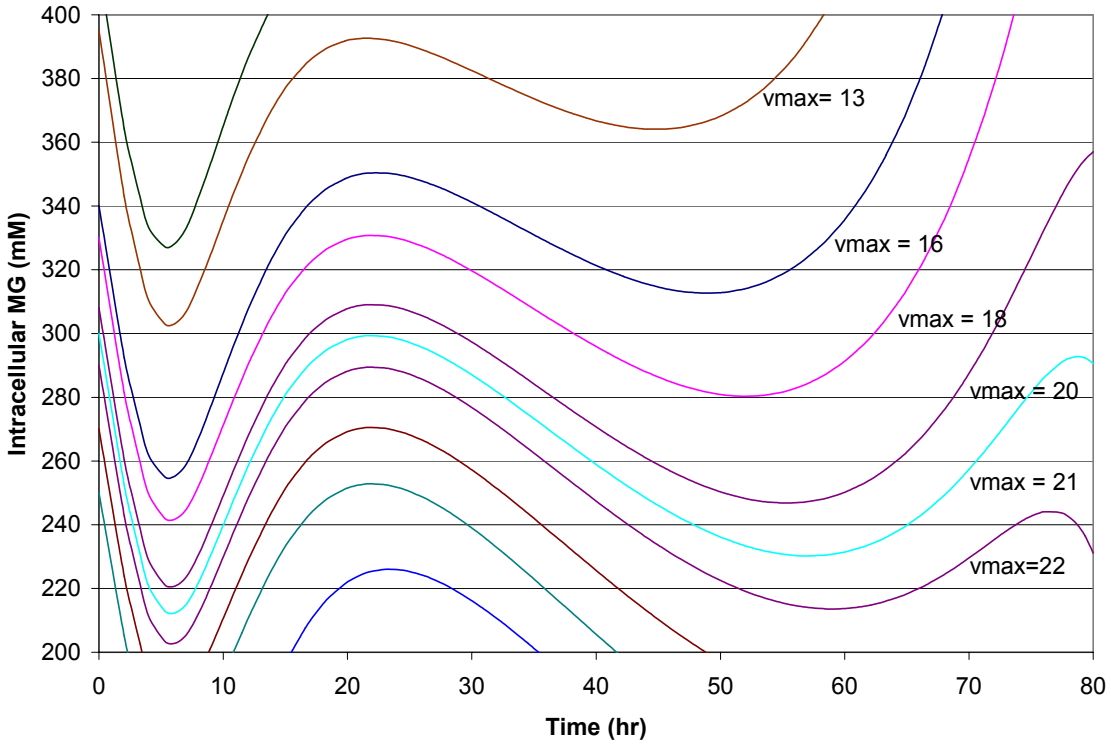

Figure 5.4 Simulated intracellular methylglyoxal concentration in batch sensitivity to vmax.

Validity Analysis

Methylglyoxal has been detected in significant amounts in a Chinese hamster ovary cell line (Chaplen et al. 1998). This is an indication that the glyoxalase pathway is insufficient in that line. Given that all transformed cell lines demonstrate considerably higher metabolic rates than their physiological counterparts, this may point to a larger trend in production cell lines. However, insufficient glyoxalase levels do not dictate the base case profile demonstrated here. Variations in enzyme amount (reflected by changes in $v_{\max}$) could result in significantly altered transient MG profiles. Figure 5-4 demonstrates that a slightly higher value of $v_{\max}$ ($v_{\max}$~21) produces a culture in which significant MG is present, but does fairly constant and thus not a direct cause of late-

batch apoptosis. Enzyme levels in the glyoxalase system are also assumed to be constant over the course of the batch. Transient variations in enzyme expression or activity are not considered.

Other model irregularities could also have a significant impact on the simulation. Formation of MG has been assumed occur as a constant fraction of glycolysis. This could change significantly during the highly variable metabolism seen in hybridoma cultures. Likewise, flux of MG out of the cell may be a variable during a batch.

Mode of Toxicity

Lactate

Lactate is widely regarded as a growth inhibiting agent in cell culture (Lao et al. 1997); this is verified in the current study at greater concentrations than have been previously screened. The current study has attempted to clarify the metabolic inhibition and osmolarity factors in this growth inhibition. In order to clarify the role of osmolarity, sodium chloride screens were performed in parallel to lactate growth and metabolism screens.

The current work verifies the proposed glycolytic inhibition of lactate by demonstrating a clear inhibition of glucose uptake rates by exogenous lactate. Sodium chloride screens, however, produced a similar growth inhibition without corresponding inhibition of glycolysis (Figure 4.17). Glucose uptake rates for sodium chloride screens were significantly higher than those of lactate for all concentrations tested, and were not significantly reduced until exogenous concentrations of 80 mM. Density profiles,

however, are very similar for the two compounds at all concentrations tested. Only the 40 mM concentration point showed significant different between lactate and sodium chloride viable density profiles. This suggests that osmolarity is a significant factor in lactate toxicity. The hypothesis that growth inhibition is a result of decreased glycolysis from lactate inhibition may be true, but this profile seems to demonstrate that osmolarity effects are significant as well, particularly at higher concentrations.

Methylglyoxal

Unlike lactate, methylglyoxal acts primarily through induction of death by apoptosis; its effect on growth is not clear. Acridine orange/ ethidium bromide staining showed that a majority of non-viable cells were apoptotic following MG exposure. The question of whether growth is inhibited during MG exposure is unclear. At an exogenous exposure concentration of 300 μM in a 24-hour screen, viable cell density was significantly reduced, but total cell density was not. This suggests that perhaps cell death was inflicted without a corresponding growth inhibition. Kinetic death screens show that significant cell death occurs in approximately five hours. Lysis of apoptotic cells following this rapid death makes actual growth difficult to quantify. Because of this uncertainty, no GI50 was assigned to MG. (Cell lysis may also inflate the apparent LC50 of MG. As a result of this, viable density information and the corresponding IC50 is used to quantify toxicity.) Growth inhibition did seem to as a result of gradual dosage. Culture viability remained high after 24-hour exposure to gradual dose, but viable density was significantly reduced.

Batch Relevance

The significant question to be answered by the conducted screens is whether either toxin is relevant during a batch production using hybridoma cells. Neutralized lactate is not likely to significantly induce cell death in a batch based on 24 hour screens. Analysis of growth inhibition effects requires slightly more speculation on the importance of intracellular versus extracellular concentrations. However, the results seem to indicate that lactate does not accumulate in amounts capable of restricting growth until late in the batch. Combined, these results seem to indicate that lactate is not a primary causal agent of growth inhibition or induction of apoptosis in a batch.

Methylglyoxal is difficult to measure in a batch. A model presented here reproduces high levels that have been detected in Chinese hamster ovary cells and predicts that MG may accumulate to toxic levels in late in batch production. In addition, the toxic screens performed here have shown that MG rapidly induces apoptosis, as is seen in a batch culture at approximately 60 hours. Based on the model and toxin profile, several MG may be responsible for batch apoptosis. Further evidence is needed to determine whether methylglyoxal actually accumulates as predicted by the model.

CHAPTER VI

CONCLUSIONS

Results Summary

Production of protein therapeutics by genetically altered cell lines is an area which promises to become increasingly important in the future. Even small advances in the efficiency of this production could result in significant financial benefit to a pharmaceutical company and a consumer. The current work focuses on examining the toxicity of several naturally occurring toxins that may have detrimental effects on this production. Exposures of 24 hours demonstrated that methylglyoxal (IC50= 0.483 mM) is toxic at approximately one one-hundredth the dose of neutralized lactic acid (IC50= 77.6 mM). Lactate was shown to be primarily toxic through growth inhibition (GI50 = 70.6 mM), while methylglyoxal was shown to significantly induce apoptosis (LC50= 0.674 mM). The effect of osmolarity in lactate toxicity was also examined, and results suggest that it may be significant over the range of concentrations tested, and is dominant at higher lactate concentrations. Toxicity results for lactate correspond well to previous work, but MG toxicity is higher than previously reported.

The current work also attempts to qualitatively describe the effects of these toxins in batch pharmaceutical production. Batch levels of lactate indicate that this toxin is not primarily responsible for growth inhibition in batch cultures. Methylglyoxal rapidly induced apoptosis, which is consistent with hybridoma batches. Methylglyoxal concentration was simulated using known data about the rate of formation as well as

batch hybridoma growth and metabolism information. The result depicts a rapid accumulation of MG late in the batch as hybridoma growth is inhibited and average cell size decreases. At the very least this model presents a scenario by which growth inhibition and decreases in mean cell size over the course of a batch could induce rapid accumulation of a toxin.

Future Work

Much of the suggested future work in this area centers on further analysis of methylglyoxal toxicity. The model for MG accumulation should be tested through several means. Validation of the model would require sophisticated measurements of intracellular concentrations over time. Improvements to the model itself could be made by measurements of glyoxalase I activity or amount over time, to more accurately portray the detoxification term.

Assuming that MG proves to be present in significant amounts (as has been shown in CHO cells) several measures could be taken to minimize its presence. The first of these is genetic modification of the cell line. Several enzymes (glyoxalase I, glyoxalase II, betaine dehydrogenase, aldose reductase, 2-oxoaldehyde dehydrogenase) are instrumental in detoxifying methylglyoxal in vivo (Vander Jagt et al. 2003) through separate pathways. Upregulating the activities of these enzymes may prove useful in maintaining low intracellular methylglyoxal levels. The potential for this technique is shown in CHO cells transfected with glyoxalase one, which have shown an increase in colony-forming ability. In addition, it may be useful to upregulate glyceraldehyde-3-phosphate dehydrogenase (G-3PDH), the glycolytic enzyme which

catalyzes the conversion of MG's triose phosphate precursor GAP to 1,3-Biphosphoglycerate.

In addition to genetic engineering, several simpler strategies could be used to minimize MG accumulation. Decreasing flux through the glycolytic pathway via a feeding strategy could decrease the amount of MG produced by the culture. Minimizing glycolytic flux or shifting metabolism towards other pathways may successfully accomplish this. Successful feeding strategies based on low glucose levels have been reported. These strategies could be duplicated along with MG measurements to determine their effectiveness in minimizing MG.

A third possible strategy for minimizing intracellular MG is the use of potential MG scavengers. Several compounds have been analyzed in the treatment of diabetes, in which MG has been linked to the development of various complications. Aminoguanidine, pyridoxamine, thiamine pyrophosphate, and metformin have all been proposed as scavengers of the compound. Addition of these compounds in a fed-batch setting may serve to detoxify methylglyoxal as it is produced, thus minimizing its batch accumulation.

In addition to direct prevention of MG buildup, it may be possible to prevent MG buildup through alteration of other batch characteristics. The base-case model depicts a scenario in which MG accumulates late in a batch through inhibition of cell growth and decreasing cell size at that time. Preventing these changes would delay the predicted spike in MG concentration.

Strategies for minimizing MG accumulation may be useful even if MG is found not to be the primary causal agent in the induction of apoptosis in a batch culture.

Indications of high levels of MG in cell lines have been reported (Chaplen et al. 1998). Consistently high levels of MG could gradually modify cellular components, exerting chronic effects on the culture. In this regard, the most efficient strategies mentioned above may be useful in consistently treating cell lines.

REFERENCES

Al-Rubeai, M., Singh, R. P. (1998). "Apoptosis in cell culture." Current Opinion in Biotechnology **9**(2): 152-156.

Altamirano, C., J. J. Cairo, et al. (2001). "Decoupling cell growth and product formation in Chinese hamster ovary cells through metabolic control." Biotechnology and Bioengineering **76**(4): 351-360.

Ayoub, F. M., R. E. Allen, et al. (1993). "Inhibition of Proliferation of Human Leukemia 60-Cells by Methylglyoxal Invitro." Leukemia Research **17**(5): 397-401.

Balcarcel, R. R. and L. M. Clark (2003). "Metabolic screening of mammalian cell cultures using well-plates." Biotechnology Progress **19**(1): 98-108.

Balcarcel, R. R. and G. Stephanopoulos (2001). "Rapamycin reduces hybridoma cell death and enhances monoclonal antibody production." Biotechnology and Bioengineering **76**(1): 1-10.

Baynes, J. W. and S. R. Thorpe (1999). "Role of oxidative stress in diabetic complications - A new perspective on an old paradigm." Diabetes **48**(1): 1-9.

Beisswenger, P. J., S. K. Howell, et al. (2003). "alpha-oxoaldehyde metabolism and diabetic complications." Biochemical Society Transactions **31**: 1358-1363.

Bonarius, H. P. J., A. Ozemre, et al. (2001). "Metabolic-flux analysis of continuously cultured hybridoma cells using (CO2)-C-13 mass spectrometry in combination with C-13-lactate nuclear magnetic resonance spectroscopy and metabolite balancing." Biotechnology and Bioengineering **74**(6): 528-538.

Brekke, O. H. and G. A. Loset (2003). "New technologies in therapeutic antibody development." Current Opinion in Pharmacology **3**(5): 544-550.

Bushell, M. E., S. L. Bell, et al. (1994). "Enhancement of Monoclonal-Antibody Yield by Hybridoma Fed-Batch Culture, Resulting in Extended Maintenance of Viable Cell-Population." Biotechnology and Bioengineering **44**(9): 1099-1106.

Chaplen, F. W. R. (1998). "Incidence and potential implications of the toxic metabolite methylglyoxal in cell culture: A review." Cytotechnology **26**(3): 173-183.

Chaplen, F. W. R., W. E. Fahl, et al. (1996). "Detection of methylglyoxal as a degradation product of DNA and nucleic acid components treated with strong acid." Analytical Biochemistry **236**(2): 262-269.

Chaplen, F. W. R., W. E. Fahl, et al. (1996). "Method for determination of free intracellular and extracellular methylglyoxal in animal cells grown in culture." Analytical Biochemistry **238**(2): 171-178.

Chaplen, F. W. R., W. E. Fahl, et al. (1998). "Evidence of high levels of methylglyoxal in cultured Chinese hamster ovary cells." Proceedings of the National Academy of Sciences of the United States of America **95**(10): 5533-5538.

Chen, K. Q., Q. Liu, et al. (2001). "Engineering of a mammalian cell line for reduction of lactate formation and high monoclonal antibody production." Biotechnology and Bioengineering **72**(1): 55-61.

Cruz, H. J., C. M. Freitas, et al. (2000). "Effects of ammonia and lactate on growth, metabolism, and productivity of BHK cells." Enzyme and Microbial Technology **27**(1-2): 43-52.

Cruz, H. J., J. L. Moreira, et al. (2000). "Metabolically optimised BHK cell fed-batch cultures." Journal of Biotechnology **80**(2): 109-118.

Davidson, S. D., D. M. Milanesa, et al. (2002). "A possible regulatory role of glyoxalase I in cell viability of human prostate cancer." Urological Research **30**(2): 116-121.

Degenhardt, T. P., S. R. Thorpe, et al. (1998). "Chemical modification of proteins by methylglyoxal." Cellular and Molecular Biology **44**(7): 1139-1145.

Doverskog, M., J. Ljunggren, et al. (1997). "Physiology of cultured animal cells." Journal of Biotechnology **59**(1-2): 103-115.

Dowd, J. E., K. E. Kwok, et al. (2000). "Increased t-PA yields using ultrafiltration of an inhibitory product from CHO fed-batch culture." Biotechnology Progress **16**(5): 786-794.

Duval, D., C. Demangel, et al. (1992). "Role of Metabolic Waste Products in the Control of Cell-Proliferation and Antibody-Production by Mouse Hybridoma Cells." Hybridoma **11**(3): 311-322.

Europa, A. F., A. Gambhir, et al. (2000). "Multiple steady states with distinct cellular metabolism in continuous culture of mammalian cells." Biotechnology and Bioengineering **67**(1): 25-34.

Fassnacht, D., S. Rossing, et al. (1998). "Effect of Bcl-2 expression on hybridoma cell growth in serum- supplemented, protein-free and diluted media." Cytotechnology **26**(3): 219-225.

Frahm, B., P. Lane, et al. (2003). "Improvement of a mammalian cell culture process by adaptive, model-based dialysis fed-batch cultivation and suppression of apoptosis." Bioprocess and Biosystems Engineering **26**(1): 1-10.

Gambhir, A., A. F. Europa, et al. (1999). "Alteration of cellular metabolism by consecutive fed-batch cultures of mammalian cells." Journal of Bioscience and Bioengineering **87**(6): 805-810.

Godbout, J. P., J. Pesavento, et al. (2002). "Methylglyoxal enhances cisplatin-induced cytotoxicity by activating protein kinase C delta." Journal of Biological Chemistry **277**(4): 2554-2561.

Godia, F. and J. J. Cairo (2002). "Metabolic engineering of animal cells." Bioprocess and Biosystems Engineering **24**(5): 289-298.

Hassell, T., S. Gleave, et al. (1991). "Growth-Inhibition in Animal-Cell Culture - the Effect of Lactate and Ammonia." Applied Biochemistry and Biotechnology **30**(1): 29-41.

Hu, Wei Shu (2003). Stoichiometry and Kinetics of Cell Growth and Product Formation: Cell and Tissue Reactor Engineering. University of Minnesota. http://hugroup.cems.umn.edu/CTRE/cd-rom/Stoichiometry%20and%20Cell%20Kinetics/Stoichiometry%20and%20Cell%20Kinetics.pdf

Irani, N., M. Wirth, et al. (1999). "Improvement of the primary metabolism of cell cultures by introducing a new cytoplasmic pyruvate carboxylase reaction." Biotechnology and Bioengineering **66**(4): 238-246.

Jang, J. D. and J. P. Barford (2000). "Effect of feed rate on growth rate and antibody production in the fed-batch culture of murine hybridoma cells." Cytotechnology **32**(3): 229-242.

Kalapos, M. P. (1999). "Methylglyoxal in living organisms - Chemistry, biochemistry, toxicology and biological implications." Toxicology Letters **110**(3): 145-175.

Kang, Y. B., L. G. Edwards, et al. (1996). "Effect of methylglyoxal on human leukaemia 60 cell growth: Modification of DNA, G(1) growth arrest and induction of apoptosis." Leukemia Research **20**(5): 397-405.

Kilhovd, B. K., I. Giardino, et al. (2003). "Increased serum levels of the specific AGE-compound methylglyoxal-derived hydroimidazolone in patients with type 2 diabetes." Metabolism-Clinical and Experimental **52**(2): 163-167.

Kromenaker, S. J. and F. Srienc (1994). "Effect of Lactic-Acid on the Kinetics of Growth and Antibody-Production in a Murine Hybridoma - Secretion Patterns During the Cell-Cycle." Journal of Biotechnology **34**(1): 13-34.

Lao, M. S. and D. Toth (1997). "Effects of ammonium and lactate on growth and metabolism of a recombinant Chinese hamster ovary cell culture." Biotechnology Progress **13**(5): 688-691.

Lapolla, A., R. Flamini, et al. (2003). "Glyoxal and methylglyoxal levels in diabetic patients: Quantitative determination by a new GC/MS method." Clinical Chemistry and Laboratory Medicine **41**(9): 1166-1173.

Lin, J. Q., M. Takagi, et al. (2002). "Possible strategy for on-line monitoring and control of hybridoma cell culture." Biochemical Engineering Journal **11**(2-3): 205-209.

Liu, B. F., S. Miyata, et al. (2003). "Methylglyoxal induces apoptosis through activation of p38 mitogen-activated protein kinase in rat mesangial cells." Kidney International **63**(3): 947-957.

Lo, T. W. C., M. E. Westwood, et al. (1994). "Binding and Modification of Proteins by Methylglyoxal under Physiological Conditions - a Kinetic and Mechanistic Study with N-Alpha-Acetylarginine, N-Alpha-Acetylcysteine, and N-Alpha-Acetyllysine, and Bovine Serum-Albumin." Journal of Biological Chemistry **269**(51): 32299-32305.

Ludemann, I., R. Portner, et al. (1994). "Effect of Nh3 on the Cell-Growth of a Hybridoma Cell-Line." Cytotechnology **14**(1): 11-20.

Martins, A., C. A. A. Cordeiro, et al. (2001). "In situ analysis of methylglyoxal metabolism in Saccharomyces cerevisiae." Febs Letters **499**(1-2): 41-44.

Mastrangelo, A. J., J. M. Hardwick, et al. (2000). "Part II. Overexpression of bcl-2 family members enhances survival of mammalian cells in response to various culture insults." Biotechnology and Bioengineering **67**(5): 555-564.

Mastrangelo, A. J., S. F. Zou, et al. (1999). "Antiapoptosis chemicals prolong productive lifetimes of mammalian cells upon Sindbis virus vector infection." Biotechnology and Bioengineering **65**(3): 298-305.

McLellan, A. C., S. A. Phillips, et al. (1992). "The Assay of Methylglyoxal in Biological-Systems by Derivatization with 1,2-Diamino-4,5-Dimethoxybenzene." Analytical Biochemistry **206**(1): 17-23.

Mercille, S. and B. Massie (1994). "Induction of Apoptosis in Nutrient-Deprived Cultures of Hybridoma and Myeloma Cells." Biotechnology and Bioengineering **44**(9): 1140-1154.

Miller, W. M., C. R. Wilke, et al. (1988). "Transient Responses of Hybridoma Cells to Lactate and Ammonia Pulse and Step Changes in Continuous Culture." Bioprocess Engineering **3**(3): 113-122.

Newland, M., M. N. Kamal, et al. (1994). "Ammonia Inhibition Hybridomas Propagated in Batch, Fed-Batch, and Continuous-Culture." Biotechnology and Bioengineering **43**(5): 434-438.

Omasa, T., K. Higashiyama, et al. (1992). "Effects of Lactate Concentration on Hybridoma Culture in Lactate-Controlled Fed-Batch Operation." Biotechnology and Bioengineering **39**(5): 556-564.

Ozturk, S. S. and B. O. Palsson (1991). "Growth, Metabolic, and Antibody-Production Kinetics of Hybridoma Cell-Culture .1. Analysis of Data from Controlled Batch Reactors." Biotechnology Progress **7**(6): 471-480.

Ozturk, S. S., M. R. Riley, et al. (1992). "Effects of Ammonia and Lactate on Hybridoma Growth, Metabolism, and Antibody-Production." Biotechnology and Bioengineering **39**(4): 418-431.

Padayatti, P. S., C. L. Jiang, et al. (2001). "High concentrations of glucose induce synthesis of argpyrimidine in retinal endothelial cells." Current Eye Research **23**(2): 106-115.

Patel, S. D., E. T. Papoutsakis, et al. (2000). "The lactate issue revisited: Novel feeding protocols to examine inhibition of cell proliferation and glucose metabolism in hematopoietic cell cultures." Biotechnology Progress **16**(5): 885-892.

Plas, D. R. and C. B. Thompson (2002). "Cell metabolism in the regulation of programmed cell death." Trends in Endocrinology and Metabolism **13**(2): 74-78.

Reichert, J. M. (2003). "Trends in development and approval times for new therapeutics in the United States." Nature Reviews Drug Discovery **2**(9): 695-702.

Sanfeliu, A., J. D. Chung, et al. (2000). "Effect of insulin stimulation on the proliferation and death of Chinese hamster ovary cells." Biotechnology and Bioengineering **70**(4): 421-427.

Sauer, P. W., J. E. Burky, et al. (2000). "A high-yielding, generic fed-batch cell culture process for production of recombinant antibodies." Biotechnology and Bioengineering **67**(5): 585-597.

Sauerwald, T. M., M. J. Betenbaugh, et al. (2002). "Inhibiting apoptosis in mammalian cell culture using the caspase inhibitor XIAP and deletion mutants." Biotechnology and Bioengineering **77**(6): 704-716.

Schmid, G. and H. W. Blanch (1992). "Extracellular and Intracellular Metabolite Concentrations for Murine Hybridoma Cells." Applied Microbiology and Biotechnology **36**(5): 621-625.

Schwabe, J. O., R. Portner, et al. (1999). "Improving an on-line feeding strategy for fed-batch cultures of hybridoma cells by dialysis and 'Nutrient-Split'-feeding." Bioprocess Engineering **20**(6): 475-484.

Simpson, N. H., A. E. Milner, et al. (1997). "Prevention of hybridoma cell death by bcl-2 during suboptimal culture conditions." Biotechnology and Bioengineering **54**(1): 1-16.

Singh, R., A. Barden, et al. (2001). "Advanced glycation end-products: a review." Diabetologia **44**(2): 129-146.

Singh, R. P., M. Alrubeai, et al. (1994). "Cell-Death in Bioreactors - a Role for Apoptosis." Biotechnology and Bioengineering **44**(6): 720-726.

Souriau, C. and P. J. Hudson (2003). "Recombinant antibodies for cancer diagnosis and therapy." Expert Opinion on Biological Therapy **3**(2): 305-318.

Terada, S., T. Komatsu, et al. (1999). "Co-expression of bcl-2 and bag-1, apoptosis suppressing genes, prolonged viable culture period of hybridoma and enhanced antibody production." Cytotechnology **31**(1-2): 141-149.

Thornalley, P. J. (1988). "Modification of the Glyoxalase System in Human Red Blood-Cells by Glucose Invitro." Biochemical Journal **254**(3): 751-755.

Thornalley, P. J. (1993). "The Glyoxalase System in Health and Disease." Molecular Aspects of Medicine **14**(4): 287-371.

Thornalley, P. J. (1995). "Advances in Glyoxalase Research - Glyoxalase Expression in Malignancy, Antiproliferative Effects of Methylglyoxal, Glyoxalase-I Inhibitor Diesters and S-D-Lactoylglutathione, and Methylglyoxal-Modified Protein-Binding and Endocytosis by the Advanced Glycation End-Product Receptor." Critical Reviews in Oncology/Hematology **20**(1-2): 99-128.

Thornalley, P. J. (1996). "Pharmacology of methylglyoxal: Formation, modification of proteins and nucleic acids, and enzymatic detoxification - A role in pathogenesis and antiproliferative chemotherapy." General Pharmacology **27**(4): 565-573.

Thornalley, P. J., L. G. Edwards, et al. (1996). "Antitumour activity of S-p-bromobenzylglutathione cyclopentyl diester in vitro and in vivo. Inhibition of glyoxalase I and induction of apoptosis." Biochemical Pharmacology **51**(10): 1365-1372.

Thornalley, P. J., N. I. Hooper, et al. (1989). "The Human Red Blood-Cell Glyoxalase System in Diabetes-Mellitus." Diabetes Research and Clinical Practice **7**(2): 115-120.

Tsuruo, T., M. Naito, et al. (2003). "Molecular targeting therapy of cancer: drug resistance, apoptosis and survival signal." Cancer Science **94**(1): 15-21.

Van Herreweghe, F., J. Q. Mao, et al. (2002). "Tumor necrosis factor-induced modulation of glyoxalase I activities through phosphorylation by PKA results in cell death and is accompanied by the formation of a specific methyl glyoxal-derived AGE." Proceedings of the National Academy of Sciences of the United States of America **99**(2): 949-954.

Vander Jagt, D. L. and L. A. Hunsaker (2003). "Methylglyoxal metabolism and diabetic complications: roles of aldose reductase, glyoxalase-1, betaine aldehyde dehydrogenase and 2-oxoaldehyde dehydrogenase." Chemico-Biological Interactions **143**: 341-351.

Walsh, G. (2000). "Biopharmaceutical benchmarks." Nature Biotechnology **18**(8): 831-833.

Xie, L. Z. and D. I. C. Wang (1994). "Fed-Batch Cultivation of Animal-Cells Using Different Medium Design Concepts and Feeding Strategies." Biotechnology and Bioengineering **43**(11): 1175-1189.

Xie, L. Z. and D. I. C. Wang (1996). "High cell density and high monoclonal antibody production through medium design and rational control in a bioreactor." Biotechnology and Bioengineering **51**(6): 725-729.

Yan, H. and J. J. Harding (1999). "Inactivation and loss of antigenicity of esterase by sugars and a steroid." Biochimica Et Biophysica Acta-Molecular Basis of Disease **1454**(2): 183-190.

Zhou, W. C., C. C. Chen, et al. (1997). "Fed-batch culture of recombinant NS0 myeloma cells with high monoclonal antibody production." Biotechnology and Bioengineering **55**(5): 783-792.

Zhou, W. C., J. Rehm, et al. (1997). "Alteration of mammalian cell metabolism by dynamic nutrient feeding." Cytotechnology **24**(2): 99-108.