View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Vanderbilt Electronic Thesis and Dissertation Archive

INTRACELLULAR HEXOKINASE LOCALIZATION IN HYBRIDOMA CULTURES: IMPLICATIONS FOR REGULATION OF METABOLISM AND CELL DEATH

By

Lindsey M. Clark

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Chemical Engineering

August, 2005

Nashville, Tennessee

Approved:

R. Robert Balcarcel

G. Kane Jennings

Robert D. Tanner

Kenneth A. Debelak

Rick Haselton

Copyright © 2005 by Lindsey M. Clark
All rights reserved

ACKNOWLEDGEMENTS

The realization of this work would not have been possible without the support of a variety of people. I would first and foremost like to express my sincere gratitude to my advisor, mentor, and now colleague, Robert Balcarcel, for his unwavering guidance throughout my development as a researcher. I thank him for pushing me to a higher level of scientific thinking, and his relentless quest for producing an extraordinary level of work is inspiring. I would also like to thank my dissertation committee, Ken Debelak, Rick Haselton, Kane Jennings, and Robert Tanner, for their time and input into this research project. A special thanks to the administration in the Vanderbilt University Department of Chemical Engineering, in particular Margarita Talavera and Douglas LeVan, for their support and encouragement throughout the trials and tribulations of graduate study. I would like to thank the Balcarcel research group for their many hours of discussions concerning research matters, which has augmented my development as a research scientist.

I would like to thank Nathan Magness and Jessica Peters for their support and encouragement throughout my last 5 years in Nashville. Their friendship is something that cannot be measured in a numerical quantity, but it possesses an infinite value which has kept me grounded during my tenure at Vanderbilt. I treasure each moment that I spend with them, and I am honored to have the opportunity to call them my friends.

Last, but certainly not least, I would like to thank my parents, Sam and Jannice, and my grandparents, John and Cookie Grissom. I cannot as eloquently as I would like convey in words the deep appreciation I feel for the love, support, and guidance they have unselfishly lavished upon me in the 27 years of my life. Without these people as a

constant in my life, I would in no way be able to accomplish these tasks, nor would I be able to enjoy my life's work near as much. My wish is that I have become the woman that they always imagined I would be, and I will continue to strive everyday to not only meet, but vastly exceed their expectations.

Thank you.

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS ... iii

LIST OF FIGURES ... viii

LIST OF TABLES ... ix

Chapter

I. HEXOKINASE BINDING TO THE MITOCHONDRIA: CONVERGENCE OF METABOLIC AND APOPTOTIC PATHWAYS ... 1

Research Motivation ... 1
Specific Research Aims ... 3
Dissertation Organization ... 4
Mitochondrial Hexokinase Links Metabolism and Apoptosis ... 4
Literature Evidence for Mitochondrial Hexokinase Association ... 4
Hexokinase Binding to the VDAC ... 6
Mitochondrial Hexokinase: Controller of Glycolysis and Apoptosis? ... 6
Chemically Induced Perturbation of Mitochondrial Hexokinase Binding ... 9
Akt/PKB Affects Glycolysis, Cell Survival, and Mitochondrial Hexokinase ... 9
Summary of Literature Support for the Specific Research Aims ... 10
Research Strategies ... 11
Quantification of Cellular Growth, Death, and Metabolism during High-Viability Cultures ... 11
Methods for Enzymatic Measurements ... 12
Induced vs. Un-induced Mitochondrial Hexokinase Detachment ... 13
Experimental Strategies: Batch and Well Plate Studies ... 14
Separation of Mitochondrial and Cytosolic Fractions ... 16
References ... 16

II. QUANTIFICATION OF GLUCOSE METABOLISM DURING HIGH-VIABILITY BATCH HYBRIDOMA CULTURES ... 23

Introduction ... 23
Materials and Methods ... 25
Cell Line and Maintenance ... 25
Batch Cultures ... 26

Cell Counting and Apoptosis Determination 27
Enzymatic Assays 27
Calculation Methods: Treatment of Experimental Data 29
Polynomial Fitting and Statistical and Error Analysis 30
Results 34
Profile of Cell Growth and Metabolism during High-Viability Batch Hybridoma Cultures 34
Statistical Comparisons among Growth, Metabolism, and Cell Death 42
Discussion 45
References 48

III. EFFECTS OF CLOTRIMAZOLE ON MITOCHONDRIAL HEXOKINASE ACTIVITY, GLYCOLYSIS AND APOPTOSIS IN HYBRIDOMA CULTURES 52

Introduction 52
Materials and Methods 55
Cell Line and Maintenance 55
Analytical Techniques 55
Cell Counting and Apoptosis Determination 55
Enzymatic Assays 56
Cell Fractionation 57
Experimental Procedures 58
Verification of Hexokinase Detachment by Clotrimazole 58
Metabolic Rate Screening (MRS) 59
Determination of Culture Viability 60
Calculation Methods 61
Intracellular Hexokinase Balance 61
Reaction Kinetics for the Hexokinase Activity Assay 62
Relating Assay Measurement to Intracellular Hexokinase Concentration 63
Rate Calculation for Metabolic Rate Screening (MRS) 63
Error Analysis and Statistical Methods 64
Results 65
Clotrimazole Decreases the Fraction of Hexokinase Bound to the Mitochondria in CRL 1606 65
Clotrimazole Causes Decreased Average Glucose Consumption over a 4-Hour Interval 70
Clotrimazole Causes Cell Death Exhibiting Apoptotic Morphology 71
Discussion 75
References 80

IV. ANALYSIS OF HEXOKINASE DETACHMENT IN BATCH HYBRIDOMA CULTURES 83

Introduction 83

Materials and Methods 85
Cell Line and Maintenance 85
Analytical Techniques 86
Cell Counting and Apoptosis Determination 86
Cell Fractionation and Hexokinase Activity Assay 86
Batch Cultures 87
Preliminary Modeling 87
Model I: Estimation of Hexokinase Detachment from the Mitochondria in Batch Cultures 87
Model II: Estimation of Changes in Total Hexokinase Activity 90
Error Analysis and Statistical Methods 91
Results 92
Preliminary Modeling Estimates 92
T-Flask Cultivation Studies 94
Spinner Flask Cultivation Studies 96
Comparison of Experimental Measurements to Model Estimates 98
Discussion 99
References 103

V. DISCUSSION OF EXPERIMENTAL FINDINGS AND CONCLUSIONS 106

Summary and Discussion of Key Findings 106
Mitochondrial-Mediated Pathway in Batch Hybridoma Cultures: Active or Inactive? 110
Effects of Clotrimazole on CRL 1606 and ER-Mediated Apoptosis 112
Future Research 114
Implications for the Biotechnology Industry 114
Concluding Remarks 115
References 117

Appendix A: Glossary of Terms 120

LIST OF FIGURES

Figure Page

2.1 Total and Viable Cell Densities of Batch Hybridoma Cell Culture............................35

2.2 Glucose and Lactate Concentration Profiles in Batch Hybridoma Culture 37

2.3 Growth and Death Rates in Batch Hybridoma Culture 40

2.4 Glucose Consumption and Lactate Production Rates in Batch Hybridoma Culture 41

2.5 Comparison of Times in which Glucose Consumption Rate, Growth Rate, and Viability Statistically Decrease in Batch Hybridoma Cultures 43

3.1 Fraction of Hexokinase Bound to the Mitochondria and Total Hexokinase Activity during 4-Hour Exposure to 0, 2, and 5 μM Clotrimazole 67

3.2 Fraction of Hexokinase Bound to the Mitochondria and Total Hexokinase Activity during 2-Hour Exposure to 0, 10, 15, and 20 μM Clotrimazole 69

3.3 Average Glucose Consumption Rate as a Function of Clotrimazole Concentration over a 4-Hour Time Interval 71

3.4 Viability by Resazurin Reduction of Hybridoma Cultures Treated with Varying Clotrimazole Concentrations...73

3.5 Viability by AO/EB of Hybridoma Cultures Treated with Varying Clotrimazole Concentrations 74

4,1 Fraction of Hexokinase Bound to the Mitochondria and Total Hexokinase Activity at 20 and 48 Hours after Subculture for CRL 1606 Cultures Cultivated in T-Flasks 95

4.2 The Ratio of Mitochondrial Hexokinase Activity to Total Hexokinase Activity and Total Hexokinase Activity in Batch CRL 1606 Cultures Cultivated in 0.5 L Spinner Flasks 97

LIST OF TABLES

Table Page

1.1 Summary of Relevant Literature Supporting the Project Aims 11

2.1 Summary of Literature Quantifying Declining Growth and Metabolism in Batch Mammalian Cell Culture 24

2.2 Summary of Statistical Techniques Applied to Reduced Batch Data............................33

2.3 Summary of Batch Hybridoma Culture Experiments 42

2.4 Summary of Times in which Maximum Growth and Glucose Consumption Occur in Batch Hybridoma Cultures 44

3.1 Multivariate ANOVA Results for Aim II Experiments 66

3.2 Trypan Blue Viability of Hybridoma Cultures Treated with Varying Clotrimazole Concentrations 72

4.1 Published Values of Fraction of Hexokinase Bound to the Mitochondria for Varying Cell Types 84

4.2 Model Predictions for the Fraction of Hexokinase Bound to the Mitochondria (Model I) and Total Hexokinase Activity (Model II) in Batch CRL 1606 Cultures Cultivated in T-Flasks and Spinner Flasks 93

4.3 Comparison of Model Estimates and Experimental Data: Decrease in Total Hexokinase and Fraction of Hexokinase Bound to the Mitochondria between 20 and 48 Hours after Subculture...99

5.1 Summary of Key Research Findings 107

5.2 Summary of Research into Apoptotic/Metabolic/Signaling Pathways and the Effects on Death in Hybridoma Cultures...112

CHAPTER I

HEXOKINASE BINDING TO THE MITOCHONDRIA: CONVERGENCE OF METABOLIC AND APOPTOTIC PATHWAYS

Research Motivation

Recombinant therapeutic proteins, including monoclonal antibodies, are manufactured from the large-scale culture of mammalian cell lines. The ability of mammalian cell lines to perform proper protein assembly, protein folding, and the intricate post-translational modifications required for protein efficacy makes them the most desirable system to utilize in this process, as opposed to plant or microbial cell lines. Despite the intrinsic ability of mammalian cells to produce therapeutic proteins, limitations to efficient small and large-scale production do exist. Mammalian cells in culture are typically plagued by slow growth rates, low productivity, inefficient metabolism (i.e., high production of toxic metabolic by-products), nutrient depletion, and cell death (apoptosis). Understanding the intracellular mechanisms that contribute to cell growth and cell death is essential in improving the cellular "factories" that will produce the maximum amount of product.[1, 2]

Surprisingly little is known about what instigates apoptotic cell death in bioprocesses or how it relates to other intracellular biochemical processes, such as protein signaling pathways or metabolism. Bioreactor stresses, such as decreased pH and nutrient depletion, are known to contribute to apoptotic cell death, but there is no clear explanation as to why cells in culture exhibit slowed growth and apoptotic cell death. Determining the cause(s) of cell death and consequently low productivity in industrial

cell culture and developing environmental and/or genetic strategies to circumvent such occurrences represents a great challenge to biotechnologists. Most research in the area of apoptotic cell death has focused on supplementation of apoptotic protein blocking agents into the culture media, up-regulation of protective proteins, such as Bcl-2, or fed-batch methodologies.[3] These strategies have been somewhat successful in extending culture viabilities and delaying cell death, but only offer a partial solution to the problem of apoptosis or identify a specific cause.

Since biopharmaceutical-producing cells manufacture their antibody products from major carbon sources in their environment (mainly glucose and glutamine) and the utilization of these sources determines cell growth and productivity, cellular metabolism is an attractive target of study for bioprocess optimization. Indeed, mammalian cells in batch cultures are known to exhibit decreasing metabolism, and there are no real explanations for this phenomenon.[4-7] The connections between glycolytic metabolism and apoptosis through the interactions between hexokinase, a glycolytic enzyme, and the mitochondria are compelling to say the least, which clearly leads to speculation that higher rates of glucose consumption may play a role as a strong survival signal for the cell and/or potentially protect the cell against mitochondrial-mediated apoptosis. A portion of the total hexokinase molecules binds to the mitochondria where in cell-free studies it has been shown that conditions are more favorable for glycolysis, and this binding may protect the cell from apoptosis by impeding the release of apoptotic proteins into the cytosol.[8, 9] If what the literature claims regarding hexokinase's role in metabolism and apoptosis holds true in a biopharmaceutical-producing cell line, then future efforts to mitigate decreasing glycolysis and cell death would be complemented by

efforts to prevent hexokinase dissociation. This traverse between metabolism and apoptosis serves as the basis for this research project and the investigation of strategies to promote increased metabolism and cell survival in bioprocesses. As a whole, the aims of this research are to quantify the effects of induced hexokinase redistribution between the mitochondrial and cytosolic fractions on cell death and glycolysis and probe a possible occurrence of hexokinase redistribution in batch cultures, which may offer an explanation to the phenomenon of decreasing glucose consumption. Hexokinase dissociation from the mitochondria may also prove to be a very early marker of an active apoptotic mechanism in batch cultures. The study of this enzyme in particular is more desirable than studying the other key regulatory enzymes in glycolysis because hexokinase is the only known glycolytic enzyme to possess mitochondrial binding characteristics.[10]

Specific Research Aims

This research precisely investigates the dynamics of hexokinase localization between the mitochondria and cytosol in batch hybridoma cultures. Preliminarily, I performed an extensive study on the physiology of the high-viability batch cultures in T-flasks and spinner flasks, and these data are presented in Chapter II. The subsequent studies are divided into three major aims:

(I) Determine the effects of clotrimazole, a compound known to detach hexokinase from the mitochondria, on glucose consumption and apoptotic cell death of hybridoma cultures.

(II) Verify the mitochondrial hexokinase dissociation effects of clotrimazole.

(III) Determine if the amount of hexokinase bound to mitochondria decreases over time in un-induced high-viability batch hybridoma cultures.

Dissertation Organization

This dissertation is organized into 5 different chapters. Chapter I contains the specific research aims and a summary of the relevant literature supporting the project ideas. Chapter II contains results from the preliminary studies of high-viability batch hybridoma culture physiology. Results and discussion for specific aims I and II are presented in Chapter III, and Chapter IV contains the results of the batch measurements of mitochondrial hexokinase activity (aim III). The data collected during various experiments are analyzed to observe any suggested correlations among hexokinase disassociation from the mitochondria, inhibition of glycolysis, and occurrence of apoptotic cell death. The regulatory properties of mitochondrial hexokinase binding in terms of controlling glucose consumption (by efficiently phosphorylating glucose) and inhibiting the release of apoptotic proteins from the mitochondria are discussed. The last chapter, V, is devoted to summarizing and discussing the major findings of this study and their implications for apoptotic and metabolic control strategies in bioprocesses.

Mitochondrial Hexokinase Links Metabolism and Apoptosis

Literature Evidence for Mitochondrial Hexokinase Association

Hexokinase (HK) is an enzyme that catalyzes the first step of glycolysis, and it exists in 4 different isoforms, designated I, II, III, and IV. HKIV is known to exist only in

liver cells, and HKIII is known to only be present in small quantities of varying cell types and lacks the N-terminal amino acid sequence necessary for regulatory binding. HKI and HKII are known to associate with the mitochondria in a variety of different cell types, particularly cancer cells.[11-13] Studies showing evidence of mitochondrial hexokinase association can be divided into 2 separate parts: (1) studies showing that hexokinase associates with the mitochondria and (2) studies showing that hexokinase binds to the voltage-dependent anion channel (VDAC) specifically. The vast majority of studies relating hexokinase and the mitochondria have been performed in a cell-free platform with isolated mitochondria, not whole-cell *in vitro* studies.

Papers showing evidence of the presence of hexokinase in the particulate fractions of cells can be found as early at 1966. The first paper showing evidence of <u>mitochondrial</u> hexokinase binding was published in *Biochemistry* in 1969 by Craven and colleagues.[14] They used a fluorescent antibody labeling technique against hexokinase and observed the distribution of intracellular hexokinase in bovine brain tissues using microscopy. It was found that the majority of the fluorescent marker was centralized to the mitochondria. Since this publication, there have been various studies claiming an association of hexokinase with the mitochondria through a variety of techniques, such as confocal microscopy of rat brain astrocytes,[15] measurement of hexokinase activity in isolated mitochondria of HeLa tumor, kidney, and brain cell types,[16-19] and inhibitory studies using hexokinase monoclonal antibodies in human brain tissues.[20]

Hexokinase Binding to the VDAC

An outer-membrane binding site for hexokinase on the VDAC of the mitochondria is well established. A specific protein involved in mitochondrial hexokinase binding (and termed hexokinase binding protein (HBP)) was first uncovered in 1979 by co-sedimentation of the protein with hexokinase from mitochondrial lysates followed by SDS-PAGE.[21] A few years later, the HBP was shown to have identically homology to the VDAC through SDS-PAGE, amino acid analysis, and the ability of the HBP to form pores.[22, 23] This protein was not identified in a transformed cell line to be the VDAC itself until years later in 1986, when Nakashima and colleagues inhibited a binding site on the VDAC of isolated rat hepatoma mitochondria and showed that supplemented hexokinase would not bind when the site was occupied.[24] Since these initial studies, there have been various review papers claiming hexokinase binding to the VDAC.[11, 13, 25, 26] A very recent study showed specificity of hexokinase I binding to the VDAC of isolated rat liver mitochondria through peptide binding competition studies to a specific domain of the VDAC protein.[27] Furthermore, hexokinase I has been shown to bind to purified VDAC protein by treatment with hexokinase followed by SDS-PAGE, and the treatment of purified VDAC reconstituted in a lipid bilayer with hexokinase induces VDAC channel closure through voltage measurements.[28] Thus, there is a clearly solid body of supporting literature for interactions between hexokinase and the mitochondrial VDAC protein.

Mitochondrial Hexokinase: Controller of Glycolysis and Apoptosis?

The mitochondria are central to both energy metabolism and apoptotic cell death, and the fact that hexokinase possesses known molecular interactions with a key apoptotic

protein on the mitochondria is indeed compelling. Cellular energy in the form of ATP is produced at the mitochondria, and proteins that participate in apoptosis are housed there as well. In mitochondrial-mediated apoptosis, pro-apoptotic proteins are released from the inner membrane space into the cytosol through a mitochondrial trans-membrane protein called the voltage-dependent anion channel (VDAC) upon reception of apoptotic-inducing stimuli. [29, 30] The opening of this protein is preceded by the opening of a protein on the inner mitochondrial membrane, termed the permeability transition pore (PTP). One of the most notable proteins released from the mitochondria during apoptosis is cytochrome c, and upon release, cytochrome-c binds with the Apaf-1 protein. In the presence of ATP or dATP, the cytochrome-c-Apaf-1 complex binds and activates procaspase-9, which in turn activates downstream caspases involved in the apoptotic pathway. [13, 31]

Cancer cells are known to exhibit an abnormal (higher) pattern of energy metabolism, potentially in part because more hexokinase is associated with the mitochondria in tumor cells as opposed to normal cells.[10] The increased efficiency of mitochondrial hexokinase as opposed to cytosolic hexokinase has been hypothesized and studied in various ways. Cell-free studies with isolated hepatocyte, hybridoma, rat liver, and rat brain mitochondria have shown that the K_m value for mitochondrial hexokinase is lower than that of cytosolic hexokinase, suggesting that cytosolic hexokinase is less efficient.[32-34] In whole-cell studies on an adenocarcinoma cell line, the levels of specific glycolytic intermediates, measured by enzymatic methods, were decreased in a dose-dependent manner with a compound that increases soluble hexokinase, suggesting that larger fractions of cytosolic hexokinase causes less efficient glucose phosphorylation.[35]

Also, one of the cell-free studies with the hybridoma cell line concluded that cytosolic hexokinase is more susceptible to inhibition by glucose-6-phosphate.[33] And also quite interestingly, inhibition of glycolysis with 2-deoxyglucose in rate brain astrocytes decreased the amount of mitochondrial hexokinase by 35%, suggesting that mitochondrial hexokinase is sensitive to shifts in nutrient availability.[15]

In terms of apoptotic cell death, there are various studies to suggest that hexokinase binding to the mitochondria actually plays a role in preventing apoptotic protein release, which would certainly indicate that higher rates of glycolysis may signal an anti-apoptotic effect since mitochondrial hexokinase is presumably more efficient than cytosolic hexokinase. Cell-free studies with isolated HeLa mitochondria treated with hexokinase showed that upon addition of Bax, an apoptotic protein that facilitates opening of the VDAC and subsequent cytochrome-c release, Bax was unable to bind and release cytochrome-c,[9] and this same kind of study has also been performed with brain mitochondria.[36] In *in vitro* hepatocyte studies, hexokinase IV actually binds to another apoptotic protein, Bad, apparently controlling its phosphorylation state (which determines protein activity), as determined using immunoprecipitation followed by SDS-PAGE. The overall picture presented from the evidence in the aforementioned studies is that when hexokinase is bound to the mitochondria, glycolysis is higher, and apoptotic proteins are antagonized from binding to the VDAC and facilitating apoptotic protein release, leading to apoptotic cell death.

Chemically Induced Perturbation of Mitochondrial Hexokinase Binding

There are very few studies that investigate chemically induced changes in mitochondrial hexokinase binding. Only clotrimazole (CTZ) and bifonazole, calmodulin antagonists known to interrupt calcium homeostasis, have been investigated in HeLa, myeloma, and carcinoma cell lines as compounds which detach hexokinase from the mitochondria.[9, 35, 37, 38] These investigations have primarily been done to identify the compounds as a potential cancer therapy, primarily since a marked reduction in viability was observed after treatment with the compounds. Furthermore, there has been at least one study investigating the effects of calcium-mobilizing hormones on mitochondrial hexokinase, and it was shown that serotonin, along with other hormones, actually increases the amount of hexokinase bound to the mitochondria in muscle by as much as 2-fold.[39] All the aforementioned studies were carried out by treatment of the cells with the compound, separation of the mitochondrial and cytosolic fractions, and measurement of hexokinase activity in each fraction by enzymatic assays.

Akt/PKB Affects Glycolysis, Cell Survival, and Mitochondrial Hexokinase

Although this research does not focus on intracellular signaling molecules, it should be noted here that the actions of one particular signaling protein, Akt/Protein Kinase B, provide strong supporting evidence for the role of mitochondrial hexokinase in maintenance of glucose metabolism and cell survival. The anti-apoptotic effects of various intracellular signaling proteins are known to be facilitated by increased glucose metabolism, and perhaps subsequently, increased mitochondrial hexokinase activity. Specifically, Akt is known to promote survival by increasing mitochondrial membrane

potential in FL5.12 cells,[40] and its anti-apoptotic effects are dependent on glucose availability.[41, 42] Active Akt prevents apoptosis even following the activation of apoptotic proteins Bid and Bax, in part by increasing mitochondrial hexokinase activity.[8, 41-43] These experiments were performed in fibroblasts over-expressing Akt by assessing apoptosis in the presence of active apoptotic proteins and measuring the hexokinase activity in sub-cellular fraction via enzymatic methods. This further supports a role for higher rates of glycolytic metabolism, perhaps instigated by higher concentrations of mitochondrial hexokinase, in cell survival.

Summary of Literature Support for the Specific Research Aims

The main literature ideas which support this research plan are summarized in Table 1.1. There are 5 main supporting claims that have previously been published: (1) decreasing glucose consumption may activate apoptotic cell death, (2) glucose consumption is known to decrease over time in batch mammalian cell cultures, (3) hexokinase is known to bind to the VDAC on the mitochondria, (4) mitochondrial hexokinase is known to phosphorylate glucose more efficiently than cytosolic hexokinase in cell-free platforms, and (5) mitochondrial hexokinase binding has been shown to prevent Bax translocation and subsequent cytochrome-c release. These primary supporting ideas coupled with the fact that there are no solid explanations for the phenomenon of decreasing glycolysis and apoptotic cell death in batch mammalian cell cultures form the foundation for the experimental strategy outlined in this research project.

Table 1.1. Summary of Relevant Literature Supporting the Project Aims. Five key literature findings supporting the research aims are listed in the left column, and the main references are listed to the right.

Primary Supporting Idea	References
Decreasing glycolysis may contribute to apoptotic cell death	Plas, et. al. 2002[44] Hammerman, et. al. 2004[45] Cohen, et. al. 2002[46] Kuznetsov, et. al. 2004[47] Downward, et. al. 2003[48]
Glycolysis decreases in batch mammalian cell culture	Ozturk, et. al. 1991[5] Goergen, et. al. 1992[6] Fitzpatrick, et. al. 1993[49] Portner, et. al. 1994[50] Neermann, et. al. 1996[7] Oh, et. al. 1996[51] Doverskog, et. al. 1997[4] Quesney, et. al. 2003[52]
Hexokinase binds to the VDAC	Nakashima, et. al. 1985, 1986[53, 54]
Mitochondrial hexokinase phosphorylates glucose more efficiently	Widjojoatmodjo, et. al. 1990[33] Laterveer, et. al. 1994[34]
Mitochondrial hexokinase binding prevents Bax translocation	Pastorino, et. al. 2002[9] Vyssokikh, et. al. 2002[36]

Research Strategies

Quantification of Cellular Growth, Death, and Metabolism during High-Viability Cultures

The preliminary phases of this project involved quantification of glycolytic metabolism, cell growth, and cell death in high-viability batch hybridoma (CRL 1606) cultures. A detailed description of growth, death, and metabolism of this cell line during the first two days of batch culture has not previously been determined, although data

concerning the metabolic and growth kinetics of various mammalian cell lines is readily available. An outline of the available literature is summarized in Chapter II. Despite the apparent availability of data, these sources do not precisely investigate the dynamic nature of metabolism and how it relates to cell death, and there are no attempts at error analysis or analysis of the times of peak metabolism. Consequently, the preliminary studies of this project were designed to provide detailed and precise data in terms of cell growth, death, glucose consumption, and lactate production in high-viability batch hybridoma cultures. For these studies, the events prior to cell death observable in regard to cellular membrane permeability (a last stage of death) were of primary interest. This study was accomplished by seeding T-flasks (175 cm^2 area) or spinner flasks at 2.0 x 10^5 cells/mL in a complete maintenance medium (IMDM) and removing a sample of media and measuring total and viable cell density every 4 hours until the viability dropped below 70%. All collected samples of the medium were stored at -20°C until the experiment was complete. All samples were analyzed for metabolite concentrations via enzymatic assays less than one week after the experiment. The raw data values of glucose concentration, lactate concentration, total cell density, and viable cell density were used to calculate the derived quantities of growth rate, death rate, glucose consumption rate, and lactate production rate.

Methods for Enzymatic Measurements

The measurements of glucose and lactate concentration and hexokinase activity were done via enzymatic assays. Enzymatic analysis generally involves the measurement of a metabolite/amino acid via an end product from a reaction or series of reactions

involving the compound of interest as a substrate. The measurement is usually achieved through spectrometry or fluorescence. In a great number of enzymatic assays, one of the end products is one of two co-enzymes, nicotinamide adenine dinucleotide phosphate (NADPH) or nicotinamide adenine dinucleotide (NAD^+), one of which is required for the reaction and both of which absorb light at 340 nm. This is the case for both the glucose and lactate enzymatic assays and the hexokinase activity assay employed in this study. Complete recipes for these assays can be found in *Methods of Enzymatic Analysis*.[55] However, these assays were formulated for use in 1.0-2.0 mL volumes, and I scaled-down the assay procedures for use in 96-well plates (200 μL volumes). Complete and detailed descriptions for each assay are in Chapter II.

Induced vs. Un-induced Mitochondrial Hexokinase Detachment

An assumption in this research is that mitochondrial hexokinase can be detached in one of 2 ways: it can be chemically induced, with compounds such as clotrimazole, or it can be un-induced, possibly occurring naturally over the course of a culture. Aims I and II focus on the effects of induced hexokinase detachment (by clotrimazole) on glucose consumption rate and apoptotic cell death in hybridoma cultures. Studying an induced system was necessary to determine whether or not hexokinase detachment was in fact a mechanism that results in an unfavorable outcome for the cell. If induced hexokinase detachment does not exert any effects on glucose consumption or cause cell death, then there would be little reason to investigate a possible occurrence of detachment in batch cultures. Aim III focuses on the un-induced case, measuring possible changes in the fraction of hexokinase bound to the mitochondria over time during the course of batch

cultures. The glucose consumption profiles quantified in the preliminary studies were used as a guide to select specific sampling times to measure mitochondrial hexokinase association in high-viability batch cultures.

Experimental Strategies: Batch and Well Plate Studies

The experimental designs in this research plan employ 4 different types of cultivation modes: T-flasks (Corning), spinner flasks (Bellco Biotechnology), and 6-well and 24-well plates (Nunc). Choice of cultivation mode for each separate experimental design was based on (1) the volume of culture that would be needed for the measurements and (2) the amount of parent culture that would be available for the measurements. The preliminary batch experiments where metabolism and growth and death kinetics were quantified were carried out in both T-flasks and spinner flasks.

Aim I experiments entailed the quantification of average glucose consumption over a 4-hour time interval for cells exposed to clotrimazole in an attempt to assess the effects of induced hexokinase detachment on glucose consumption rate. These experiments were carried out in 24-well plates to allow for a maximum number of replicates for each tested concentration, and a specialized low-glucose medium formulation (RPMI) was used in the experiments to eliminate the need for sample dilution (and accordingly reduce error) for the metabolite assays. A 4-hour time interval of study and a high seeding density (1.75×10^6 cells/mL) were chosen in an effort to measure a detectable glucose concentration difference for rate calculation and error analysis. To quantify the effects of various clotrimazole concentrations (presumably inducing various levels of hexokinase detachment) on cell death, experiments were

carried out in 6-well plates over a 24-hour period to assess long-term effects of induced hexokinase detachment on cell viability and apoptosis. Again, a 6-well plate format was chosen as the most logical mode of cultivation based on the culture volume needed for this experiment.

The Aim II experiments were carried out in T-flasks (75 cm^2 area). A parent culture was split into 2 separate cultures, one receiving clotrimazole treatment and the other receiving DMSO solvent only. Cells were seeded at 7.5 x 10^5 cells/mL, and the culture was sampled and fractionated for either a 2 or 4-hour period. Fractionated samples were stored at -80°C for later hexokinase activity analysis. Cultivation in well plates was not appropriate for these experiments due to the large sampling size and frequency, which required relatively larger culture volumes.

The Aim III experiments were carried out in T-flasks (175 cm^2 area) and spinner flasks in a similar fashion to the batch cultures in the preliminary studies. However, samples for glucose and lactate concentrations were not removed, but fractionated cell homogenates were sampled according to a pre-determined time schedule based on known profiles of glucose consumption in previous batch culture studies. The fractionated samples were stored at -80°C, and hexokinase activity was assayed in all samples together less that one week after completion of the experiment.

The measurements performed in all studies focus specifically on high-viability cultures, since it is the premise of this research that commitment to apoptotic death potentially occurs prior to the "visible" occurrence of apoptosis (i.e., when cell death is apparent by the dye-based methods used in this study).

Separation of the Mitochondrial and Cytosolic Fractions

In order to measure hexokinase activity in the mitochondrial and cytosolic fractions, each fraction must be separated using a population of cells (7.5 x 10^6 to 1 x 10^7 total cells). The fractions were separated via centrifugation of homogenates in a sucrose buffer, a method based on that of the Pierce Mitochondrial Isolation Kit (#89874) and the BioVision Mitochondria/Cytosol Fractionation Kit (#K256-100). This is a well-characterized method that has been used in various studies for mitochondrial hexokinase activity determination and is outlined in a number of laboratory manuals and papers.[9, 37, 43, 56, 57] The method is based on the differences in sedimentation rates of each organelle in a gravitational field. The buffers supplied in the kits were not used since they were shown to interfere with the enzymatic hexokinase activity assay during assay development (components such as CHAPS, HEPES, and EDTA are known to interfere with enzyme activity). However, it is known that a sucrose buffer is suitable for this method, which does not interfere with the hexokinase enzymatic assay.[56] Cell lysis was carried out by mechanical homogenation prior to fractionation.

References

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

2. Wurm, F., *Production of Recombinant Protein Therapeutics in Cultivated Mammalian Cells.* Nature Biotechnology, 2004. **22**(11): p. 1393-1398.

3. Arden, N. and M. Betenbaugh, *Life and death in mammalian cell culture: strategies for apoptosis inhibition.* Trends in Biotechnology, 2004. **22**(4): p. 174-180.

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

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

6. Goergen, J.L., A. Marc, and J.M. Engasser, *Comparison of Specific Rates of Hybridoma Growth and Metabolism in Batch and Continuous Cultures.* Cytotechnology, 1992. **10**(2): p. 147-155.

7. Neermann, J., Wagner, R., *Comparative analysis of glucose and glutamine metabolism in transformed mammalian cell lines, insect and primary liver cells.* Journal of Cellular Physiology, 1996. **166**(1): p. 152-169.

8. Gottlob, K., et al., *Inhibition of early apoptotic events by Akt/PKB is dependent on the first committed step of glycolysis and mitochondrial hexokinase.* Genes & Development, 2001. **15**(11): p. 1406-1418.

9. Pastorino, J.G., N. Shulga, and J.B. Hoek, *Mitochondrial binding of hexokinase II inhibits Bax-induced cytochrome c release and apoptosis.* Journal of Biological Chemistry, 2002. **277**(9): p. 7610-7618.

10. Nakashima, R.A., L.J. Scott, and P.L. Pedersen, *The Role of Mitochondrial Hexokinase Binding in the Abnormal Energy-Metabolism of Tumor-Cell Lines.* Annals of the New York Academy of Sciences, 1986. **488**: p. 438-450.

11. Wilson, J., *An introduction to the isoenzymes of mammalian hexokinase types I-III.* Biochemical Society Transactions, 1997. **25**(1): p. 103-107.

12. Wilson, J.E., *Isozymes of mammalian hexokinase: structure, subcellular localization and metabolic function.* Journal of Experimental Biology, 2003. **206**(12): p. 2049-2057.

13. Pastorino, J.G. and J.B. Hoek, *Hexokinase II: The integration of energy metabolism and control of apoptosis.* Current Medicinal Chemistry, 2003. **10**(16): p. 1535-1551.

14. Craven, P., Goldblat.PJ, and R. Basford, *Brain Hexokinase - Preparation of Inner and Outer Mitochondral Membranes.* Biochemistry, 1969. **8**(9): p. 3525-&.

15. Lynch, R., K. Fogarty, and F. Fay, *Modulation of Hexokinase Association with Mitochondria Analyzed with Quantative 3-Dimensional Confocal Microscopy.* Journal of Cell Biology, 1991. **112**(3): p. 385-395.

16. Parry, D.M. and P.L. Pedersen, *Intracellular-Localization and Properties of Particulate Hexokinase in the Novikoff Ascites Tumor - Evidence for an Outer Mitochondrial-Membrane Location.* Journal of Biological Chemistry, 1983. **258**(18): p. 904-912.

17. Parry, D.M. and P.L. Pedersen, *Intracellular-Localization of Rat-Kidney Hexokinase - Evidence for an Association with Low-Density Mitochondria.* Journal of Biological Chemistry, 1984. **259**(14): p. 8917-8923.

18. Parry, D.M. and P.L. Pedersen, *Glucose Catabolism in Brain - Intracellular-Localization of Hexokinase.* Journal of Biological Chemistry, 1990. **265**(2): p. 1059-1066.

19. Vriezen, N. and J.P. van Dijken, *Subcellular localization of enzyme activities in chemostat-grown murine myeloma cells.* Journal of Biotechnology, 1998. **61**(1): p. 43-56.

20. Golestani, A. and M. Nemat-Gorgani, *Hexokinase 'binding' of normal and tumoral human brain mitochondria.* Molecular and Cellular Biochemistry, 2000. **215**(1-2): p. 115-121.

21. Felgner, P., J. Messer, and J. Wilson, *Purification of a Hexokinase-Binding Protein from the Outer Mitochondrial-Membrane.* Journal of Biological Chemistry, 1979. **254**(12): p. 4946-4949.

22. Linden, M., P. Gellerfors, and B. Nelson, *Pore Protein and the Hexokinase-Binding Protein from the Outer-Membrane of Rat-Liver Mitochondria are Identical.* FEBS Letters, 1982. **141**(2): p. 189-192.

23. Fiek, C., et al., *Evidence for Identity Between the Hexokinase-BIinding Protein and the Mitochondrial Porin in the Outer-Membrane of Rat-Liver Mitochondria.* Biochimica et Biophysica ACTA, 1982. **688**(2): p. 429-440.

24. Nakashima, R.A., et al., *Hexokinase Receptor Complex in Hepatoma Mitochondria - Evidence from N,N'-Dicyclohexylcarbodiimide-Labeling Studies for the Involvement of the Pore-Forming Protein Vdac.* Biochemistry, 1986. **25**(5): p. 1015-1021.

25. Arora, K.K., D.M. Parry, and P.L. Pedersen, *Hexokinase Receptors - Preferential Enzyme Binding in Normal-Cells to Nonmitochondrial Sites and in Transformed-Cells to Mitochondrial Sites.* Journal of Bioenergetics and Biomembranes, 1992. **24**(1): p. 47-53.

26. Golshani-Hebroni, S.G. and S.P. Bessman, *Hexokinase binding to mitochondria: A basis for proliferative energy metabolism.* Journal of Bioenergetics and Biomembranes, 1997. **29**(4): p. 331-338.

27. Al jamal, J., *Involvement of porin N,N-dicyclohexylcarbodiimide-reactive domain in hexokinase binding to the outer mitochondrial membrane.* Protein Journal, 2005. **24**(1): p. 1-8.

28. Azoulay-Zohar, H., et al., *In self-defence: Hexokinase promotes voltage-dependent anion channel closure and prevents mitochondria-mediated apoptotic cell death.* Biochemical Journal, 2004. **377**: p. 347-355.

29. Green, D.R., Reed, J.C., *Mitochondria and Apoptosis.* Science, 1998. **281**(5381): p. 1309-1312.

30. Green, D. and G. Kroemer, *The pathophysiology of mitochondrial cell death.* Science, 2004. **305**(5684): p. 626-629.

31. Alnemri, E.S., *Hidden powers of the mitochondria.* Nature Cell Biology, 1999. **1**(2): p. E40-E42.

32. Adams, V., et al., *Activation of Low Km Hexokinases in Purified Hepatocytes by Binding to Mitochondria.* Biochimica Et Biophysica Acta, 1988. **932**(2): p. 195-205.

33. Widjojoatmodjo, M.N., A. Mancuso, and H.W. Blanch, *Mitochondrial Hexokinase-Activity in a Murine Hybridoma.* Biotechnology Letters, 1990. **12**(8): p. 551-556.

34. Laterveer, F., et al., *The Kinetic Consequences of Binding of Hexokinase-I to the Mitochondrial Outer-Membrane.* Biochimica et Biophysica ACTA-Bioenergetics, 1994. **1188**(3): p. 251-259.

35. Penso, J. and R. Beitner, *Clotrimazole decreases glycolysis and the viability of lung carcinoma and colon adenocarcinoma cells.* European Journal of Pharmacology, 2002. **451**(3): p. 227-235.

36. Vyssokikh, M., Zorova L, Zorov D, Heimlich G, Jurgensmeier JM, Brdiczka D, *Bax releases cytochrome c preferentially from a complex between porin and adenine nucleotide translocator. Hexokinase activity suppresses this effect.* Molecular Biology Reports, 2002. **29**(1-2): p. 93-96.

37. Penso, J. and R. Beitner, *Clotrimazole and bifonazole detach hexokinase from mitochondria of melanoma cells.* European Journal of Pharmacology, 1998. **342**(1): p. 113-117.

38. Penso, J. and R. Beitner, *Detachment of glycolytic enzymes from cytoskeleton of Lewis lung carcinoma and colon adenocarcinoma cells induced by clotrimazole and its correlation to cell viability and morphology.* Molecular Genetics and Metabolism, 2002. **76**(3): p. 181-188.

39. Bassukevitz, Y., M. Chenzion, and R. Beitner, *Ca2+-Ionophore A23187 and the Ca2+-Mobilizing Hormones Serotonin, Vasopressin, and Bradykinin Increase Mitochondrially Bound Hexokinase in Muscle.* Biochemical Medicine and Metabolic Biology, 1992. **47**(2): p. 181-188.

40. Plas, D.R., et al., *Akt and Bcl-x(L) promote growth factor-independent survival through distinct effects on mitochondrial physiology.* Journal of Biological Chemistry, 2001. **276**(15): p. 12041-12048.

41. Majewski, N., et al., *Akt inhibits apoptosis downstream of BID cleavage via a glucose-dependent mechanism involving mitochondrial hexokinases.* Molecular and Cellular Biology, 2004. **24**(2): p. 730-740.

42. Rathmell, J., Fox CJ, Plas DR, Hammerman PS, Cinalli RM, Thompson CB, *Akt-directed glucose metabolism can prevent Bax conformation change and promote growth factor-independent survival.* Molecular and Cellular Biology, 2003. **23**(20): p. 7315-7328.

43. Majewski, N., et al., *Hexokinase-mitochondria interaction mediated by Akt is required to inhibit apoptosis in the presence or absence of Bax and Bak.* Molecular Cell, 2004. **16**(5): p. 819-830.

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

45. Hammerman, P., C. Fox, and C. Thompson, *Beginnings of a signal-transduction pathway for bioenergetic control of cell survival.* TRENDS in Biochemical Sciences, 2004. **29**(11): p. 586-592.

46. Cohen, I., M. Castedo, and G. Kroemer, *Tantalizing Thanatos: unexpected links in death pathways.* Trends in Cell Biology, 2002. **12**(7): p. 293-295.

47. Kuznetsov, A., et al., *Regulating cell survival by controlling cellular energy production: novel functions for ancient signaling pathways?* FEBS Letters, 2004. **577**(1-2): p. 1-4.

48. Downward, J., *Cell biology - Metabolism meets death.* Nature, 2003. **424**(6951): p. 896-897.

49. Fitzpatrick, L., H.A. Jenkins, and M. Butler, *Glucose and Glutamine-Metabolism of a Murine B-Lymphocyte Hybridoma Grown in Batch Culture.* Applied Biochemistry and Biotechnology, 1993. **43**(2): p. 93-116.

50. Portner, R., et al., *Estimation of Specific Glucose-Uptake Rates in Cultures of Hybridoma Cells.* Journal of Biotechnology, 1994. **34**(3): p. 237-246.

51. Oh, G.S., et al., *Interactive dual control of glucose and glutamine feeding in hybridoma cultivation.* Journal of Fermentation and Bioengineering, 1996. **81**(4): p. 329-336.

52. Quesney, S., et al., *Kinetics and metabolic specificities of Vero cells in bioreactor cultures with serum-free medium.* Cytotechnology, 2003. **42**(1): p. 1-11.

53. Nakashima, R., P. Mangan, and P. Pedersen, *Evidence for a relationship between Hexokinase-Binding and the Mitochondrial VDAC Protein in AS-30D Hepatoma-Cells - DCCD Labels the Pore Protein and Inhibits Hexokinase Binding.* Biophysical Journal, 1985. **47**(2): p. A238-A238.

54. Nakashima, R., et al., *Hexokinase Rexeptor Complex in Hepatoma Mitochondria - Evidence from N,N'-Dicyclohexylcarbodiimide-Labeling Studies for the Involvement of the Pore-Forming Protein VDAC.* Biochemistry, 1986. **25**(5): p. 1015-1021.

55. Bergmeyer, H.U., *Methods of Enzymatic Analysis*. Methods of Enzymatic Analysis, ed. H.U. Bergmeyer. Vol. 3. 1974, New York: Academic Press, Inc.

56. Sharpe, P.T., *Methods of Cell Separation*. Laboratory Techniques in Biochemistry and Molecular Biology, ed. R.H.a.P.H.v.K. Burdon. Vol. 18. 1988, Amsterdam: Elsevier.

57. Patel, D., *Separating Cells*. 2001, New York: Springer.

CHAPTER II

QUANTIFICATION OF GLUCOSE METABOLISM DURING HIGH-VIABILITY BATCH HYBRIDOMA CULTURES

Introduction

The physiology of batch mammalian cell cultures in terms of growth, death, and metabolism is available in various research articles. However, the quality of the data in these published works is not sufficient for quantitative analyses, and there have been no instances of statistical analyses describing the times in which maximum rates occur or analyses to show that the rates are in fact decreasing. Furthermore, the physiology of the particular hybridoma cell line used in this study has not been established during the first 48 hours in batch culture. The preliminary phases of this project involve the quantification of cellular metabolism in high-viability (>70%) batch hybridoma cell cultures. The data from these preliminary experiments will be used as a guide for selection of key time points in which to study hexokinase activity in the mitochondrial and cytosolic fractions in Aim III.

The major research articles investigating growth and metabolic kinetics in batch mammalian cell culture are summarized in Table 2.1. All of these studies report decreasing glucose consumption rate prior to cell death in the batch cultures. However, none of these studies include an error analysis on the calculated rates, and the quality of the data is arguably low, considering samples where removed from the batch only every 12-24 hours. These studies do not really constitute reliable data sets for proper error and statistical analyses, in particular to make quantitative observations concerning peak times

for metabolic rates and make observations concerning the relationship between changes in metabolism and other physiological descriptors.

Table 2.1. Summary of Literature Quantifying Declining Growth and Metabolism in Batch Mammalian Cell Culture. All studies in the table show a phenomenon of declining glucose consumption rate prior to cell death in batch cultures. No error analysis was performed in any of the studies. Sampling times for metabolites were once or twice daily. *NS=Not Specified.

Cell Line	Cultivation Method	Sampling interval (hrs)	Error Analysis	Reference
Hybridoma	Bioreactor	12	None	Ozturk, et. al. 1991[1]
Hybridoma	Bioreactor	12	None	Georgen, et. al. 1992[2]
Hybridoma	T-flask	24	None	Fitzpatrick, et. al. 1993[3]
Hybridoma	T-flask	24	None	Portner, et. al. 1994[4]
Hybridoma	Spinner Flask	12	None	Oh, et. al. 1996[5]
Hybridoma	T-flask	NS*	None	Neermann, et. al. 1996[6]
Hybridoma	Bioreactor	NS*	None	Doverskog, et. al. 1997[7]
Vero	Bioreactor	24	None	Quesney, et. al. 2003[8]

A phenomenon of increasing metabolism has not been observed in batch mammalian cell culture, and decreasing metabolism usually coincides with slowing growth rates and/or growth arrest. However, decreasing glycolytic rates in batch mammalian cell culture have been documented previously in batch and serum-stimulated batch hybridoma cultures where the growth rate was kept at a high level, [2, 7] and the uncoupling of growth and metabolism is also demonstrated in the existence of multiple steady-states at the same growth rate.[7, 9-11] Thus, decreasing metabolism can occur despite sustained growth by growth-factor (insulin) stimulation.[12]

The purpose of this work is to quantify glucose metabolism (i.e., glucose consumption and lactate production rates) and cell growth kinetics during high-viability batch hybridoma cultures by gathering precise data in 4-hour time intervals. Not only

does the phenomenon of decreasing glycolysis need to be quantitatively and precisely measured, it must also be confirmed for this particular hybridoma cell line. Here, a very comprehensive data set showing the physiology of four batch hybridoma cultures in terms of growth, death, glucose uptake rate, and lactate production rate was gathered to quantify the time-profile of metabolism prior to visible cell death. Using mass balance equations on extracellular metabolites in conjunction with the concentration measurements in the batch cultures, I quantify the profile of glucose uptake and lactate production rate in this hybridoma cell line and perform error and statistical analyses to show decreasing metabolism. The discovery of an appreciable decrease in metabolic rates in the early stages (24 hours or less) of batch cultures will be used to discuss the implications in terms of progression to apoptotic cell death. Potential causes of the phenomenon of decreasing metabolism are also discussed.

Materials and Methods

Cell Line and Maintenance

The cell line used was a murine hybridoma (ATCC CRL-1606) that secretes an IgG against human fibronectin. During maintenance and all experiments, the hybridomas were cultivated in a serum-free, hydrolysate-free IMDM formulation, comprised of glutamine-free basal Iscove's Modified Dulbecco's Medium (IMDM, Mediatech) and supplemented with 4.0 mM glutamine (Mediatech), 10 mg/L insulin (Sigma), 5 mg/L holo-transferrin (Sigma), 2.44 μL/L 2-aminoethanol (Sigma), 3.5 μL/L 2-mercaptoethanol (Sigma), and 10 U/ml penicillin – 10 μg/ml streptomycin (Mediatech).

The hybridomas were cultivated in T-flasks (Corning) and maintained in an incubator (Forma Scientific) at 37°C, 10% CO_2 and 95% humidity during maintenance.

Batch Cultures

Batch culture experiments were performed in both T-175 cm^2 culture flasks (Corning) and spinner flasks (Bellco Biotechnology). Initially, the cultures were seeded at a density of 2.0 x 10^5 vcell/mL in complete IMDM and a volume of either 60.0 mL for the T-flasks or 500 mL for the spinner flasks. At t=0 (immediately after seeding), 1.0 mL was sampled into two separate culture tubes (0.5 mL each), trypan blue cell counts were performed (2 slides) with 0.1 mL from each tube, and the remaining culture (0.8 mL) was combined into a 1.2 mL micro-centrifuge tube and centrifuged at 200*g* for 15 minutes (Forma Scientific Centrifuge). The supernatant was transferred to another micro-centrifuge tube and stored at -20°C. The sampling and counting procedure was repeated every four hours until the viability dropped below 70%. Between sampling, the cultures were incubated at 37°C, 10% CO_2, and 95% humidity. Analysis of the supernatants for metabolite concentrations was performed after all samples had been collected. Samples were stored for less than one week prior to metabolite analysis and were subjected to one or less freeze-thaw cycle. All cultures were passaged every 24 hours for at least 2 weeks prior to the experiments, except for the batch designated T-48. This inoculum was maintained every 48 hours 8 days prior to the experiment.

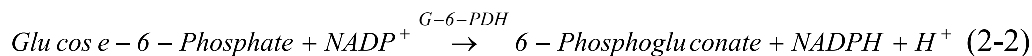
Cell Counting and Apoptosis Determination

Cell number and viability were determined via trypan blue dye exclusion using a hemocytometer. The viability was calculated as the number of viable cells divided by the number of total cells. A minimum of 2 slides were counted for each sample. Apoptosis was characterized using the acridine orange/ethidium bromide fluorescence assay.[13] A total of 400 cells were counted for each slide for the determination of apoptosis. The percentage of each type of cell, whether it was deemed to be early apoptotic, late apoptotic, necrotic, or viable, was determined by dividing the number of each type of cell by the total number of cells counted (400).

Enzymatic Assays

Glucose and lactate concentrations were determined via enzymatic assays based on the recipes developed by Bergmeyer and Lowry,[14] which I reformulated for use in a 96-well plate (Nunc) format.[15] The glucose assay, developed for a range of 0 to 5 mM, consists of the coupled reactions:

$$Glucose + ATP \xrightarrow{Hexokinase} Glucose-6-Phosphate + ADP \quad (2\text{-}1)$$

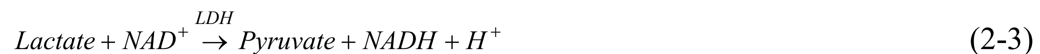


$$Glucose-6-Phosphate + NADP^{+} \xrightarrow{G-6-PDH} 6-Phosphogluconate + NADPH + H^{+} \quad (2\text{-}2)$$

In the first reaction, glucose is phophorylated by hexokinase, and in the second reaction, phosphorylated glucose is converted to 6-phosphogluconate, which is accompanied by the reduction of NADP. The amount of NADPH formed is proportional to the amount of glucose substrate, and the NADPH concentration is detected by UV/vis spectrometry at 340 nm. A calibration line was set up for glucose concentrations in the range of 0-5 mM, and the optical density measurement from the unknown samples was used in the

calibration line to calculate the unknown concentration. The assay was carried out in 96-well plates at room temperature with a reaction volume of 200 μL (30 μL unknown sample/standard, 15 μL hexokinase stock, and 155 μL assay buffer). The assay components consisted of 12 mM ATP, 2.0 mM $NADP^+$, 0.8 mM $MgCl_2$, 0.4 mM DTT, 0.5 U/mL hexokinase, and 0.24 U/mL G-6-PDH in a 20 mM tris buffer solution at pH 8.0. Unknown samples were diluted in glucose-free RPMI medium, and calibration standards were made in the same glucose-free RPMI solvent to remove any effects of phenol red on the optical density measurement. Dilution factors for the unknown samples varied from 4-8X, depending on the expected glucose concentration.

The lactate assay was carried out in a similar approach for a range of 0-3 mM. The reaction describing this assay is a reduction of lactate to pyruvate accompanied by the formation of NADH:



$$Lactate + NAD^+ \xrightarrow{LDH} Pyruvate + NADH + H^+ \quad (2\text{-}3)$$

The final assay concentrations for the lactate assay were 9.0 mM NAD^+ and 50 U/mL LDH in a hydrazine-glycine buffer at pH 9.5. The reaction volume was 200 μL (15 μL unknown sample/standard, 10 μL LDH stock, and 170 μL assay buffer). Dilution factors for the unknown samples ranged from 2-8X, depending on the expected lactate concentration, and dilutions and lactate standards were prepared in DI H_2O as a solvent. For the both the lactate and glucose assays, the incubation time was 90 minutes. All absorbance measurements were performed using a μQuant Microplate Spectrophotometer (Bio-Tek Instruments).

Calculation Methods: Treatment of Experimental Data

Raw concentration and cell density data were used to calculate the specific metabolic rates for glucose consumption and lactate production over time in batch cultures. The rate is calculated from a mass balance using equation 2-4:

$$\frac{dC_i}{dt} = q_i n_V \tag{2-4}$$

where q_i is the uptake or production rate of a metabolite *i,* C_i is the concentration of the metabolite, n_v is the viable cell density of the culture during the time of analysis, and t is the time. If metabolite concentration and growth profiles are known for a batch system over a specified amount of time, then a polynomial regression can be performed and the first derivative can be used to describe the rate of change of the measured metabolite, and thus q can be calculated from equation (2-5). This is the method of calculation that will be used in this study.

$$q_i(t) = \frac{dC_i}{dt} * \frac{1}{n_V(t)} \tag{2-5}$$

Growth and death rates are determined from total and viable cell balances (2-6 and 2-7)

$$\frac{dn_T}{dt} = \mu n_V = \mu f_V n_T \tag{2-6}$$

$$\frac{dn_V}{dt} = \mu n_V - k_d n_V \tag{2-7}$$

where n_T is the total cell density in cells/mL, μ is the growth rate in hr^{-1}, f_v is the fraction viable (n_V/n_T), and k_d is the death rate in hr^{-1}. Again, since the concentration of total and viable cells is known over time, then a polynomial regression can be performed and the first derivative used to calculate growth and death rates from equations 2-8 and 2-9:

$$\mu(t) = \frac{dn_T / dt}{n_v(t)} \tag{2-8}$$

$$k_d(t) = \mu(t) - \frac{dn_V / dt}{n_V(t)} \tag{2-9}$$

Third or fourth order polynomials (depending on the correlation coefficient) were fitted to data for total cell density (cells/mL), viable cell density (vcells/mL), glucose concentration (mM), and lactate concentration (mM) using least-squares polynomial fit procedures and error analysis based on covariant terms in an error matrix (described below). The coefficients for each term in the polynomial were generated, and these models were used in the calculations for growth, death, and metabolic rates as a function of time.

The first derivative of the functions describing glucose consumption and growth rate were calculated using the product rule (2-10 and 2-11) in order to apply statistical methods and calculate the maximum values.

$$\frac{dq_{glc}}{dt} = -\frac{d^2C_{glc}}{dt^2}\frac{1}{n_V} - \frac{dC_{glc}}{dt}\frac{d(1/n_V)}{dt} \tag{2-10}$$

$$\frac{d\mu}{dt} = \frac{d^2n_T}{dt^2}\frac{1}{n_V} + \frac{dn_T}{dt}\frac{d(1/n_V)}{dt} \tag{2-11}$$

Polynomial Fitting and Statistical and Error Analysis

Error on the raw data values for cell density was calculated as the standard deviation of the 2 slides measured. The error on the raw glucose and lactate concentration values was determined by the uncertainty in the calibration line. For the calibration lines

on the enzymatic assays, the optical density (OD, corresponding to NADPH or NADH concentration) is linearly related to the metabolite concentration C.

$$OD = mC + b \qquad (2\text{-}12)$$

where m is the slope and b is the intercept. The calibration line does not intercept the origin since blank wells do have some absorbance, but the OD of the blank was subtracted from each OD value for the standards for calibration purposes. The error on any calculated concentration determined from the calibration line is described by equation 2-13,

$$\sigma = \frac{s_r}{m}\sqrt{\frac{1}{r} + \frac{1}{n} + \frac{(\overline{OD}_i - \overline{OD})^2}{m^2 S_{xx}}} \qquad (2\text{-}13)$$

where s_r is the standard deviation about the regression, r is the number of replicates measured, n is the number of calibration points, OD_i (bar) is the measured quantity, OD (bar) is the average optical density from all calibration points, and S_{xx} is the standard deviation of the measured concentrations in the calibration, given by equation 2-14.

$$S_{xx} = \sum (C_i - \overline{C})^2 \qquad (2\text{-}14)$$

The calculation method in its entirety can be found in *Principles of Instrumental Analysis*.[16]

Polynomial curves were fitted to the raw data values (C_{glc}, C_{lac}, n_T, and n_V) using a determinant solution method described in Chapters 6 and 7 of *Data Reduction and Error Analysis for the Physical Sciences*.[17] The method presented in these chapters is a least-squares fit using the method of determinants. In brief, the method sets up a matrix solution using the raw data values, and calculates the coefficients of each term of the polynomial to a specified degree. For the uncertainty, the chapter includes the calculation of an error matrix, the elements of which are the variance and covariance values for each

of the coefficients. The error matrix is used to calculate the uncertainty for each value of the independent variable. Thus, for any calculated result, the uncertainty can be determined from an equation which uses the partial derivatives of the dependent variable with respect to each polynomial coefficient. The calculation method is quite lengthy and not presented in detail here. The error bars for q_{glc} (glucose consumption), q_{lac} (lactate production), μ (growth rate), and k_d (death rate) were all derived from this method. The degree of the polynomial needed for each raw data set was determined by the value of the correlation coefficient (R^2) and the significance of each coefficient as determined by ANOVA in JMPIN 4.0 software.

Statistical treatment of the data was accomplished by either Model I ANOVA, which analyzes treatment effects, followed by Tukey's Test or a student's t-test. Normality of the data was verified through calculations of skewedness and kurtosis. Statistical tests were done in Microsoft Excel, although all calculations were verified in JMPIN 4.0 statistical software. Table 2.2 summarizes the statistical comparisons applied to this data set.

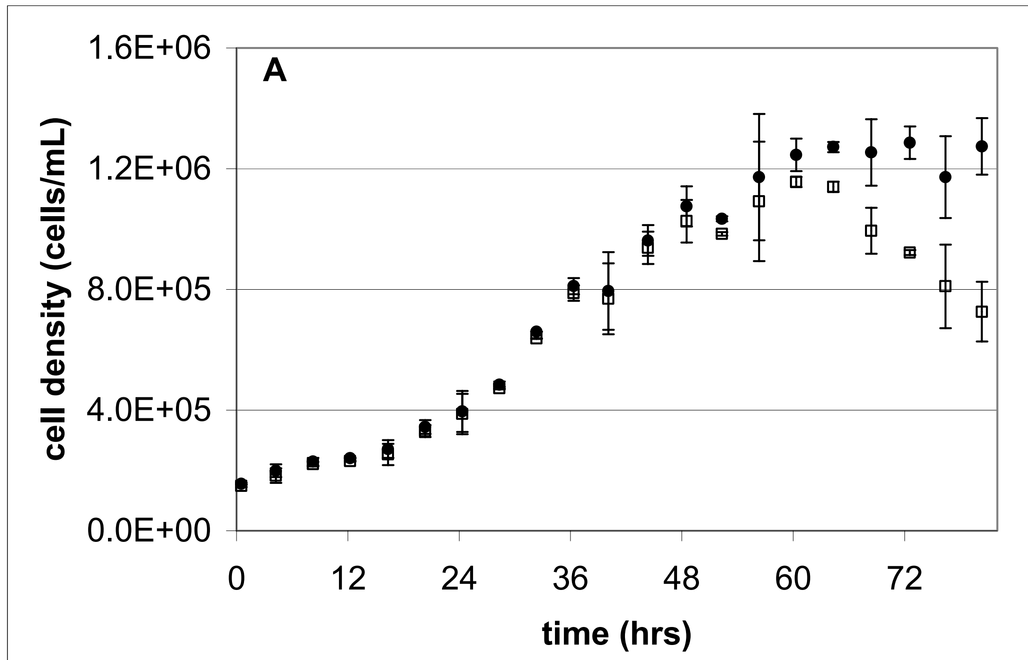
Table 2.2. Summary of Statistical Techniques Applied to Reduced Batch Data. Comparisons were made to statistically show that glucose consumption rate decreases early in the batch cultures and prior to decreases in growth rate and viability. *MSD=minimum significant difference.

comparison	statistical test	sample size	degrees of freedom	basis of comparison
Determination of time in which functions are statistically decreasing	Model I ANOVA/ Tukey's Test	2 or 3	1 or 2	F statistic; MSD*
Comparison of statistically decreasing times	Two-sample t-test	8	6	t statistic
Comparison of peak times for q_{glc} and μ	Two-sample t-test	8	6	t statistic
Comparison of **difference** in peak times for q_{glc} and μ	One-sample t-test	4	3	t statistic

Results

Profile of Cell Growth and Metabolism during High-Viability Batch Hybridoma Cultures

Figures 2.1 through 2.4 illustrate the kinetics of growth and metabolism in one batch experiment for cells cultivated in a T-flask. Since all the batch experiments exhibited very similar physiological phenomena, the experimental data from this one experiment is presented in detail, and the results from all 4 replicates are summarized in Table 2.2. Figure 2.1 shows the profile of total and viable cell densities for high-viability (>70%) batch hybridoma cell cultures. Figure 2.1A is the raw data values with errors (standard deviations on the measurements from 2 counted slides). The increasing nature of the error bars with time is due to dilution errors during high-density, late-stage culture. When a polynomial regression is performed and the error is calculated again, the error bars are reduced (Figure 2.1B). In some cases, the data were truncated slightly in order to improve the polynomial fit. The average standard deviation for the errors on the raw data for the cell counts was about 5.4×10^4, and the average standard deviation for the values in the polynomial fit was about 2.2×10^4, which represents over a 50% reduction in error.



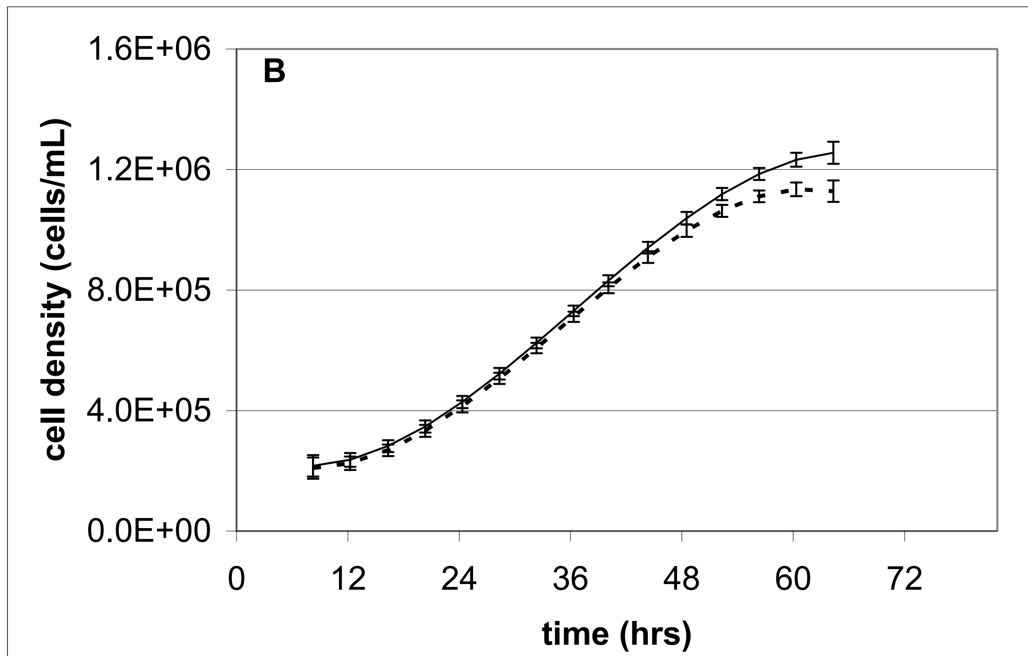
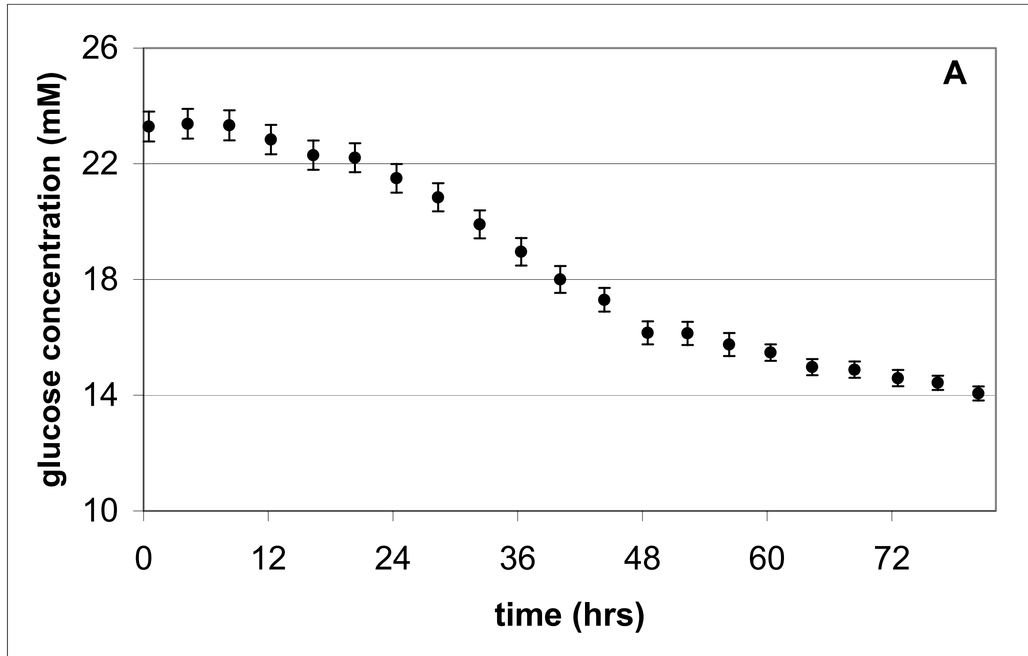
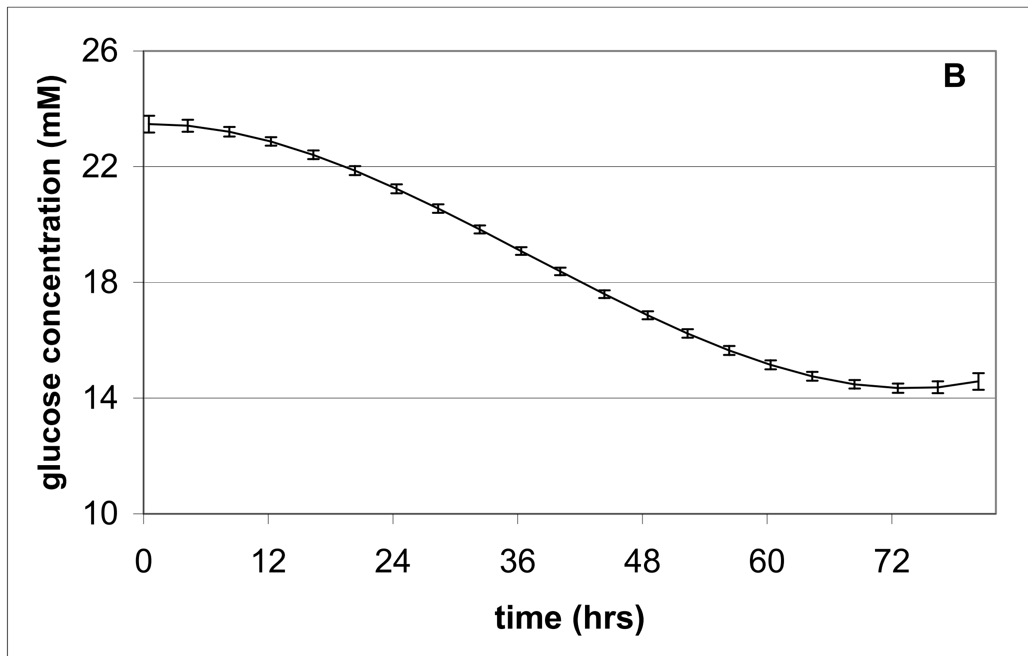


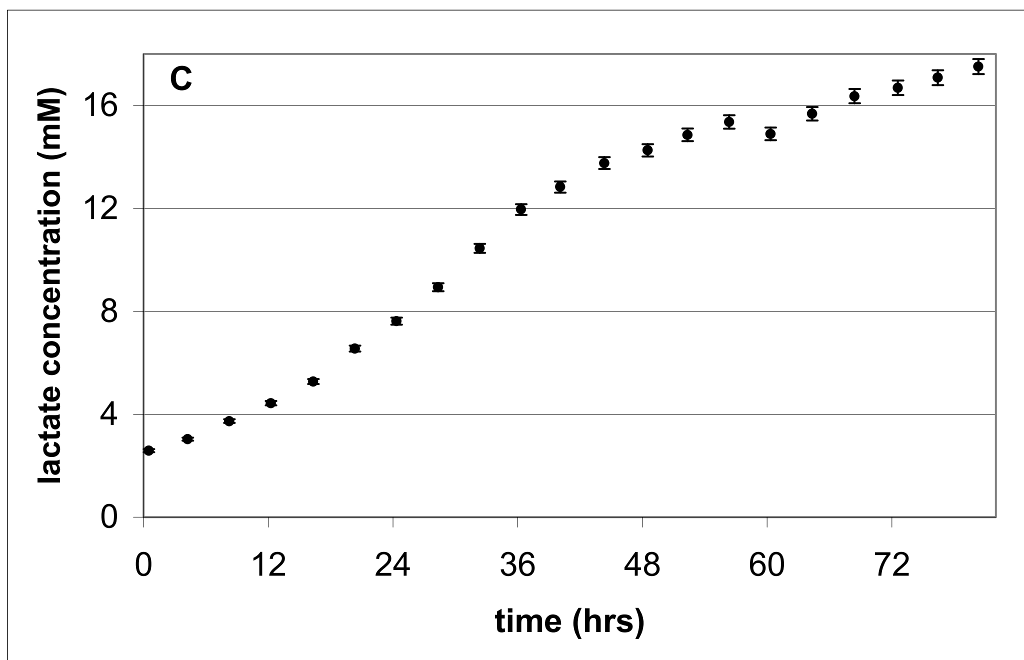
Figure 2.1. Total and Viable Cell Densities of Batch Hybridoma Culture. The raw data measurement values (A) for both total cell density (●) and viable cell density (□) are somewhat noisy, but this noise is reduced in error estimated from the polynomial fits (B).

The profile of growth kinetics in batch hybridoma cultures can also be observed in Figure 2.1. The cells exhibit a lag phase of growth, which ends about 12 hours after subculture at 2.0 x 10^5 cells/mL. The cells continue to grow until about 48 hours, when the growth rate (or more appropriately, division rate) has slowed considerably and apoptotic cell death begins, as evidenced by the deviation in total and viable cell density curves. In batch culture, this particular cell line achieves densities ranging from 1.3-3.0 x 10^6 cells/mL, depending on the inoculum and mode of cultivation.

The raw data values for glucose and lactate concentration in this batch culture are shown in Figure 2.2, A and C, and the corresponding polynomial fits, 3rd and 4th order for glucose and lactate, respectively, are shown in Figure 2.2, B and D. Again, the polynomial fit analysis reduces the estimates of errors significantly. The average error on the glucose concentration is reduced from 0.41 to 0.17, and the average lactate error is reduced from 0.19 to 0.08. The initial presence of a small amount of lactate (~3 mM) is the result of passaging.


26
22
18
14
10
A
glucose concentration (mM)
0
12
24
36
48
60
72
time (hrs)


26
22
18
14
10
B
glucose concentration (mM)
0
12
24
36
48
60
72
time (hrs)



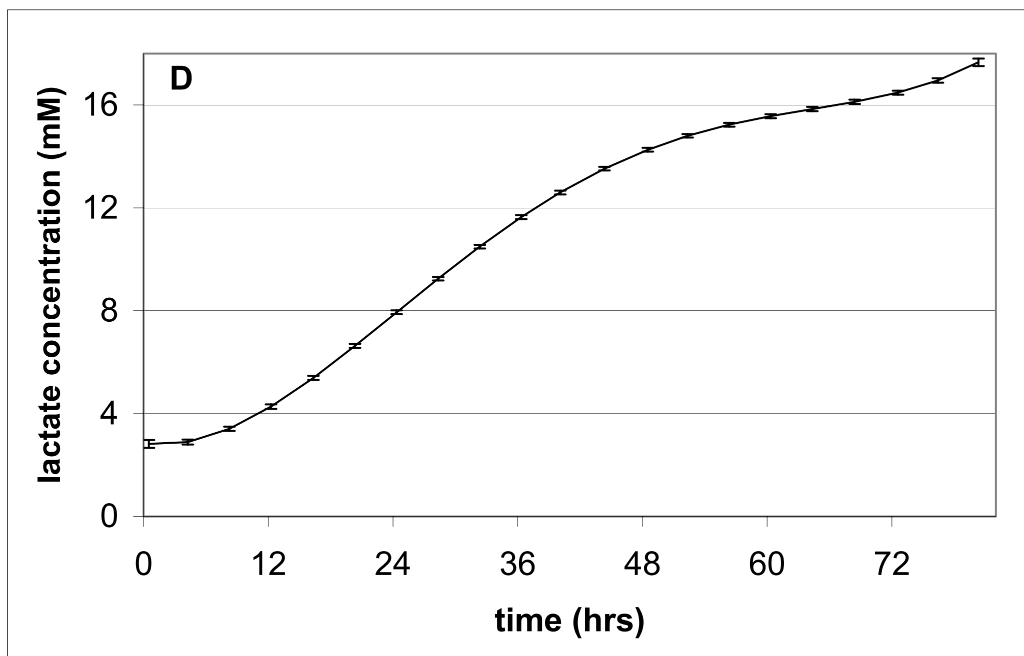


Figure 2.2. Glucose and Lactate Concentration Profiles in Batch Hybridoma Culture. Glucose concentration-time data (A) was fitted with a 3rd order polynomial (C), and lactate concentration-time data (B) was fitted with a 4th order polynomial (D).

Using the polynomial equations for C_{glc}, C_{lac}, n_V, and n_T, the growth rate, death rate, glucose consumption rate, and lactate production rate were calculated according to the mass balance equations. Figure 2.3 illustrates the resulting profile of growth rate and death rate in this batch culture. Clearly, growth rate reaches a maximum early in the culture and exhibits a decreasing trend throughout the batch. Death rate begins slowly ascending after about 48 hours. No real significance is attributed to the non-zero death rate at 8-12 hours after subculture, at which time the culture is still in the lag phase of growth. Although mammalian cells in culture are known to exhibit primarily apoptotic cell death,[18-20] the occurrence of apoptosis was verified using the AO/EB assay in the spinner flasks and in other T-flask experiments (separate from the experiments shown here) for this cell line. By 72 hours after subculture in T-flasks, the cells were approximately 60% viable, and 36% apoptotic, and 4% necrotic. And by 52 hours after subculture in the spinner flasks, the cells were approximately 37% viable, 59% apoptotic, and 4% necrotic.

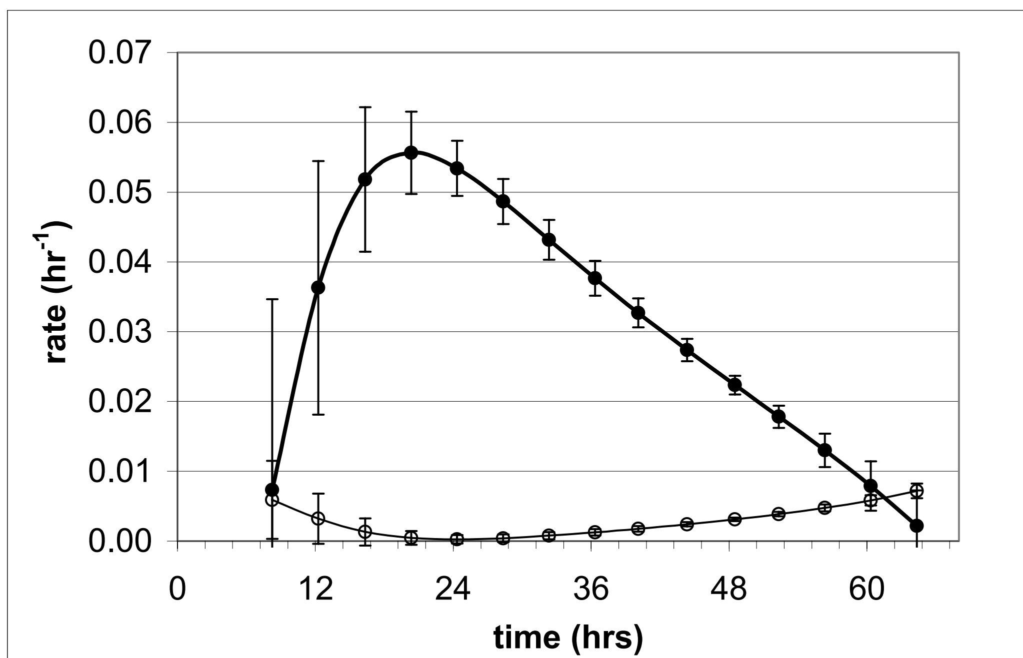


Figure 2.3. Growth and Death Rates in Batch Hybridoma Culture. Growth rate (●) peaks early in the culture and steadily declines over the course of the batch. Death rate (○) begins slowly ascending after about 48 hours.

Similar to the profile of growth rate, metabolic rates, specifically glucose consumption and lactate production, reach a maximum very early in the batch culture and decrease throughout the duration of the batch (Figure 2.4). The absolute magnitude of the metabolic rates and the extent of the decrease throughout the batch is a function of both the inoculum maintenance schedule and mode of cultivation (Table 2.2).

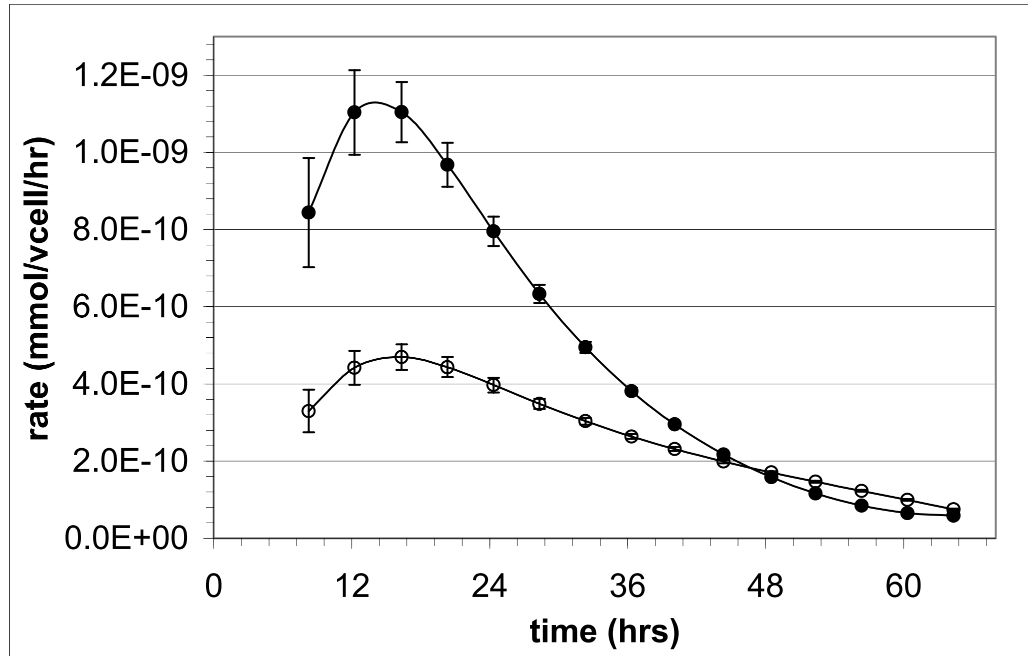


Figure 2.4. Glucose Consumption and Lactate Production Rates in Batch Hybridoma Culture. Metabolic rates peak near the end of the lag phase of growth (~12-20 hours after subculture) and continue to decline until apparent cell death (~48 hours after subculture). Metabolic data is shown for glucose consumption (○) and lactate production (●) rate.

Table 2.3 summarizes the major metabolic and growth rate observations from all 4 replicate experiments. The inoculum variability between the T-48 flask and the other three cultures is quite apparent. Maximum glucose consumption rates occurred between 15 and 20 hours for all batches, and maximum growth rates occurred after 20 hours. The glucose consumption rate decreases about 2.5-3.7X between 15 and 48 hours, depending on the inoculum. Lactate production rate, although not listed in the table, decreased much more variably, ranging from 4X to 18X lower between 15 and 48 hours depending on inoculum, which indicates variable metabolic shift for each culture. These physiological

changes occur well in advance of the apparent decreases in viability, which happens 48-68 hours after subculture.

Table 2.3. Summary of Batch Hybridoma Culture Experiments. The peak glucose consumption rates were about 5 x 10^{-10} mmol/vcell/hr. except for the 48-hour inoculum, which exhibited much higher metabolism. Errors are the standard deviations calculated from the polynomial fits. Peak metabolic rates were determined by setting the first derivative of the GUR and growth rate functions to zero. **mmol/vcell/hr x 10^{-10}, **q_{glc} at 44 hrs is listed here since the data were truncated*

experiment	peak q_{glc} *	time (hrs)	peak μ (hr^{-1})	time (hrs)	q_{glc} at 48 hours *	time f_v ↓ (hrs)
T-24 Flask	4.70±0.34	15.84	0.056±0.006	20.35	1.71±0.04	64
T-48 Flask	7.41±0.35	20.65	0.052±0.002	26.97	1.99±0.03	68
Spinner 1	4.74±0.37	15.67	0.065±0.007	22.88	1.87±0.08**	48
Spinner 2	5.23±0.34	15.79	0.066±0.005	23.09	2.15±0.05	48

Statistical Comparisons among Growth, Metabolism, and Cell Death

Enabled by calculated rates that have improved error estimates, statistical tests were performed to garner information on the times in which glucose consumption, growth, and viability are reaching a maximum and statistically decreasing. This allows for observations to be made concerning the order of physiological events prior to apoptotic cell death and also allows for statistical significance of the decreasing nature of growth and metabolism prior to cell death to be established. Initially, a Model I ANOVA was performed by comparing each time point between 12 and 48 hours for each separate experiment. All ANOVA tests were found to be significant based on calculated F values that were far greater than the critical F ($p<0.0001$ in most cases). Tukey's Test, comparing each pair of means, identified the times in which we can say statistically that each function was decreasing. The results are summarized in Figure 2.5. Clearly, GUR statistically decreases prior to growth rate or viability.

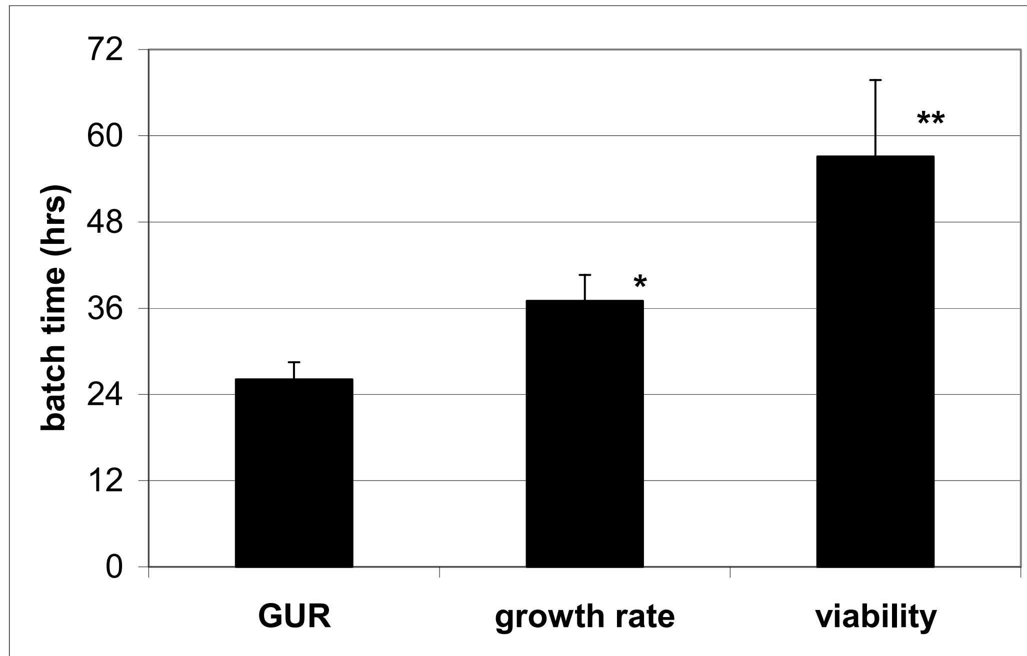


Figure 2.5. Comparison of Times in which Glucose Consumption Rate, Growth Rate, and Viability Statistically Decrease in Batch Hybridoma Cultures. For each separate experiment, comparisons were made among the various time points by one-way ANOVA followed by Tukey's Test. Data shown is the time in which each physiological descriptor was statistically lower than the maximum value. Error bars are the standard deviations of the 4 replicate experiments. Statistical comparison was performed via a one-tailed student's t-test. The α level was not adjusted to reduce Type I error since the comparison of means was not for comparison of specific treatments, but rather physiological occurrences. Sample size was 3 for the q_{glc} ANOVA and 2 for both the growth rate and viability ANOVA tests. *$p<0.0112$, **$p<0.0025$

Comparison of the peak times of glucose consumption rate and growth rate can be performed by either a one-sample or two-sample t-test (Table 2.4). For the two-sample t-test, the peak times of glucose consumption rate for each flask were averaged, and this was also done for the peak times of growth rate. The two means were compared, yielding a t-statistic of 3.46 ($t_{critical}$=2.45). This was significant at the 99.9% confidence level. For the one-sample, the difference in the times of peak glucose consumption and growth was calculated for each flask, and the difference was compared to zero. This yielded a t-

statistic of 9.79 ($t_{critical}$=5.84), which is also significant at the 99.9% confidence level. Thus, two-different t-tests show that glucose consumption reaches a maximum statistically prior to growth rate.

Table 2.4. Summary of Times in which Maximum Growth and Glucose Consumption Occur in Batch Hybridoma Cultures. The maximum values were determined by setting the derivative to zero and noting the time in which the maximum occurs. The data in the table was used for both a one-sample and two-sample t-test. Means for the time of peak q_{glc} and μ were compared to each other, and the difference in times was compared to zero. ***p<0.001*

experiment	time of peak q_{glc} (hrs)	time of peak μ (hrs)	t_{μ}-t_{qglc}
T-24 Flask	15.84	20.35	4.51
T-48 Flask	20.65	26.97	6.32
Spinner 1	15.67	22.88	7.20
Spinner 2	15.79	23.09	7.30
Average	**16.99±2.44**	**23.32±2.73****	**6.34±1.29****

Discussion

The key findings and observations in this chapter are (1) glucose consumption and lactate production are observed to decrease statistically over time in batch hybridoma (CRL 1606) cell cultures, (2) decreases in metabolism occur well in advance of apoptotic cell death, and (3) glucose consumption rate statistically reaches a maximum prior to growth rate. Although growth and metabolic kinetic data has been previously published, the application of error analysis by polynomial fitting and statistical analyses on peak rates in this project is a novel approach to analyzing physiological data. A second approach to function fitting by generalized logistic equations was attempted,[21] but did not provide acceptable fits to the data by visual inspection and also did not provide any method of error analysis, which was essential for this study. Here, I have presented firm statistical evidence that glucose consumption rate begins declining before growth rate and viability, and this certainly provides a firm basis for speculation that declines in metabolism in the early stages of batch culture may contribute to unfavorable physiological outcomes, such as apoptotic cell death.

This study precisely illustrates a statistical decrease in glucose consumption and lactate production very early in batch hybridoma cultures (26 hours after subculture, on average), which has not been previously determined. The quantification of decreasing metabolism very early after subculture has several implications in terms of the progression to apoptotic cell death in batch hybridoma cultures. Literature reviews have hypothesized that decreasing glucose consumption may not provide the mitochondria with enough substrates to maintain permeability, thus eventually causing apoptotic protein release and subsequent death.[22, 23] It has also been hypothesized that given the

fact that Akt functions to promote cell survival by increasing glucose transporters and hexokinase activity in hematoma cells over-expressing Akt,[24] apoptotic proteins, such as Bax, might function as a metabolic sensor, and when metabolism reaches a certain threshold, the apoptotic mechanism is activated.[23] This is pure speculation, of course, but the fact that we can now prove a statistical decline in metabolism prior to cell death is persuasive circumstantial evidence to support such hypotheses for continued studies.

Statistically, growth rate is reaching a maximum and decreasing after glucose consumption, but it should be noted that the growth rate measurement is actually division rate. The division rate of the cells may be expected to lag glucose consumption rate since cells in the S and G2/M phase will complete the cell cycle in the coming hours, presumably allowing growth rate to appear high. A real measure of growth rate is to see whether cells in G0/G1 actually are growing more slowly or not, but this was not pursued in this study. So, there might actually not be an uncoupling of growth and metabolism in this cell line for the batch mode of cultivation, although we do statistically observe an apparent uncoupling using the measurements of this particular data set.

Glutamine is known to be the second largest carbon source for mammalian cell lines after glucose.[25] To verify that glutamine consumption is not increasing to compensate for the decrease in glucose consumption, glutamine/glutamate was measured in the spinner flask cultures (data not shown). The calculated rate exhibited the same peak followed by a steady decrease, identical to the glucose and lactate rates.

Changing environmental conditions, such as pH and osmolality, are known to contribute to decreasing glucose consumption rate.[26-28] The pH and osmolality in batch T-flask cultures was measured in this study (albeit in separate T-flask cultures from the

metabolic studies), and osmolality decreased only 6 mOsm/kg solvent between subculture and 48 hours. The pH decreased from 7.3 at subculture, to 7.14 at 24 hours, to 6.92 at 48 hours. A study regarding the effects of pH on glucose uptake rate in continuous steady-state hybridoma cultures found little dependence on glucose uptake for pH values 7.0 and above,[26] and the pH in the cultures in this study remains above 7.0 for most of the batch. Thus, during the beginning of the decline in metabolism, pH was presumably stable.

It should be noted that the magnitude of metabolism is not necessarily a factor here, nor is the presence or absence of metabolic shift as evidenced from calculation of the q_{lac}/q_{glc} ratio for each batch. In fed-batch studies, glucose metabolism is maintained at lower levels, and in fact this feeding maintains viability a bit longer despite reduced glucose metabolism.[29] Thus, regardless of inoculum variations and feeding of nutrients, the phenomenon of metabolic decline still occurs in advance of cell death.

The findings in this chapter suggest a possible role for declining metabolism in the activation of the apoptotic pathway since glucose consumption is indeed observed to be declining along with or in advance of declining growth and in advance of death. The question as to what is causing such a decline remains, and there are numerous possible hypotheses for this question. This research project moved forward to investigating one possible cause: hexokinase detachment from the mitochondria. Interestingly during some preliminary work, I discovered that this cell line is sensitive to an indirect Akt inhibitor, LY294002. Supplementation of the culture medium with this compound caused apoptotic cell death as evidenced by AO/EB and decreased glucose consumption in 4-hour metabolic rate screens in concentrations up to 10 μM (data not shown). The Akt pathway

is known to exert cell protective effects by increasing cellular metabolism, and the fact that this cell line is sensitive to inhibition of this pathway suggests that the pathway is active in this cell line and it plays a role in cell survival and homeostasis. And intriguingly, it has been reported that addition of LY294002 in MEF cell lines also decreases mitochondrial hexokinase activity.[30] This work implied a susceptibility of the hybridoma cells to mitochondrial hexokinase detachment. In conclusion, decreasing glucose consumption and susceptibility to LY294002 provided the basis for my investigation of hexokinase detachment in hybridoma batch culture and its possible role in declining glycolysis and apoptotic cell death.

References

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

2. Goergen, J.L., A. Marc, and J.M. Engasser, *Comparison of Specific Rates of Hybridoma Growth and Metabolism in Batch and Continuous Cultures.* Cytotechnology, 1992. **10**(2): p. 147-155.

3. Fitzpatrick, L., H.A. Jenkins, and M. Butler, *Glucose and Glutamine-Metabolism of a Murine B-Lymphocyte Hybridoma Grown in Batch Culture.* Applied Biochemistry and Biotechnology, 1993. **43**(2): p. 93-116.

4. Portner, R., et al., *Estimation of Specific Glucose-Uptake Rates in Cultures of Hybridoma Cells.* Journal of Biotechnology, 1994. **34**(3): p. 237-246.

5. Oh, G.S., et al., *Interactive dual control of glucose and glutamine feeding in hybridoma cultivation.* Journal of Fermentation and Bioengineering, 1996. **81**(4): p. 329-336.

6. Neermann, J., Wagner, R., *Comparative analysis of glucose and glutamine metabolism in transformed mammalian cell lines, insect and primary liver cells.* Journal of Cellular Physiology, 1996. **166**(1): p. 152-169.

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

8. Quesney, S., et al., *Kinetics and metabolic specificities of Vero cells in bioreactor cultures with serum-free medium.* Cytotechnology, 2003. **42**(1): p. 1-11.

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

10. Follstad, B.D., R. R. Balcarcel, G. Stephanopolos, D. I. C. Wang, *Metabolic Flux Analysis of Hybridoma Continuous Culture Steady State Multiplicity.* Biotechnology and Bioengineering, 1999. **63**(6): p. 675-683.

11. Guardia, M.J., et al., *Cybernetic modeling and regulation of metabolic pathways in multiple steady states of hybridoma cells.* Biotechnology Progress, 2000. **16**(5): p. 847-853.

12. Zeng, A.P. and W.D. Deckwer, *Mathematical-Modeling and Analysis of Glucose and Glutamine Utilization and Regulation in Cultures of Continuous Mammalian-Cells.* Biotechnology and Bioengineering, 1995. **47**(3): p. 334-346.

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

14. Bergmeyer, H.U., *Methods of Enzymatic Analysis*. Methods of Enzymatic Analysis, ed. H.U. Bergmeyer. Vol. 3. 1974, New York: Academic Press, Inc.

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

16. Skoog, D., *Principles of Instrumental Analysis*. 3rd. ed. 1985, Philadelphia: Saunders College Publishing.

17. Bevington, P., *Data reduction and error analysis for the physical sciences*. 1969, New York: McGraw-Hill.

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

19. Singh, R.P., et al., *Apoptosis and its control in cell culture systems.* Cytotechnology, 1997. **23**(1-3): p. 87-93.

20. Mastrangelo, A.J.a.M.J.B., *Implications and Applications of Apoptosis in Cell Culture.* 1995. **6**(2): p. 198-202.

21. Jolicoeur, P. and J. Pontier, *Population-Growth and Decline - A 4-Parameter Generalization of the Logistic Curve.* Journal of Theroretical Biology, 1989. **141**(4): p. 563-571.

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

23. Hammerman, P., C. Fox, and C. Thompson, *Beginnings of a signal-transduction pathway for bioenergetic control of cell survival.* TRENDS in Biochemical Sciences, 2004. **29**(11): p. 586-592.

24. Rathmell, J., Fox CJ, Plas DR, Hammerman PS, Cinalli RM, Thompson CB, *Akt-directed glucose metabolism can prevent Bax conformation change and promote growth factor-independent survival.* Molecular and Cellular Biology, 2003. **23**(20): p. 7315-7328.

25. Stephanopoulos, G., Aristidou, A. A., Nielsen, J., *Metabolic Engineering: Principles and Methodologies*. 1998, San Diego: Academic Press. 725.

26. Miller, W.M., H.W. Blanch, and C.R. Wilke, *A Kinetic-Analysis of Hybridoma Growth and Metabolism in Batch and Continuous Suspension-Culture - Effect of Nutrient Concentration, Dilution Rate, and Ph.* Biotechnology and Bioengineering, 1988. **32**(8): p. 947-965.

27. Miller, W.M., H. W. Blanch, C. R. Wilke, *A Kinetic Analysis of Hybridoma Growth and Metabolism in Batch and Continuous Suspension Culture: Effect of*

Nutrient Concentration, Dilution Rate, and pH. Biotechnology and Bioengineering, 2000. **67**(6): p. 853-871.

28. Ozturk, S.S., M.R. Riley, and B.O. Palsson, *Effects of Ammonia and Lactate on Hybridoma Growth, Metabolism, and Antibody-Production.* Biotechnology and Bioengineering, 1992. **39**(4): p. 418-431.

29. Xie, L.Z. and D.I.C. Wang, *Applications of Improved Stoichiometric Model in Medium Design and Fed-Batch Cultivation of Animal-Cells in Bioreactor.* Cytotechnology, 1994. **15**(1-3): p. 17-29.

30. Majewski, N., et al., *Hexokinase-mitochondria interaction mediated by Akt is required to inhibit apoptosis in the presence or absence of Bax and Bak.* Molecular Cell, 2004. **16**(5): p. 819-830.

CHAPTER III

EFFECTS OF CLOTRIMAZOLE ON MITOCHONDRIAL HEXOKINASE ACTIVITY, GLYCOLYSIS, AND APOPTOSIS IN HYBRIDOMA CULTURES

Introduction

Despite the implications of mitochondrial hexokinase playing a significant regulatory role in both glycolytic and apoptotic pathways, published research evaluating the *in vitro* physiological effects of intracellular hexokinase re-distribution between the mitochondrial and cytosolic fractions is sparse. In 1992, a published work investigated the response of rat diaphragm muscle to calcium-immobilizing hormones via treatment, separation of the fractions, and enzymatic assay of hexokinase activity and found that mitochondrially bound hexokinase was increased.[1] A follow-up study by Penso and colleagues evaluated the effects of calmodulin antagonists clotrimazole and bifonazole on mitochondrial hexokinase activity in melanoma cells and discovered that interruption of calcium transport imparted the opposite effect and mitochondrial hexokinase activity was decreased.[2] Clotrimazole has since been utilized in cell free platforms to study the response of mitochondria to hexokinase detachment in terms of permeability and apoptotic protein release and also to illustrate the requirement of mitochondrial hexokinase in the anti-apoptotic effects of Akt.[3, 4] Clotrimazole has additionally been used to exhibit the inhibition of Bax-dependent apoptotsis by mitochondrial hexokinase binding.[4]

It has been shown that clotrimazole treated carcinoma cells exhibit decreased amounts of glycolytic intermediates, namely glucose 1,6-bisphoshpate and fructose 1,6-

bisphosphate, and ATP levels in a dose and time-dependent manner via treatment and enzymatic assay of the intermediates.[5] This suggests a decrease in glycolytic activity, although glucose consumption rates were not evaluated in this study. And the effects of hexokinase gene transfection on growth rate and average lactate production have been evaluated for an NID-3T3 cell line, and growth rate appears to be slightly enhanced while the average lactate production rate increased 2-fold, suggesting increased glycolysis.[6] The amount of HK in the mitochondrial fraction also increased 2-fold while the increase in cytosolic HK was nominal, indicating that mitochondrial HK has large glycolytic control in this cell line. The effects on glucose consumption were also not evaluated in this study. Although cell-free studies on isolated mitochondria show that hexokinase more efficiently phosphorylates glucose when attached to the mitochondria, there are no *in vitro* studies to support such a claim.[7-9] If mitochondrial hexokinase is more efficient than cytosolic hexokinase (i.e., lower K_m value and less inhibition by glucose-6-phosphate), then detaching hexokinase from the mitochondria *in vitro* would cause a measurable decrease in glucose consumption in cases where the enzyme exerts a high level of control on the pathway. And since metabolic control through fed-batch cultivations is widely used in industry, these results have particular relevance to bioprocesses since perturbation of mitochondrial hexokinase could potentially serve as a target to control glycolysis.

Furthermore, if mitochondrial hexokinase plays a role in maintaining VDAC closure and preventing apoptotic protein release, then detachment of this enzyme *in vitro* would induce apoptotic cell death. A dose-dependent decrease in viability as determined by trypan blue dye exclusion has been documented for clotrimazole treated melanoma and carcinoma cultures,[2, 5, 10] although the mode of death has not been quantified.

Additionally, the kinetics of induced mitochondrial hexokinase detachment by clotrimazole have not been evaluated on any cell line.

The activity of the bax-induced pathway or the function of mitochondrial hexokinase is not known for the hybridoma CRL 1606 cell line. The general objective of this work is to explore the activity of the bax-induced apoptotic pathway in CRL 1606 and quantify the effects that perturbation of this pathway has on cell death and glycolysis by inducing mitochondrial hexokinase detachment. If the Aim III studies show that hexokinase is indeed becoming detached during batch culture, then the effects that this detachment has on death and glucose consumption must be quantified to make the claim that such an occurrence in batch cultures is detrimental to the cells. Here, clotrimazole will be used as a probe to induce hexokinase detachment. If it is shown that clotrimazole treatment exerts no effect on this cell line, this would indicate that either clotrimazole does not detach hexokinase from the mitochondria or the cells do not require hexokinase to be bound to the mitochondrial to maintain homeostasis. In the following studies, I induce mitochondrial hexokinase detachment in hybridoma cultures via supplementation of clotrimazole to the culture medium and measure apoptotic morphology and calculate short-term glucose consumption rates. I also verify the antagonistic effects of clotrimazole on mitochondrial hexokinase binding. These experiments satisfy Aims I and II of this research project. Results are used to discuss the potential role of mitochondrial hexokinase in the regulation of glycolysis and apoptotic cell death.

Materials and Methods

Cell Line and Maintenance

The cell line used was a murine hybridoma (ATCC CRL-1606) that secretes an IgG against human fibronectin. During maintenance and all experiments, the hybridomas were cultivated in a serum-free, hydrolysate-free IMDM formulation, comprised of glutamine-free basal Iscove's Modified Dulbecco's Medium (IMDM, Mediatech) and supplemented with 4.0 mM glutamine (Mediatech), 10 mg/L insulin (Sigma), 5 mg/L holo-transferrin (Sigma), 2.44 μL/L 2-aminoethanol (Sigma), 3.5 μL/L 2-mercaptoethanol (Sigma), and 10 U/ml penicillin – 10 μg/ml streptomycin (Mediatech). The hybridomas were cultivated in T-flasks (Corning) and maintained in an incubator (Forma Scientific) at 37°C, 10% CO_2 and 95% humidity during maintenance.

Analytical Techniques

Cell Counting and Apoptosis Determination

Cell number and culture viability were determined via trypan blue dye exclusion using a hemocytometer. Culture viability was calculated as the number of viable cells divided by the number of total cells. A minimum of 2 slides were counted for each sample. Apoptotic morphology by the presence of DNA fragmentation was characterized using the acridine orange/ethidium bromide fluorescence assay.[11] A total of 400 cells were counted for each slide for the determination of apoptosis. The percentage of each type of cell, whether it was deemed to be early apoptotic, late apoptotic, necrotic, or

viable, was determined by dividing the number of each type of cell by the total number of cells counted. Differentiation of each cell type was determined by the visual inspection of fragmented DNA and color.

Enzymatic Assays

Glucose concentrations for the metabolic rate screening experiments were determined enzymatically via the exact method previously described in Chapter II. Samples were not diluted since the highest expected glucose concentration was 4.0 mM, which is less than the detection limit of 5.0 mM.

Hexokinase activity in the mitochondrial and cytosolic fractions was determined enzymatically using previously described methods which I re-formulated for use in a 96-well plate format.[12] The reaction scheme for the assay is the same as the glucose assay (equations 2-1 and 2-2 in Chapter II). The final concentrations of components in the assay buffer were 216 mM glucose, 1.1 mM ATP, 1.2 mM NADP, 10 mM $MgCl_2$, and 1.0 U/mL G6PDH in 40 mM triethanolamine buffer (pH=8.0). After addition of 15 μL of undiluted sample to 155 μL of assay buffer, the reaction was initiated by the addition of 30 μL of 1.44 M glucose solution (in triethanolamine buffer). The absorbance, which corresponds to the concentration of NADPH, was measured every minute over a 10-minute period at a 340 nm wavelength using the kinetic feature on a μQuant UV/vis plate reader (Bio-Tek Instruments). The concentration of NADPH in the sample was determined by Beer's Law,

$$A = \varepsilon b C \qquad (3\text{-}1)$$

where A is the measured absorbance of the well, ε is the emissivity of NADPH at 340 nm (6.22 $mM^{-1}cm^{-1}$), b is the path length of the well (0.606 cm) and C is the concentration of NADPH (mM). Samples were analyzed in triplicate at room temperature (25°C). Hexokinase activity (units) at the specified conditions was calculated as the initial velocity of the reaction (i.e., slope of the resulting line), which has the units of μmol of NADPH formed per minute per liter of reaction volume in the well at 25°C and pH 8.5.

Cell Fractionation

Mitochondrial and cytosolic fractions were separated via centrifugation of homogenates in a sucrose buffer. First, 7.5 x 10^6 total cells were sampled and centrifuged for 15 minutes at 200*g* and 25°C to pellet the cells, the spent medium was removed, and the cells were re-suspended in 200 μL ice-cold 250 mM sucrose buffer (pH=7.2, prepared in PBS) supplemented with 10 μL/mL buffer Halt® protease inhibitor cocktail (Pierce). Next, the cells were homogenized for 2 minutes using a Kontes homogenizer. The 2-minute time was verified as the minimum time necessary for complete cell lysis. The lysate mixture was then centrifuged at 700*g* for 10 minutes and 4°C to pellet cell debris (nuclei, membranes, etc..), and the supernatant was removed and transferred to an additional tube. The cell debris was discarded. The sample was then centrifuged again for 15 minutes at 12,000*g* and 4°C to pellet the mitochondria. The supernatant (cytosolic fraction) was transferred to another tube, and the mitochondrial fraction was re-suspended in 100 μL sucrose buffer. Samples were stored at -80°C for later hexokinase activity analysis.

Experimental Procedures

Clotrimazole (CTZ, Sigma), a specific inhibitor of Ca^{2+}-activated potassium channels, was used to induce hexokinase detachment from the mitochondria. Clotrimazole has been identified as a calmodulin antagonist, which disrupts intracellular calcium homeostasis, including calcium transport in the mitochondria. This calcium transport is necessary for enhanced mitochondrial hexokinase binding.[13-15] Admittedly, clotrimazole may have other unknown effects on intracellular homeostasis, but this approach is the most direct way known to detach hexokinase. A 25 mM clotrimazole stock was prepared in dimethyl sulfoxide (DMSO, Sigma) and used for all experiments. All cultures received 0.08% (v/v) DMSO regardless of clotrimazole concentration for all experiments except the resazurin reduction viability experiment.

Verification of Hexokinase Detachment by Clotrimazole

The kinetic measurements of hexokinase activity in the mitochondrial and cytosolic fractions for clotrimazole concentrations from 0 to 20 μM were performed in T-75 cm^2 culture flasks (Corning). Test medium stocks with 2X the amount of clotrimazole to be tested were prepared in complete IMDM maintenance medium. The flasks were seeded at an initial volume of 24.0 mL and a density of 1.0 x 10^6 vcells/mL by adding 12.0 mL of a 2.0 x 10^6 vcell/mL culture to 12.0 mL of the test medium stock. The seeding culture was obtained from a 1.0 x 10^6 vcell/mL parent flask by centrifuging the contents of the flask at 200*g* for 15 minutes (Forma Scientific Centrifuge), removing the spent media, and re-suspending with complete IMDM. Immediately after seeding, 7.5 mL of culture from each flask was sampled from each flask and fractionated. The flask was

incubated at 37°C, 10% CO_2 and 95% humidity, and the sampling and fractionation procedures were repeated at 1 hour and either 2 hours or 4 hours after seeding. After fractionation, the mitochondrial and cytosolic fractions were stored at -80°C for later hexokinase activity analysis. Each concentration was tested in triplicate with a separate control.

Metabolic Rate Screening (MRS)

The metabolic rate screening experiments were performed as previously described.[16] Briefly, hybridoma cells were cultivated in T-175 cm^2 flasks, centrifuged at 200*g* for 10 minutes, and re-suspended in a low-glucose RPMI 1640 test medium (Sigma) containing various concentrations of clotrimazole (8 to 16 μM). These concentrations were chosen as a subset of the 2-20 μM range previously tested in an attempt to cause the most amount of detachment with the least amount of cell death in a 4-hour period. The RPMI test medium was fully supplemented similar to the IMDM maintenance medium. The cells were then seeded into a 24-well plate at a density of 1.75×10^6 vcells/mL and incubated for 4 hours at 37°C, 95% humidity, and 10% CO_2. Samples for initial concentrations of glucose were removed from the cultures used to seed the well plate. Samples from each well were removed after 4 hours, placed in separate micro-centrifuged tubes, centrifuged, and supernatants were transferred to a new set of micro-centrifuge tubes. All samples were stored at -20°C for later glucose concentration analysis. Cell counts were performed by trypan blue dye exclusion on 2 wells of each concentration at the conclusion of the experiment. The experiment was performed 4 times, for a total of 16 replicates for each clotrimazole concentration. This experimental

platform is more desirable than T-flask studies because it provides quick, multi-replicate, quantitative information concerning the metabolic effects of clotrimazole.

Determination of Culture Viability

The initial viability measurements using trypan blue dye exclusion and AO/EB for a 0-30 μM clotrimazole concentration range and 4-hour exposure period were performed in 24-well plates (Nunc) as part of a metabolic rate screening experiment as described above. The results of the metabolic screen for these concentrations are not presented in this study, but the viability measurements are presented to illustrate cell death by the trypan blue marker. Measurement of cell viability using resazurin reduction was performed in 96-well plates (Nunc) at a density of 7.5×10^5 cells/mL and clotrimazole concentrations of 0-30 μM using previously published methods.[17] The apoptosis measurements by AO/EB were performed in triplicate in 6-well plates (Nunc). Test medium stocks were prepared in the same manner as the MRS experiments above, at 2X the concentration to be tested. Cells were seeded at a volume of 2 mL and a density of 7.5×10^5 vcells/mL by adding 1.0 mL of culture to 1.0 mL of 2X test medium stock. The seeding culture was obtained from an ~8×10^5 vcells/mL parent flask by centrifuging the contents of the flask at 200*g* for 15 minutes, removing the spent media, and re-suspending with complete IMDM. At 2, 4, 8, and 16 hours after seeding, a volume of 100 μL was removed from each well and separately mixed with AO/EB dye for counting.

Calculation Methods

Intracellular Hexokinase Balance

To equate the hexokinase concentrations between the mitochondrial and cytosolic fractions and derive the relationship between the measured activities in the buffer to the intracellular activities, the molar amounts of hexokinase in each fraction must be determined and related to the molar amounts in the assay buffer. A mass balance can be written on the total intracellular hexokinase:

$$n_T C_{tHK} = n_T C_{cHK} V_c + n_T C_{mtHK} S_{mt} \tag{3-2}$$

where n_T (cells/mL) is the number of total cells, C_{tHK} (mmol/cell) is the total concentration of hexokinase, C_{cHK} (mmol/$L_{cytosol}$) is the concentration of hexokinase in the cytosol, V_c ($L_{cytosol}$/cell) is the volume of cytosol, C_{mtHK} (mmol/cm^2) is the concentration of hexokinase per area of mitochondria, and S_{mt} (cm^2/cell) is the area of mitochondria. The first term on the right side of equation 3-2 represents the amount of unbound hexokinase, and the second term represents the amount of bound hexokinase (in total moles). Thus, the ratio of bound hexokinase to total hexokinase can be described by equation 3-3.

$$\frac{bound\ HK}{total\ HK} = \frac{n_T C_{mtHK} S_{mt}}{n_T C_{cHK} V_c + n_T C_{mtHK} S_{mt}} \tag{3-3}$$

Below, I will derive how I can relate this relationship to the reaction kinetics of the hexokinase activity assay.

Reaction Kinetics for the Hexokinase Activity Assay

For the reaction in the sucrose buffer, we can assume Michaelis-Menten kinetics for the rate of disappearance of the glucose substrate, [18]

$$-\frac{dC_{glc}}{dt} = \frac{V_{max} C_{glc}}{K_m + C_{glc}} \tag{3-4}$$

where C_{glc} (mM) is the concentration of glucose, V_{max} (mM/min) is the maximal velocity of the reaction, and K_m (0.1 mM) is the Michaelis constant for glucose.[12] At sufficiently high substrate concentrations ($>>K_m$), the rate of disappearance of glucose is approximately equal to the maximal velocity.

$$-\frac{dC_{glc}}{dt} \cong V_{max} \tag{3-5}$$

But the rate of glucose disappearance is equal to the rate of NADPH formation by mass balance (1:1 molar ratio), assuming that the second reaction is non-limiting (see equation 2-2).

$$-\frac{dC_{glc}}{dt} = \frac{dNADPH}{dt} \tag{3-6}$$

Furthermore, the initial rate of the reaction, V_{max}, is proportional to the enzyme concentration, E, by a reaction constant, k.[18]

$$V_{max} = kE \tag{3-7}$$

So combining equations 3-5, 3-6, and 3-7, we get

$$\frac{dNADPH}{dt} = kE \tag{3-8}$$

Equation 3-8 is an expression for the concentration of hexokinase enzyme, E, valid in both the mitochondrial and cytosolic fractions. We can also multiply each side of equation 3-8 by the volume to get the total moles of enzyme:

$$V_{buffer}\frac{dNADPH}{dt} = V_{buffer}kE \quad (3\text{-}9)$$

Relating Assay Measurement to Intracellular Hexokinase Concentration

The total moles from equation 3-9 are equivalent to the total moles from the intracellular hexokinase balance in 3-3.

$$\frac{bound\ HK}{total\ HK} = \frac{n_T C_{mtHK} S_{mt}}{n_T C_{cHK} V_c + n_T C_{mtHK} S_{mt}} = \frac{V_{buffer} kE_{mito}}{V_{buffer} kE_{mito} + V_{buffer} kE_{cyto}} \quad (3\text{-}10)$$

Substituting equation 3-8 into 3-10, we get

$$\frac{bound\ HK}{total\ HK} = \frac{V_{buffer} \Delta NADPH_{mito}}{V_{buffer} \Delta NADPH_{mito} + V_{buffer} \Delta NADPH_{cyto}} \quad (3\text{-}12)$$

Equation 3-12 describes the calculation for the relationship between the mitochondrial bound and total hexokinase concentrations, which will be valid only for the measurement of the initial reaction rates. If the buffer volume is the same for each fraction, then V_{buffer} will cancel out of the equation. It should be noted that the dilution factor for the addition of sample into the assay buffer in the well plate was also considered. In the hexokinase activity assay, the change in NADPH is measured, and the volume of the buffer for each fraction is known. Thus, the fraction bound is calculated by equation 3-12. It should be noted that during assay development, the sum of the measured activities in the fractions was tested against the total activity in a sample that was not fractionated, and the results were consistent.

Rate Calculations for Metabolic Rate Screening (MRS)

Rates for each well of the metabolic screening experiment were calculated as the change in concentration divided by the viable cell density of the seed culture and the time

interval of 4 hours. Average rates for control and clotrimazole-supplemented cultures were taken as the average and standard deviation from a conglomeration of 16 wells (4 wells from each of the 4 replicate experiments).

Error Analysis and Statistical Methods

Statistical treatment of the clotrimazole-induced mitochondrial hexokinase detachment experiments was accomplished by multivariate ANOVA and Model I ANOVA followed by the Tukey-Kramer Method. The outcome of the multivariate ANOVA is an F statistic, which is compared to a critical F value to determine statistical difference for each source of variation. The Tukey-Kramer Method compares all sets of means to each other using the calculated minimum significant difference (MSD). If the difference in any pair of means is greater than the MSD, then the means are said to be statistically different.

Statistical treatment of the metabolic rate screening data was accomplished by (1) randomized block analysis to test for any potential influence of inoculum variations on the calculated glucose consumption rate and (2) Model I ANOVA followed by either Dunnett's Test to test the effects of clotrimazole on glucose consumption rate as compared to the control or the Tukey-Kramer Method. A significant F value from the ANOVA indicates that there is a significant difference between the means of 2 or more groups at the 95% confidence level. In the randomized block, F values were generated for both the inoculum and CTZ and then compared to a critical F value based on the degrees of freedom. For the follow-up Dunnett's Test, a value for the minimum significant difference is calculated based on the mean square within and compared to the differences

in the means of each test group and the control. If the difference in means is greater than the minimum significant difference, then there is a statistically significant difference in the means.

Errors on the trypan blue and resazurin reduction viability measurements were calculated as the standard deviation of 2 independent replicates. Errors on the fraction of each cell type determined by AO/EB were calculated as the standard deviation from 3 independent replicates. Comparison of viabilities for each concentration at each time point was done using Model I ANOVA followed by the Tukey-Kramer Method. All calculations and statistical tests were performed using Microsoft Excel and the results of the statistical tests were verified using JMP 4.0 statistical software.

Results

Clotrimazole Decreases the Fraction of Hexokinase Bound to the Mitochondria in Hybridoma Cultures

The experiments quantifying the kinetics of hexokinase detachment from the mitochondria by clotrimazole treatment involved 3 different sources of variance: inoculum, time, and clotrimazole treatment. Initially, the effects of all 3 sources of variation on the fraction of total hexokinase bound to the mitochondria were determined statistically using multivariate ANOVA (Table 3.1). The effects of both time and clotrimazole treatment are significant sources of variation. The 2 and 15 μM concentrations showed a significant inoculum variation.

Table 3.1. Multivariate ANOVA Results for Aim II Experiments. The effects of clotrimazole treatment, time, and inoculum were tested. The effects of inoculum were insignificant for 2, 10 and 20 μM concentrations, while the effects of clotrimazole treatment and time were largely significant for all concentrations. *$p<0.05$

	F statistics		
clotrimazole (μM)	**treatment**	**time**	**inoculum**
2	24.4*	26.3*	0.8
5	406.7*	13.0*	50.0*
10	2090.4*	91.3*	2.6
15	1378.8*	26.9*	33.6*
20	311.8*	10.5*	0.4

A portion of hexokinase does bind to the mitochondria in hybridoma CRL 1606 cultures, as indicated by the *in vitro* measurement of hexokinase activity in the mitochondrial fraction. That portion is estimated to be between 20% and 30% of the total amount of hexokinase, which corroborates estimates for another hybridoma cell line.[7] Furthermore, clotrimazole reduces the amount of hexokinase associated with the mitochondrial fraction (Figures 3.1 and 3.2). For the 0+ and 1 hour time points, the fraction of hexokinase bound to the mitochondria was statistically similar across the 0-5 μM concentration range (Figure 1A). However by 4 hours after exposure, there is a dose-dependent trend in the fraction of hexokinase bound to the mitochondria among the 0, 2, and 5 μM concentrations according to the Tukey-Kramer Method, which indicated statistical difference among the means at the 95% confidence level. Furthermore, the 0 and 2 μM concentrations exhibited a statistically significant increase in total hexokinase activity between 1 and 4 hours (Figure 1B). The 5 μM concentration exhibited statistically constant values for both fraction bound and total hexokinase activity over the 0-4 hour time interval studied.

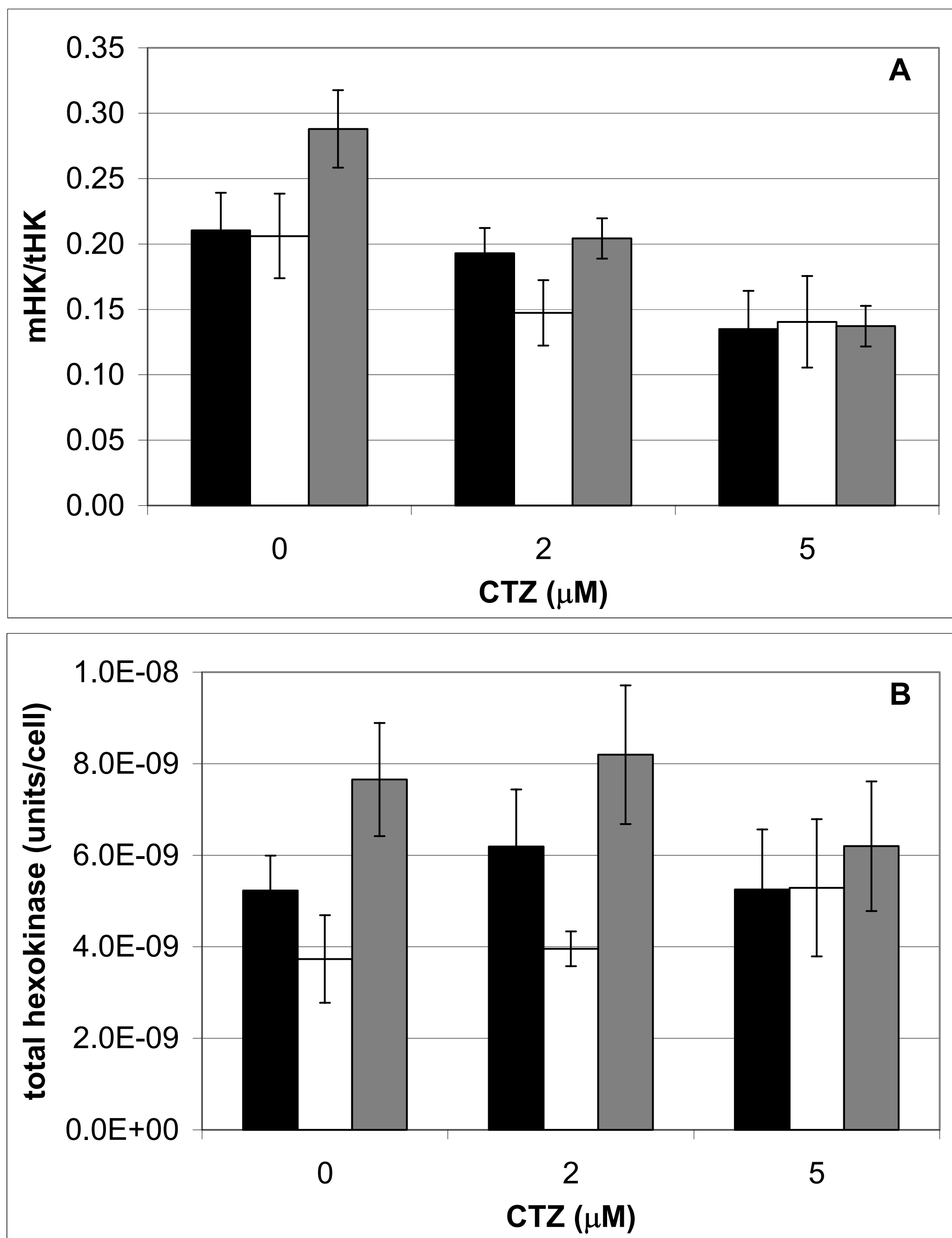


Figure 3.1. Fraction of Hexokinase Bound to the Mitochondria and Total Hexokinase Activity during 4-Hour Exposure to 0, 2, and 5 μM Clotrimazole. Statistical analysis with the Tukey-Kramer Method reveals that there is statistical difference in the fraction of hexokinase bound to the mitochondria among the 0, 2, and 5 μM concentrations 4 hours after exposure (A), but clotrimazole treatment did not affect the total hexokinase activity (B). Error bars represent the standard deviation of three replicates. Data is shown for 0+ (black bars), 1 hour (white bars), and 4 hours (grey bars) after clotrimazole exposure.

Figure 3.2 illustrates the fraction of hexokinase bound to the mitochondria and the total hexokinase activity for cultures exposed to 0, 10, 15, and 20 μM clotrimazole over a 2-hour period. For these larger concentrations, there is no dose-dependency in the fraction bound among the 10, 15, and 20 μM concentrations for any of the time points studied as determined by comparison of means from the Tukey-Kramer Method (Figure 3.2A). The fraction of hexokinase bound to the mitochondria for all tested concentrations was statistically decreased from the control to a level of about 10%. Clotrimazole treatment did not exert any effect on the total hexokinase activity (Figure 3.2B), but it should be noted that the control cultures and the 2 μM clotrimazole-treated culture from Figure 3.1B exhibited a small decrease in total hexokinase activity 1 hour after exposure and then an increase 2 or 4 hours after exposure.

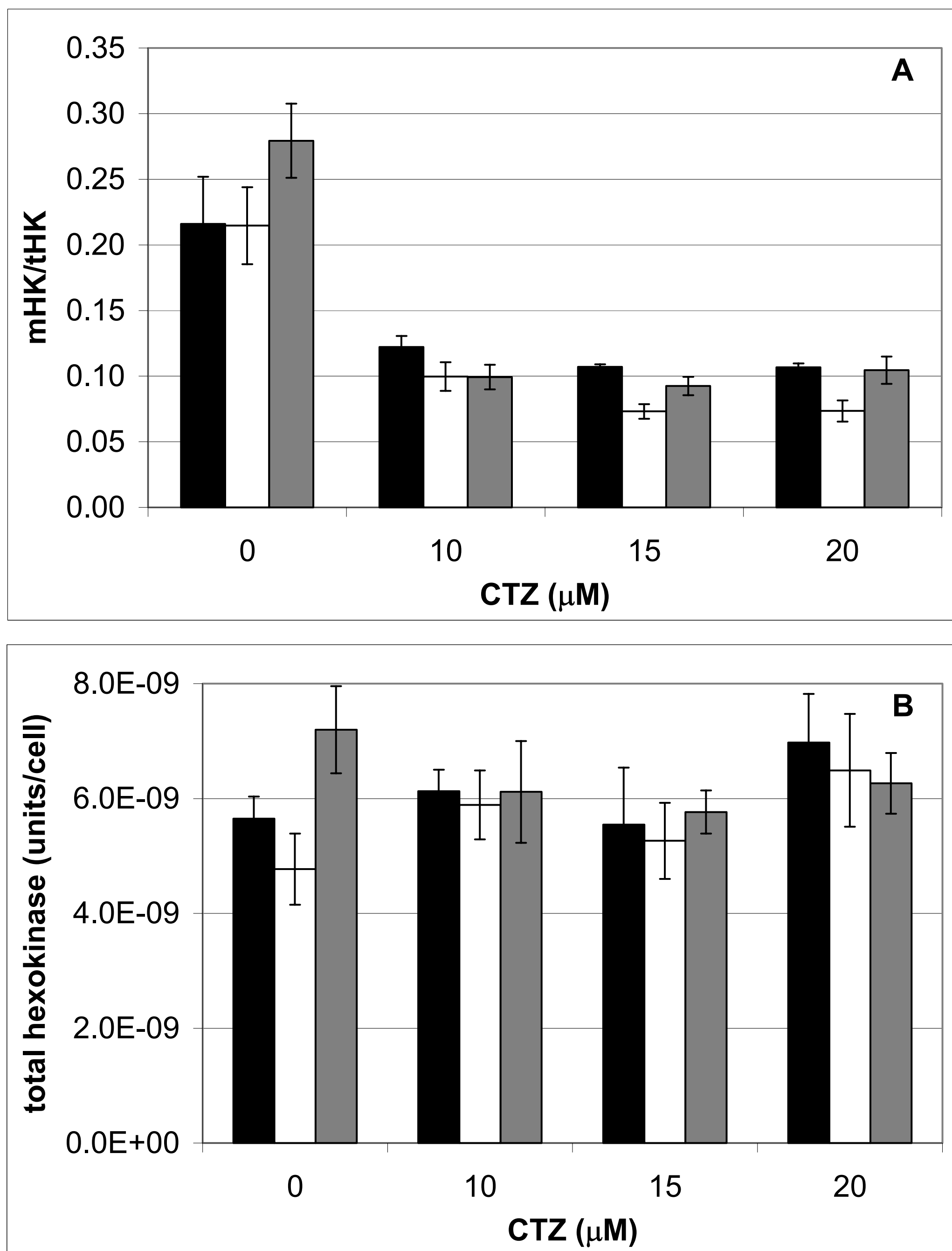


Figure 3.2. Fraction of Hexokinase Bound to the Mitochondria and Total Hexokinase Activity during 2-Hour Exposure to 0, 10, 15, and 20 μM Clotrimazole. Statistical analysis with the Tukey-Kramer Method reveals that there is no statistical differences in the fraction of hexokinase bound to the mitochondria among the 10, 15, and 20 μM concentrations 2 hours after exposure (A), but the fraction of hexokinase bound to the mitochondria for each concentration was statistically decreased from the control. Total hexokinase activity remained fairly constant over the 2-hour time interval (B). Error bars represent the standard deviation of three replicates. Data is shown for 0+ (black bars), 1 hour (white bars), and 2 hours (grey bars) after clotrimazole exposure.

Clotrimazole Causes Decreased Average Glucose Consumption Rate over a 4-Hour Interval

Previous studies have reported dose-dependent decreases in glycolytic intermediates for carcinoma cells exposed to clotrimazole concentrations up to 50 μM for 0.5-2 hours.[5] However, here I show that the average glucose consumption rate as a function of clotrimazole concentration over a 4-hour period is decreased 12-22% in a dose-*independent* manner for an 8-16 μM concentration range (Figure 3.3). The concentrations were chosen as a subset of the 2-20 μM range in an attempt to produce a large decrease in mitochondrial hexokinase activity in conjunction with the largest observable effects on glucose consumption. A randomized block analysis of the data yielded F values of 4.9 and 13.2 for the inoculum and clotrimazole, respectively. Comparing to the critical F values of 8.7 and 9.7, respectively, it is clear that the changes in glucose consumption can be attributed solely to clotrimazole treatment since the effects of inoculum are insignificant. The results of a Model I ANOVA yielded an F value of 11.1, and compared to the critical F of 2.33, this shows there is a significant difference between two or more means of the test groups. A follow-up with Dunnett's test confirmed that all experimental test groups are significantly different from the control with 95% confidence. Furthermore, a follow-up with the Tukey-Kramer Method did reveal that the 12 μM concentration caused a statistically decreased glucose consumption rate as compared to the other concentrations, but the difference was relatively small.

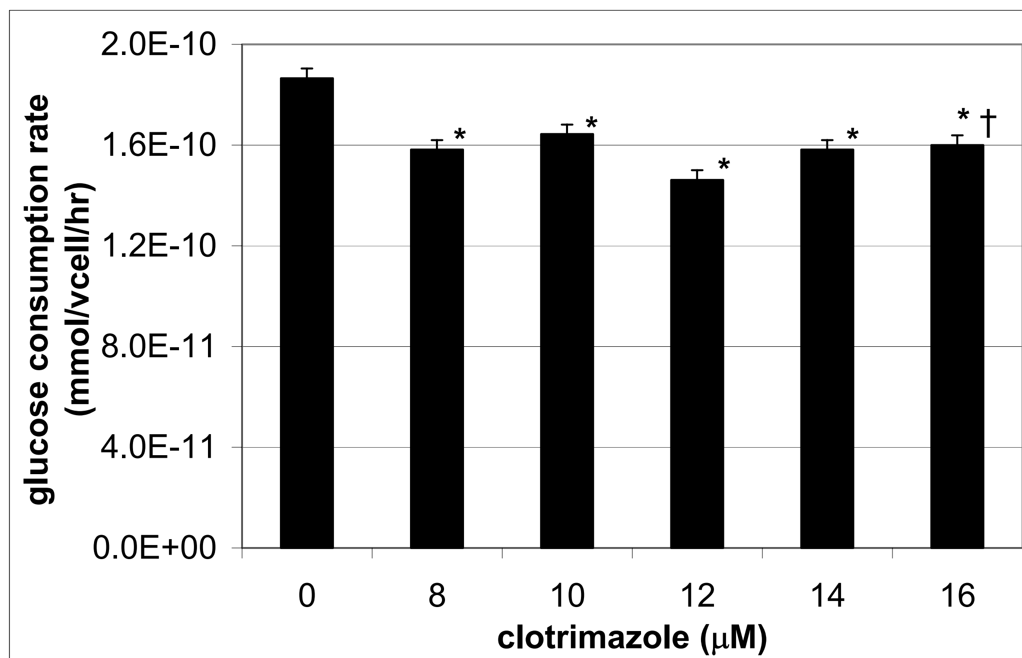


Figure 3.3. Average Glucose Consumption Rate as a Function of Clotrimazole Concentration over a 4-Hour Time Interval. Hexokinase detachment induced by CTZ leads decreased glucose consumption in this hybridoma cell line. The decrease ranges from about 12 to 22% and does not follow a dose-dependent trend. All test cultures exhibited glucose consumption that was statistically decreased from the control. **p<0.05, †16 μM treatment resulted in viabilities significantly decreased from the control after 4 hours*

Clotrimazole Causes Cell Death Exhibiting Apoptotic Morphology

The effects of clotrimazole on cell viability were quantified using 3 different dyes to make a comprehensive assessment of cell death: trypan blue dye exclusion, resazurin reduction (Alamar Blue), and AO/EB. The trypan blue dye assessment was used for an initial short-term (4 hour exposure) measurement of viability for a concentration range of 0-30 μM, and the data show significant decreases in viability for the 15, 20, and 30 μM concentrations at the 95% confidence level (Table 3.2). This data was additionally assessed with AO/EB after the trypan blue counts. The 30 μM concentration proved to be

a particularly lethal dosage, resulting in approximately 60% necrosis after 4 hours (AO/EB data not shown).

Table 3.2. Trypan Blue Viability of Hybridoma Cultures Treated with Varying Clotrimazole Concentrations. Cells were cultivated in 24-well plates as part of an MRS experiment. Statistical treatment was performed with Model I ANOVA followed by Dunnett's Test. *$p<0.05$

Clotrimazole (μM)	Viability
0	0.97±0.003
5	0.94±0.011
10	0.93±0.001
15	0.84±0.008*
20	0.67±0.072*
30	0.12±0.003*

Subsequent testing of viability focused on clotrimazole concentrations from 0 to 20 μM since the 30 μM concentration caused a significant amount of death exhibiting necrotic morphology (i.e., absence of fragmented DNA). Cell death was further quantified using resazurin reduction after 16 hours of exposure (Figure 3.4). Clearly, there is a dose-dependency for culture viability after this exposure time.

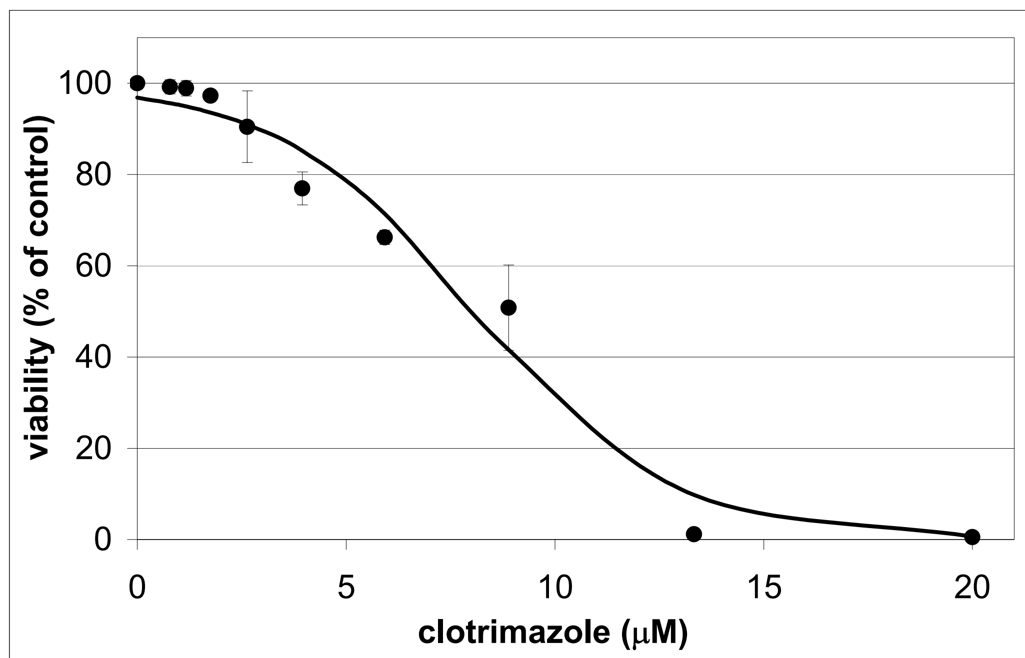
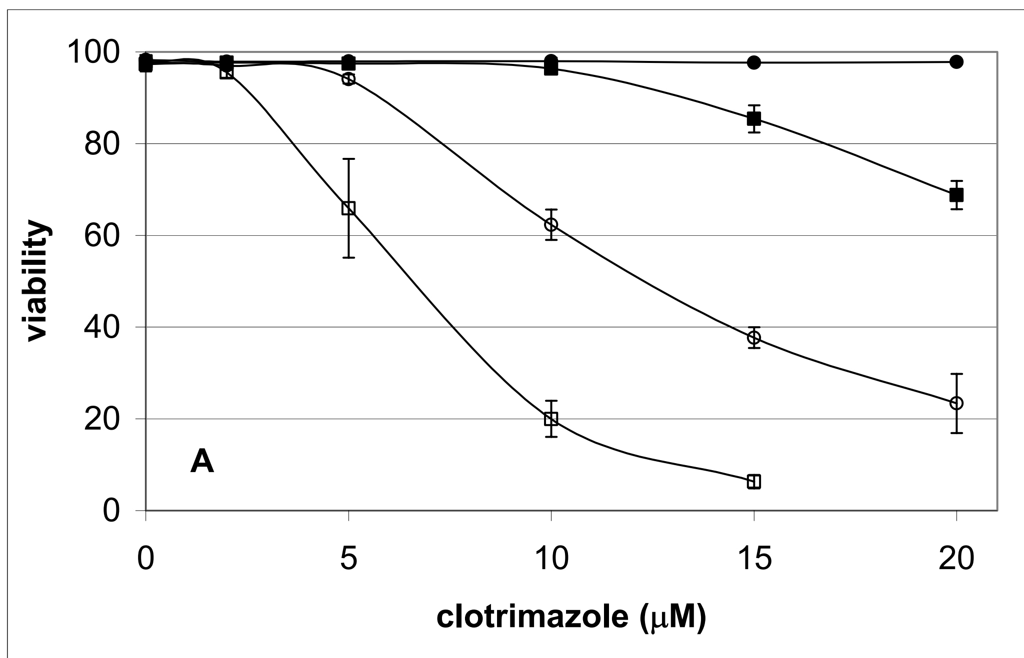


Figure 3.4. Viability by Resazurin Reduction of Hybridoma Cultures Treated with Varying Clotrimazole Concentrations. Data shown is for a 16 hour exposure. The black circles represent the raw data points, and the black line represents the best fit line.

Lastly, cell viability was determined over a 16-hour exposure time using the AO/EB fluorescence assay to again verify dose-dependent decreases in viability for clotrimazole treatment and also to qualify the presence of apoptotic morphology (Figure 3.5). Again, dose-dependent decreases in viability as determined by the Tukey-Kramer Method are witnessed. And from Figure 3.5B, it was determined that the primary mode of cell death was apoptosis based on the presence of DNA fragments in the majority of the dead cells counted. The 15 and 20 μM concentrations did exhibit some necrosis, but on average less than 15%.



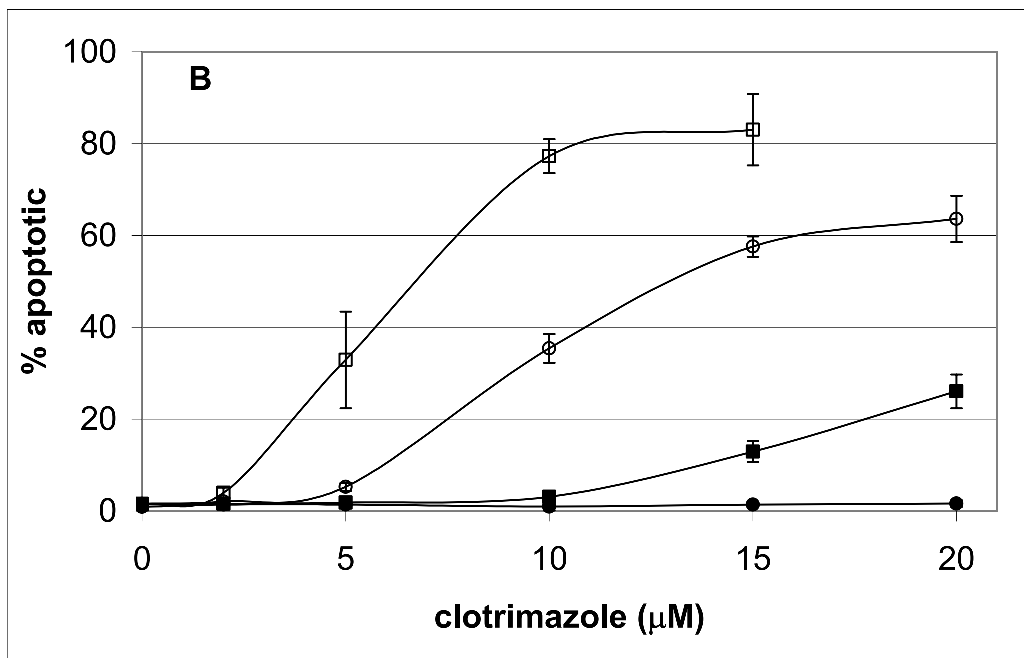


Figure 3.5. Viability by AO/EB of Hybridoma Cultures Treated with Varying Clotrimazole Concentrations. Data shown is after 2 (●), 4 (■), 8 (○), and 16 (□) hours of exposure. The overall viability (A) is reduced in a dose- and time-dependent manner. Based on the appearance of fragmented DNA, most cell death was deemed apoptotic (B).

Discussion

The results presented here reveal that the addition of clotrimazole to biopharmaceutical-producing hybridoma CRL 1606 cultures has a negative physiological impact in terms of apoptotic cell death and decreased glucose consumption. The quantification of the kinetics of induced hexokinase detachment from the mitochondria by clotrimazole, the changes in viability over longs times and assessment of apoptotic morphology, and the quantification of effects on glucose uptake rate are all novel contributions of this work. Furthermore, the metabolic and death effects of mitochondrial hexokinase detachment by clotrimazole in a biopharmaceutical-producing cell line have not been measured until now. The data from this study supports Aims I and II of this research: the effects clotrimazole on short-term glucose consumption and cell death have been quantified and the action of hexokinase re-localization to the cytosol by clotrimazole treatment has been verified. Below, I elaborate on the results and the potential implications that the data impart concerning the effects of clotrimazole treatment and mitochondrial hexokinase on both glucose consumption and cell survival.

Clotrimazole treatment decreases the *in vitro* amount of hexokinase activity associated with the mitochondrial fraction (Figures 3.1 and 3.2). The amount of bound hexokinase never reaches zero, even for the highest tested clotrimazole concentration. This result corroborates previously published studies, which shows a "leveling off" of HK detachment between 15 and 40 μM, then a continuation of the decreasing trend.[2] Three potential explanations for this result are offered here. First of all, lack of complete detachment could be explained by the action of the molecule. Clotrimazole detaches mitochondrial hexokinase by inhibiting calcium transport across the mitochondrial

membrane. However, this is probably not a complete inhibition—some transport probably remains. Relatively little is known about the role of calcium in mitochondrial hexokinase binding, but it has been said to only "enhance" binding.[15] Thus, even if there is absolutely no calcium transport (i.e., there is complete inhibition by clotrimazole), some basal level of hexokinase could still remain bound. Secondly, experimental error could potentially be to blame for the lack of complete detachment. If there is not a complete separation of the mitochondrial and cytosolic fractions, then ultimately we will never see all hexokinase in the cytosolic fraction since there will always be some contamination of HK in the mitochondrial fraction. However, there is no solid reason to believe this is occurring, particularly since the method has been proven to yield highly efficient separations and the lack of complete detachment corroborates previously published studies. And lastly, the fact that we do not see the mitochondrial HK activity reach zero could simply be due to investigating concentrations that are lower than required for complete detachment.

In terms of the kinetics of hexokinase detachment via clotrimazole treatment, the action of the compound appears to be very rapid and reach saturation. This evidenced by the significantly decreased fraction of bound hexokinase at the initial time point for all treated cultures and a relatively constant value of fraction bound 1 and 2 hours after exposure for the higher clotrimazole concentrations. This mechanism of hexokinase detachment by clotrimazole is not well described—the compound is known to be a calmodulin antagonist, which among a few actions, is a protein known to bind calcium ions and maintain intracellular calcium transport. Disruption of this calcium transport across the mitochondrial membrane is thought to detach hexokinase.[2] If clotrimazole

indeed has this effect in biopharmaceutical-producing cell lines, such as the hybridoma used here, then this suggests that calcium homeostasis is critical to cell survival.

The data provide strong indications that clotrimazole-induced hexokinase detachment precedes cell death since 2 hours after exposure, viabilities of the clotrimazole-treated cultures were comparable to the control (Figure 3.5), yet each culture had lesser amounts of mitochondrial hexokinase e.g. fraction of hexokinase bound to the mitochondria at 1 hour (Figures 3.1 and 3.2). The data suggest that the decrease in amount of mitochondrial hexokinase bound in response to clotrimazole is an effect that occurs within about an hour time scale, as opposed to 10's of hours and, more importantly, at a timescale that is faster than that observed for changes in metabolism, growth, and death in batch culture systems. The data also suggest that complete hexokinase detachment is not needed to induce cell death. At the higher clotrimazole concentrations (15 and 20 μM), the fraction of hexokinase bound to the mitochondria was about 10% 2 hours after exposure, yet apoptotic cell death presumably began occurring as early as four hours after exposure. Also, a relatively high viability is observed for the cultures receiving 2 μM clotrimazole even up to 16 hours after exposure despite seemingly having a larger fraction of unbound VDAC sites as compared to the control. Perhaps there is a certain percentage of VDAC sites that must be open to induce apoptotic death. Or if mitochondrial hexokinase is not mitigating cell survival in this case, then this suggests that calcium transport can be interrupted to a certain extent without inducing cell death.

The fact that we see clotrimazole inducing death that exhibits apoptotic morphology is a significant finding. Does this mean that hexokinase detachment causes

apoptosis? This is a distinct possibility, although it must be conceded that clotrimazole may induce other intracellular events that cause apoptosis. The mechanistic action of clotrimazole as a specific calmodulin inhibitor allows these studies to identify targets which are important for cell survival and potentially glycolysis in this cell line. In addition to intracellular calcium homeostasis, calmodulin has been identified to be involved with the regulation a number of proteins, including cyclic nucleotide-dependent phosphodiesterase, nitric oxide synthase, calcineurin, and many kinases.[19] Thus, since it is obvious that clotrimazole is detrimental to cell survival, then all the aforementioned proteins are targets that may be critical to cell survival for this hybridoma cell line.

Results from the metabolic rate screen reveal that clotrimazole causes less than a 20% average decrease in glucose consumption over a 4-hour period, and the change in glucose consumption did not correlate with clotrimazole concentration in the 8-16 μM range. Whether or not the detachment of hexokinase by clotrimazole is the exact cause of the measured decrease in glucose consumption is not entirely clear, although it can certainly be said that cultures treated with clotrimazole are known to exhibit both decreased mitochondrial hexokinase activity and decreased glucose consumption in the hours prior to death. The most likely explanation for the observed dose-*independent* decrease in glucose consumption rate is the apparent dose-*independency* of mitochondrial hexokinase detachment by clotrimazole treatment over a similar concentration range. From Figure 3.2, we can see that the 10 and 15 μM concentrations, which most closely match the concentration range for the MRS experiments, do not exhibit statistically different amounts of mitochondrial hexokinase activity during the 2-hour exposure period. Thus, if 8-16 μM clotrimazole causes similar decreases in mitochondrial

hexokinase detachment, then a dose-*independent* decrease in glucose consumption rate over the same concentration range would be a plausible result. Secondly, it can be considered that hexokinase re-location to the cytosol may result in a rapid decrease in glucose consumption, but metabolic control dictates a quick return to a hypothetical setpoint based on current cellular energy demands by increasing the intracellular metabolite pool. This would certainly explain the similar average glucose uptake rates for each clotrimazole concentration over the 4-hour time period. And lastly, based on the varying effects of calmodulin inhibition, it must be considered that something other than clotrimazole-induced hexokinase detachment may have caused the 12-22% decrease, although it is impossible to know exactly what protein target would have been responsible for decreased glucose consumption and cell death.

Despite 8-16 μM clotrimazole causing dose-independent decreases in mitochondrial hexokinase activity and short-term glucose consumption, it should be noted that the 10 and 15 μM concentrations used in the AO/EB viability measurements (Figure 3.5) caused dose-dependent decreases in culture viability in the 4-hour and 8-hour exposure times. Thus, the same amount of mitochondrial hexokinase detachment by clotrimazole leads to different amounts of cell death at later times, suggesting that something other than mitochondrial hexokinase detachment is controlling the cell death response.

In summary, the clotrimazole treatment of CRL 1606 does have negative consequences for culture viability and reduces glucose consumption. Furthermore, decreases in mitochondrial hexokinase activity by clotrimazole treatment precede or coincide with these physiological changes. Clotrimazole treatment resulted in a dose-

dependent decrease in viability, while the decrease in glucose consumption and mitochondrial hexokinase acitivity was dose-*independent*. The data imply that hexokinase detachment and reduced glucose consumption both occur prior to the visible occurrence of cell death (from the dye-based methods used in this study). The true *in vitro* metabolic significance of mitochondrial hexokinase needs further investigation in terms of studying a wider concentration range and dynamic metabolic responses rather than average rates over a specified time interval. The implications of clotrimazole toxicity (presumably by interrupting calcium homeostasis) and quantification of a detectable decrease in average glucose consumption for a biopharmaceutical-producing cell line extend far beyond hexokinase detachment from the mitochondria, although this event was the primary focus of Aims I and II of this research project. The overall result from this work is that the event of reduced mitochondrial hexokinase activity does precede cell death and coincides with decreased glucose consumption, which is very significant evidence to implicate a regulatory role for mitochondrial hexokinase in metabolic and apoptotic pathways in this hybridoma cell line.

References

1. Bassukevitz, Y., M. Chenzion, and R. Beitner, *Ca2+-Ionophore A23187 and the Ca2+-Mobilizing Hormones Serotonin, Vasopressin, and Bradykinin Increase Mitochondrially Bound Hexokinase in Muscle.* Biochemical Medicine and Metabolic Biology, 1992. **47**(2): p. 181-188.

2. Penso, J. and R. Beitner, *Clotrimazole and bifonazole detach hexokinase from mitochondria of melanoma cells.* European Journal of Pharmacology, 1998. **342**(1): p. 113-117.

3. Majewski, N., et al., *Hexokinase-mitochondria interaction mediated by Akt is required to inhibit apoptosis in the presence or absence of Bax and Bak.* Molecular Cell, 2004. **16**(5): p. 819-830.

4. Pastorino, J.G., N. Shulga, and J.B. Hoek, *Mitochondrial binding of hexokinase II inhibits Bax-induced cytochrome c release and apoptosis.* Journal of Biological Chemistry, 2002. **277**(9): p. 7610-7618.

5. Penso, J. and R. Beitner, *Clotrimazole decreases glycolysis and the viability of lung carcinoma and colon adenocarcinoma cells.* European Journal of Pharmacology, 2002. **451**(3): p. 227-235.

6. Fanciulli, M., et al., *Glycolysis and Growth-Rate in Normal and in Hexokinase-Transfected NIH-3T3 Cells.* Oncology Research, 1994. **6**(9): p. 405-409.

7. Widjojoatmodjo, M.N., A. Mancuso, and H.W. Blanch, *Mitochondrial Hexokinase-Activity in a Murine Hybridoma.* Biotechnology Letters, 1990. **12**(8): p. 551-556.

8. Pedersen, P.L., et al., *Mitochondrial bound type II hexokinase: a key player in the growth and survival of many cancers and an ideal prospect for therapeutic intervention.* Biochimica Et Biophysica Acta-Bioenergetics, 2002. **1555**(1-3): p. 14-20.

9. Laterveer, F., et al., *The Kinetic Consequences of Binding of Hexokinase-I to the Mitochondrial Outer-Membrane.* Biochimica et Biophysica ACTA-Bioenergetics, 1994. **1188**(3): p. 251-259.

10. Penso, J. and R. Beitner, *Detachment of glycolytic enzymes from cytoskeleton of Lewis lung carcinoma and colon adenocarcinoma cells induced by clotrimazole and its correlation to cell viability and morphology.* Molecular Genetics and Metabolism, 2002. **76**(3): p. 181-188.

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

12. Bergmeyer, H.U., *Methods of Enzymatic Analysis*. Methods of Enzymatic Analysis, ed. H.U. Bergmeyer. Vol. 3. 1974, New York: Academic Press, Inc.

13. Pastorino, J.G. and J.B. Hoek, *Hexokinase II: The integration of energy metabolism and control of apoptosis.* Current Medicinal Chemistry, 2003. **10**(16): p. 1535-1551.

14. Salgueiro-Pagadigorria, C., et al., *Naproxen affects Ca2+ fluxes in mitochondria, microsomes and plasma membrane vesicles.* Chemico-Biological Interactions, 2004. **147**(1): p. 49-63.

15. Gunter, T., et al., *Calcium and mitochondria.* FEBS Letters, 2004. **567**(1): p. 96-102.

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

17. Yang, Y., Balcarcel, R. R., *96-well plate assay for sublethal metabolic activity.* Assay and Drug Development Technologies, 2004. **2**(4): p. 353-361.

18. Fogler, H.S., *Elements of Chemical Reaction Engineering*. 3rd. ed. 1999, Upper Saddle River, New Jersey: Prentice Hall.

19. Sigma-Aldrich, *Sigma-Aldrich Website*. 2005.

CHAPTER IV

ANALYSIS OF HEXOKINASE DETACHMENT IN BATCH HYBRIDOMA CULTURES

Introduction

The measurement of hexokinase activity in the cellular sub-fractions during batch hybridoma cell cultures has not been quantified, although a few studies have investigated total hexokinase activity in batch cultures. A 1993 study proposed that the decrease in hexokinase activity at late stage batch culture may be responsible for the low utilization of glucose at the end of the batch.[1] This study included enzymatic measurements of hexokinase activity at 2, 4, and 7 days after subculture and found that by 48 hours, total hexokinase activity was decreased about 40%. However, no statistical significance was established for the measurements. Various articles discuss the activity of hexokinase, both total and in the sub-fractions, in mammalian cell types collected at various stages of culture, but not over time (Table 4.1). Clearly, quite a large range of the fraction of total hexokinase bound to the mitochondria has been determined based on the cell type studied.

Table 4.1. Published Values of Fraction of Hexokinase Bound to the Mitochondria for Varying Cell Types. All studies used cellular fractionation followed by enzymatic assay to determine the hexokinase activity in each fraction (mitochondrial and cytosolic).

cell type	mHK/tHK	reference
tumor	0.50	Parry, et. al., 1983[2, 3]
kidney	0.80	Parry, et. al., 1984[3]
hepatoma	0.78	Nakashima, et. al., 1986[4, 5]
hybridoma	0.25	Widjojoatmodjo, et. al., 1990[5]
NIH-3T3	0.40	Fanciulli, et. al., 1994[6]
myeloma	0.20	Vriezen, et. al., 1998[7]

Data and statistical analyses in Chapter II illustrated a phenomenon of decreasing glucose metabolism prior to cell death in batch hybridoma cultures. The specific cause of this decline is unclear, as is the particular apoptotic pathway responsible for instigating cell death. The results in Chapter III suggest that the biopharmaceutical-producing hybridoma cell line used in this study is sensitive to the Bax-induced apoptotic pathway, although it is not known if this pathway is involved in the natural apoptotic death that occurs in batch culture.

The prospect of having variable amounts of hexokinase bound to mitochondria during batch culture make such a phenomenon a logical one to study given its prominent role both glycolysis and a major apoptotic pathway. Mitochondrially bound hexokinase presumably has preferential access to ATP, which may stimulate glycolysis.[8, 9] Thus, discovery of a systematic detachment of hexokinase from the mitochondria during batch culture may certainly explain the steady decrease in glycolysis and would also provide insight as to the particular pathway responsible for apoptosis.

In the Aim III phase of this project, hexokinase activities in the mitochondrial and cytosolic fractions were measured during high-viability hybridoma cultures. These

cultures were grown in the same manner as the cultures in Chapter II, and they presumably exhibit the same decreasing glucose consumption profile prior to apoptotic cell death. Preliminarily, models (based on Michaelis-Menten kinetics) to estimate the decrease in the fraction of hexokinase bound to the mitochondria and potentially the decrease in total hexokinase were developed. The experimental studies were performed in both T-flasks and spinner flasks. The experimental data and model estimates were compared. The data are used to discuss the potential roles of mitochondrial and cytosolic hexokinase in cell survival and mediation of glucose consumption in batch hybridoma cultures.

Materials and Methods

Cell Line and Maintenance

The cell line used was a murine hybridoma (ATCC CRL-1606) that secretes an IgG against human fibronectin. During maintenance and all experiments, the hybridomas were cultivated in a serum-free, hydrolysate-free IMDM formulation, comprised of glutamine-free basal Iscove's Modified Dulbecco's Medium (IMDM, Mediatech) and supplemented with 4.0 mM glutamine (Mediatech), 10 mg/L insulin (Sigma), 5 mg/L holo-transferrin (Sigma), 2.44 μL/L 2-aminoethanol (Sigma), 3.5 μL/L 2-mercaptoethanol (Sigma), and 10 U/ml penicillin – 10 μg/ml streptomycin (Mediatech). The hybridomas were cultivated in T-flasks (Corning) and maintained in an incubator (Forma Scientific) at 37°C, 10% CO_2 and 95% humidity during maintenance.

Analytical Techniques

Cell Counting and Apoptosis Determination

Cell number and viability were determined via trypan blue dye exclusion using a hemocytometer. The viability was calculated as the number of viable cells divided by the number of total cells. A minimum of 2 slides were counted for each sample. Apoptotic morphology by the presence of DNA fragmentation was characterized using the acridine orange/ethidium bromide fluorescence assay.[10] A total of 400 cells were counted for each slide for the determination of apoptosis. The percentage of each type of cell, whether it was deemed to be early apoptotic, late apoptotic, necrotic, or viable, was determined by dividing the number of each type of cell by the total number of cells counted. Differentiation of each cell type was determined by the visual inspection of fragmented DNA and color.

Cell Fractionation and Hexokinase Activity Assay

The mitochondrial and cytosolic fractions were separated by the exact method described in Chapter III. Fractionated samples were stored at -80°C for later hexokinase activity analysis. Hexokinase activity in the mitochondrial and cytosolic fractions was determined by the exact method also described in Chapter III.

Batch Cultures

Batch experiments were performed either in T-flasks (Corning) or spinner flasks (Bellco Biotechnology) with an initial seeding density of 2.0 x 10^5 vcells/mL. After the initial seeding, the cultures were counted using trypan blue dye exclusion method and incubated at 37°C, 10% CO_2, and 95% humidity in an incubator. At specific time points during the early and late stages of each culture, the cultures were counted again by trypan blue due exclusion (2 slides), and a total of 1e7 cells was sampled into a centrifuge tube. The sample was centrifuged at 200*g* for 15 minutes (Forma Scientific Centrifuge) to pellet the cells. The mitochondrial and cytosolic fractions in this sample were fractionated in a sucrose buffer and stored at -20°C for later hexokinase activity analysis. The speed of the impeller in the spinner flask studies was set to approximately 60 RPM. Cell death exhibiting apoptotic morphology was verified in the spinner flask studies by AO/EB counting after the viability dropped below 50%.

Preliminary Modeling

Model I: Estimation of Hexokinase Detachment from the Mitochondria in Batch Cultures

Prior to experimentation, I developed a model to estimate the expected decrease in the fraction of hexokinase bound to the mitochondria in batch cultures based on the measured profile of glucose concentration in batch cultures. This model was based on the following primary assumptions: (1) the total concentration of hexokinase does not change over time, (2) initially (just after subculture), 25% of the total amount of hexokinase is bound (estimate from Table 4.1), (3) mitochondrial and cytosolic hexokinase possess

different kinetic properties (i.e., k and K_m values)[11, 12], (4) the detachment is described by non-reversible, first order kinetics, and (5) the rate of glucose consumption is equal to the rate of glucose phosphorylation to glucose-6-phosphate. First, I assumed a first-order rate of detachment for mitochondrial hexokinase (equation 4-1).

$$C_{mHK} \xrightarrow{k} C_{cHK} \quad (4\text{-}1)$$

For the purposes of this model, both mitochondrial and cytosolic hexokinase concentrations are expressed on a per volume of cytosol basis (mmol/$L_{cytosol}$). Writing the rate law, we get

$$\frac{dC_{mHK}}{dt} = -\frac{dC_{cHK}}{dt} = -kC_{mHK} \quad (4\text{-}2)$$

where C_{mHK} is the concentration of mitochondrial hexokinase, C_{cHK} is the concentration of cytosolic hexokinase, and k (hr^{-1}) is the first-order rate constant. Assuming the rate of detachment is constant, equation 4-2 can be integrated to yield equation 4-3,

$$C_{mHK} = C_{mo}e^{-kt} \quad (4\text{-}3)$$

where C_{mo} (mmol/$L_{cytosol}$) is the initial concentration of mitochondrial hexokinase. Assuming that the total mass of hexokinase remains constant, the concentration of cytosolic hexokinase is equal to the sum of the initial concentration and the change in the concentration of mitochondrial hexokinase,

$$C_{cHK} = C_{co} + C_{mo} - C_{mo}e^{-kt} \quad (4\text{-}4)$$

where C_{co} (mmol/$L_{cytosol}$) is the initial concentration of cytosolic hexokinase. The conversion of intracellular glucose to glucose-6-phosphate can be assumed to be modeled by Michaelis-Menten enzyme kinetics. Since the intracellular pool of glucose is very low,[13] we can use the simplified form for low substrate concentration (equation 4-5),[14]

$$\frac{dC_{glc}}{dt} = -\frac{V_{max} C_{glc}}{K_m} \quad (4\text{-}5)$$

where C_{glc} (mmol/$L_{cytosol}$) is the intracellular glucose concentration, V_{max} (mmol/$L_{cytosol}$/hr) is the maximal rate of reaction, and K_m (mmol/$L_{cytosol}$) is the Michaelis-Menten constant. However, we do know that V_{max} is equal to a reaction rate k times the enzyme concentration. Assuming that both mitochondrial and cytosolic hexokinase have different k and K_m values, we can write an expression for the overall rate of glucose phosphorylation,

$$\frac{dC_{glc}}{dt} = C_{glc}\left[\frac{k_1 C_{mHK}}{K_{mm}} + \frac{k_2 C_{cHK}}{K_{cm}}\right] \quad (4\text{-}6)$$

where k_1 and k_2 (hr^{-1}) are the reaction rate constants for product formation for mitochondrial and cytosolic hexokinase, respectively, and K_{mm} and K_{cm} (mmol/$L_{cytosol}$) are the Michaelis constants for mitochondrial and cytosolic hexokinase, respectively. We also know from the literature (and from my own data) that during mid-exponential phase hybridoma cultures, the fraction of total hexokinase bound to the mitochondria is around 0.20-0.25.[5] Thus,

$$\frac{C_{mo}}{C_{mo} + C_{co}} = 0.25 \quad (4\text{-}7)$$

Substituting 4-3, 4-4, and 4-7 into 4-6, we get

$$\frac{dC_{glc}}{dt} = C_{glc}\left[\frac{k_1 C_{mo} e^{-kt}}{K_{mm}} + \frac{k_2 C_{mo}(4 - e^{-kt})}{K_{cm}}\right] \quad (4\text{-}8)$$

Equation 4-8 was rearranged and integrated from C_{glco} (initial glucose concentration) to C_{glc} and 0 to t to yield the model,

$$ln(C_{glc}) = Ae^{-kt} + Bt + C \quad (4\text{-}9)$$

where A, B, and C are described by equations 4-10 through 4-12.

$$A = \frac{k_1 C_{mo} / K_{mm} + k_2 C_{mo} / K_{cm}}{-k} \quad (4\text{-}10)$$

$$B = \frac{4 k_2 C_{mo}}{K_{cm}} \quad (4\text{-}11)$$

$$C = \frac{k_1 C_{mo} / K_{mm} - k_2 C_{mo} / K_{cm}}{k} + ln(C_{glco}) \quad (4\text{-}12)$$

The outcome of this model is to calculate k, the average rate of detachment, and estimate the ratio of the amount of mitochondrial hexokinase to the total amount of hexokinase. Furthermore, the estimates of A, B, and C yield an insight as to how much more efficient mitochondrial hexokinase must be as compared to cytosolic hexokinase.

The model was performed using the known glucose concentration profiles from Chapter II, which were converted to mmol/L of cytosol using an estimated cell volume (V_{cell}~1375 μm^3)[15] and the viable cell density, which was measured. The model fitting was performed using MatLab and the LSQNONLIN non-linear curve fitting function. The unknowns, A, B, C, and k, were the output parameters of the program. The fraction of hexokinase bound was estimated by equation 4-13 (using 4-3 and 4-4).

$$\frac{mtHK}{tHK} = \frac{C_{mHK}}{C_{mHK} + C_{cHK}} = 0.25e^{-kt} \quad (4\text{-}13)$$

Model II: Estimation of Changes in Total Hexokinase Activity

It is possible that the intracellular location of hexokinase has no effect on its activity. If this is the case, then the decrease in glucose uptake may possibly be explained by an overall decrease in the total amount of hexokinase. To estimate the relative decrease in the total amount of hexokinase that would contribute to the observed decrease in glucose consumption, I simplified the above model (equation 4-6) to equation 4-14,

$$\frac{dC_{glc}}{dt} = \frac{k'C_{tHK}C_{glc}}{K_m} \quad (4\text{-}14)$$

where C_{tHK} (mmol/$L_{cyotsol}$) is the total concentration of hexokinase, k′ (hr^{-1}) is the rate constant for product formation from the hexokinase-glucose complex, and K_m (mmol/$L_{cytosol}$) is the Michaelis constant. Rearranging, we have

$$k'C_{tHK} = \frac{dC_{glc}}{dt} * \frac{K_m}{C_{glc}} \quad (4\text{-}15)$$

The left hand side of equation 4-15 is equal to the activity of the enzyme. Since glucose concentration as a function of time is known, it can be converted to a per cell basis using the viable cell density. The K_m value was estimated to be 0.1 mM.[16]

Error Analysis and Statistical Methods

Statistical comparisons for the fraction of hexokinase bound to the mitochondria and total hexokinase between 20 and 48 hours for the T-flask measurements was performed by the student's t-test. The errors on the measurements for these experiments were calculated as the standard deviation of 6 replicates.

A statistical comparison among the time points in which hexokinase activity was measured in the spinner flasks was performed using a Model I ANOVA. If statistical significance was established by calculating an F value greater than the critical F at the specified degrees of freedom, the ANOVA was followed-up with Tukey's Test, which compares the differences in means for each pair to a least significant difference. If the difference in means for a pair is greater than the least significant difference, then there is a statistically significant difference. The errors on each measurement for the spinner flask experiments were calculated as the standard deviation of the 3 independent replicates.

Results

Preliminary Model Estimates

Prior to measuring the hexokinase activity in the mitochondrial and cytosolic fractions in batch cultures, I formulated two models to estimate (1) the observable decrease in the **fraction of hexokinase bound** to the mitochondria that would be necessary to produce the calculated decrease in glucose uptake during batch cultures and (2) the observable decrease in the **total hexokinase activity** that would be necessary to produce the calculated decrease in glucose consumption during batch cultures assuming that hexokinase does not possess varying kinetic properties by virtue of its intracellular location. The glucose concentration curves from the four batch experiments in Chapter II were used for these models. I should note here that glucose concentration profiles were not determined for the batch experiments used to measure hexokinase activity in this chapter.

Based on the first model, the output of which is an estimate for first-order rate of detachment (k) of mitochondrial hexokinase, the fraction of total hexokinase bound would have to decrease from about 0.18 to 0.12 between the early and late stages of culture for T-flask cultures and 0.19 to 0.13 for spinner flask cultures (Table 4.2). This represents slightly over a 30% decrease for both modes of cultivation. The model estimated an average k value (first-order rate of detachment) of 0.015 hr^{-1}. This number is the average from all four flasks.

The second model is a variation on the first, and estimates the decrease in total hexokinase activity over time that would be necessary for the commensurate decrease in

glucose consumption. According to the model, the expected decrease in total hexokinase activity for T-flask cultures is about 60%, although the spinner flask models only estimate about a 23% reduction in total hexokinase activity between 20 and 48 hours. The variability of the model estimates is due to inoculum variations of the batch experiments (i.e., differing glucose concentration profiles). It should also be noted that the magnitude of the total hexokinase activity/cell is not the same as the experimental measurements since the models were carried out using estimates for Michaelis-Menten constants as well as cell volume. Hence, only the relative decrease over time is of interest, not the absolute magnitude.

Table 4.2. Model Predictions for the Fraction of Hexokinase Bound to the Mitochondria (Model I) and Total Hexokinase Activity (Model II) in Batch CRL 1606 Cultures Cultivated in T-Flasks and Spinner Flasks. Data show the results of both models. Error bars represent the standard deviation of the two spinner flask experiments. * $x10^{-11}$

Time (hours)	Model I mHK/tHK	Model II total hexokinase (units/cell)*
	T-Flasks	
20	0.18±0.04	4.3±1.3
48	0.12±0.06	1.7±0.1
	Spinner Flasks	
8	0.22±0.002	3.0±0.6
20	0.19±0.004	3.5±0.3
32	0.16±0.005	2.7±0.2
40	0.14±0.005	2.4±0.04
48	0.13±0.006	2.7±0.4

Thus, based on these models, cultures grown in both T-flasks and spinner flasks should exhibit an approximately 30% reduction in the fraction of hexokinase bound to the mitochondria, respectively, between 20 and 48 hours after subculture. Next, experiments

are performed to measure the fraction of hexokinase bound over the course of batch cultures and compare the model estimates.

T-Flask Cultivation Studies

The first experimental part of this study investigated the differences in the fraction of hexokinase bound to the mitochondria for cells cultivated in T-flasks. Only 2 time points were measured: 20 hours and 48 hours after subculture. This is the minimal amount of data needed to make a claim that hexokinase detaches from the mitochondria over time in batch cultures, and also the relatively small culture volume of a T-flask limited the number of sampling times. Figure 4.1 illustrates the fraction of total hexokinase bound to the mitochondria and the total hexokinase activity per cell at 20 and 48 hours after subculture for hybridomas cultivated in T-flasks. These time points were chosen to coincide with (1) the approximate time of peak metabolism and (2) the metabolism just prior to visible apoptotic cell death. The data represent 6 replicates. The first observation that can be made from this data is that less than 25% of the total amount of hexokinase appears to be bound to the mitochondria, even at the early stages of culture. Secondly, there does appear to be a 17% reduction in the fraction of hexokinase bound to the mitochondria, though this is lower than expected from the model estimates. Furthermore, the total amount of hexokinase appears to remain constant, although no time points were measured between 20 and 48 hours. The average growth rate for these cultures between 20 and 48 hours was 0.045 ± 0.003 hr^{-1}, similar to the T24 flask in Chapter II which exhibited an average growth rate of 0.043 hr^{-1} during the same time period.

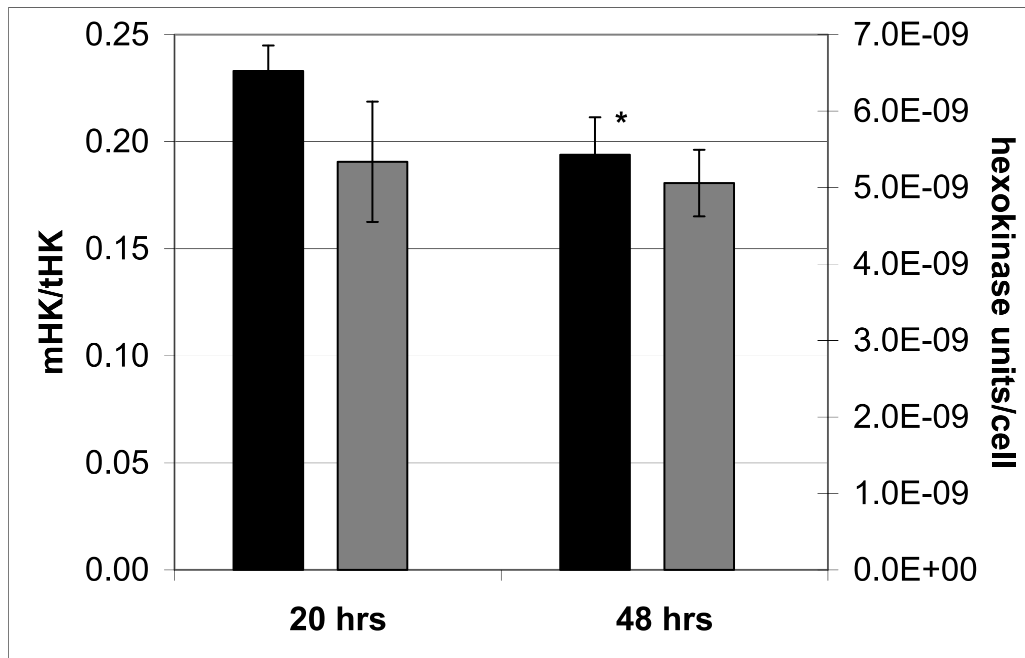


Figure 4.1. Fraction of Hexokinase Bound to the Mitochondria and Total Hexokinase Activity at 20 and 48 Hours after Subculture for CRL 1606 Cultures Cultivated in T-Flasks. Less than 25% of the total hexokinase is bound 20 hours after subculture (black bars). There is statistical evidence of decreased mitochondrial hexokinase association between 20 and 48 hours after subculture, but the decrease appears to be fairly slight (~17% decrease). Statistical treatment via the student's t-test showed statistical difference at the 95% confidence level. There is no statistical evidence of a decrease in total hexokinase activity between 20 and 48 hours (grey bars). Error bars represent the standard deviation of 6 replicates. *$p<0.05$.

It is clear from the T-flask data that more time points are needed for a thorough analysis of the changes in hexokinase distribution in batch cultures. This could only be accomplished by performing measurements in a spinner flask due to the large sample size (1e7 total cells) required for the fractionation procedure and the hexokinase activity assay. Thus, I amended the experimental protocol to cultivations in spinner flasks and increased sampling times.

Spinner Flask Cultivation Studies

The data set for detachment of hexokinase from mitochondria in batch culture was next expanded by using the same experimental procedure, but with an increased number of time points and an alternate cultivation method. Spinner flasks were used in this study because they hold much larger culture volumes as compared to T-flasks, which allows for a greater number of measurements to be made. As we can see from Figure 4.2A, a sort of equilibrium between mitochondrial and cytosolic hexokinase is maintained throughout the batch cultures given the fact that the fraction of total hexokinase bound does not change. Application of a Model I ANOVA resulted in an insignificant F value, indicating that no pairs of time points are significantly different. The fraction of total cells which apoptotic morphology (DNA fragmentation) was over 75% at the late stages of cultures, as verified by AO/EB counting. The average growth rate for these cultures over the 20-48 hour period was 0.054 ± 0.004 hr^{-1}, which is notably higher than the spinner flask cultures from Chapter II which exhibited an average growth rate of 0.040 ± 0.004 hr^{-1} over the same time period.

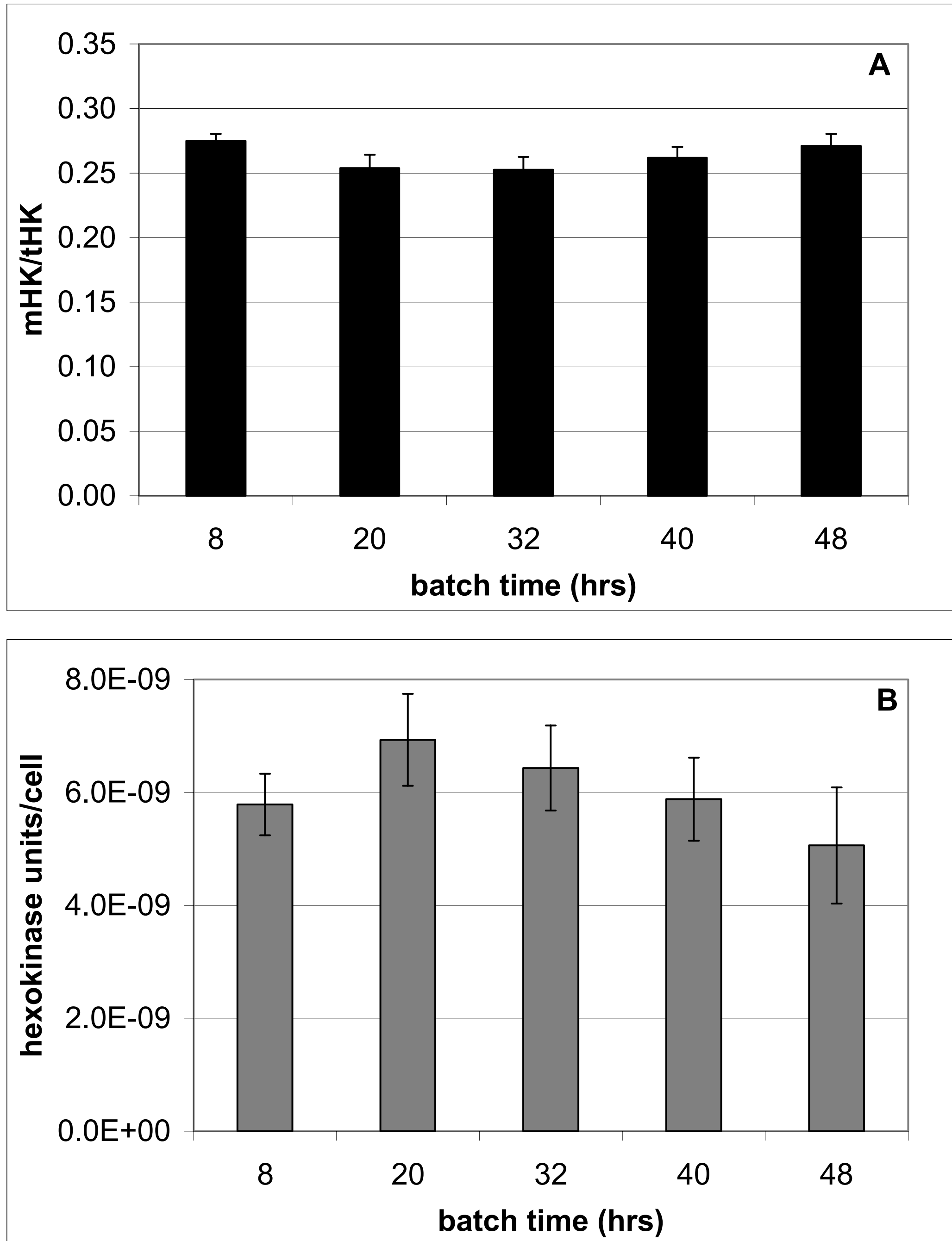


Figure 4.2. The Ratio of Mitochondrial Hexokinase Activity to Total Hexokinase Activity and Total Hexokinase Activity in Batch CRL 1606 Cultures Cultivated in 0.5 L Spinner Flasks. The fraction of hexokinase bound to the mitochondria remains static over the course of batch culture (A). However, the total hexokinase activity per cell exhibits a decreasing trend, although statistical significance could not be established (B). Error bars represent the standard deviation of 3 independent replicates.

Even though the fraction of hexokinase bound to the mitochondrial appears to remain static, there is evidence that the total amount of hexokinase is decreasing over time in batch cultures (Figure 4.2B). Summing the measured activities in the mitochondrial and cytosolic fractions, there appears to be a 27% decrease in the total hexokinase activity. It should be noted that statistical comparisons were not significant in any case. In both the T-flask and spinner flask cultures, total mitochondrial hexokinase activity decreased ~22% between 20 and 48 hours.

Comparison of Experimental Measurements to Model Estimates

Using the model predictions and experimental data at 20 and 48 hours after subculture, comparisons can be made on the expected decrease in the fraction of hexokinase bound to the mitochondria and the total hexokinase activity. The comparisons are summarized in Table 4.3. The decrease in the fraction of hexokinase bound between 20 and 48 hours after subculture is only about 17% for the T-flask cultures and there is a slight (yet insignificant) increase for the spinner flask cultures. These values are clearly lower than expected. The change in total hexokinase in the spinner flasks did correspond nicely to model estimates, however. The T-flasks exhibited almost no change in total hexokinase between early and late-stage culture.

Table 4.3. Comparison of Model Estimates and Experimental Data: Decrease in Total Hexokinase and Fraction of Hexokinase Bound to the Mitochondria between 20 and 48 Hours after Subculture. The estimated decrease in total hexokinase is much greater for the T-flask cultures since these cultures exhibited a larger decrease in glucose metabolism over the 20-48 hour time interval.

	T-Flasks		**Spinner Flasks**	
	Total Hexokinase	*mHK/tHK*	*Total Hexokinase*	*mHK/tHK*
Model (Predicted)	-58.6%	-35.7%	-22.1%	-32.9%
Experimental	-5.2%	-16.8%	-27.0%	+6.7%

Discussion

The data presented in this chapter represent novel work on the investigation of changes in hexokinase association with the mitochondria in batch hybridoma cell cultures. There are three major findings from this Aim III study:

- The fraction of hexokinase bound to the mitochondria for hybridoma cultures grown in a T-flask decreases an average of 17% between 20 and 48 hours after subculture, while spinner flask cultures exhibited no statistical change in the fraction bound over time. These measurements did not match model predictions.
- No statistical change in total hexokinase activity is evident for cultures grown in T-flasks or spinner flasks.
- Cultures grown in both T-flasks and spinner flasks exhibited a 22% decrease in total mitochondrial hexokinase activity (on a per cell basis) between 20 and 48 hours after subculture, but the decrease was **not statistically significant.**

The T-flask cultures appear to exhibit a small decrease in the fraction of hexokinase bound to the mitochondria (~17%) between early and late stage culture, but the spinner flask cultures showed a consistent (and higher) ratio of mitochondrial to total hexokinase activity between 8 and 48 hours after subculture. This clearly suggests that there are some inoculum differences based on the mode of cultivation. Hybridomas cultivated in spinner flasks have different levels of dissolved oxygen, grow more quickly, and die more quickly than those cultivated in T-flasks. Perhaps coincidentally, the average growth rate for the spinner flask cultures between 20 and 48 hours was ~17% higher than the T-flask cultures. However, it is not clear if mitochondrial hexokinase is related to growth in any way. The effects of differing environments are clearly seen in the mitochondrial hexokinase activity. Higher growth rates in the spinner flask cultures imply higher rates of glycolysis and higher flux through the TCA cycle, and this could subsequently mean higher amounts of hexokinase bound to the mitochondria. If in fact the spinner flasks are more highly oxygenated due to constant stirring, then this would imply that higher levels of dissolved oxygen effect the fraction of hexokinase bound to the mitochondria. I did not measure dissolved oxygen levels, so it is not known if the spinner flasks really do provide higher levels of oxygenation. Thus, it is possible that the mitochondrial-mediated pathway (potentially activated by hexokinase dissociation) is more active when cultures are grown in T-flasks if there is in fact less dissolved oxygen available. However, further study would be needed to investigate this point and make a claim regarding its validity.

In terms of total hexokinase activity and the activities in each separate fraction, the T-flasks exhibited no change in hexokinase activity between 20 and 48 hours, but the

spinner flask data does provide evidence that the total amount of hexokinase (both mitochondrial and cytosolic) is decreasing over time, although statistical significance could not be established. The profile corresponds to that of the assumed glucose consumption (i.e., peaks at ~16-20 hours after subculture and declines throughout the duration of the batch). This decrease does in fact correlate with model estimates, which predicted a 22% decrease in total hexokinase activity between early and late stages of culture, while the measurements illustrated about a 27% decrease between early and late stages of culture. However, I must claim here that total hexokinase activity is remaining constant throughout all batch cultures since no measurements were statistically different. More replicates would be needed to determine if a decrease in total hexokinase activity does in fact correspond to (and could potentially be the cause of) the decrease in glucose consumption rate.

In comparison to the expected decreases in glucose consumption during these batch cultures, the hexokinase activity appears to change very little between 20 and 48 hours. In Chapter II, the glucose consumption in the T-24 flask decreased about 62% between 20 and 48 hours, yet a similar culture in this Aim III study exhibited only a 17% decrease in the fraction of hexokinase bound to the mitochondria and no change in the total hexokinase activity. And the spinner flask cultures from Chapter II showed an almost 60% decrease in glucose consumption between 20 and 48 hours, yet there was no change at all in the fraction of hexokinase bound. Thus, the decrease in glucose consumption in the both spinner flask and T-flask cultures appears to occur despite nearly constant levels of total hexokinase activity. This suggests that something even more upstream than the hexokinase-catalyzed reaction is responsible for decreasing glucose

consumption, and the most probable target is the availability of glucose transporters on the cell membrane. A decreasing concentration of these transporter proteins would logically cause a decrease in glucose consumption.

These cultures die by apoptosis despite little or no change in mitochondrial hexokinase throughout the high-viability period. The implication of these results is that mitochondrial hexokinase is not critical during high-viability batch hybridoma cultures. However, I must concede that the pathway may still be active and may even be the predominate death pathway since systematic detachment may occur **immediately** before the onset of death. But in the hours leading up to death, there is apparently no change in mitochondrial hexokinase. Perhaps the hypothesis that changes in cellular physiology that occur during high-viability periods of culture may contribute to later apoptotic cell death is not a valid one, although some environmental, genetic, and/or molecular factor must be instigating cell death, either at early or late stage culture. And other apoptotic pathways do exist that are potentially active and may cause death in batch hybridoma cultures. These will be outlined in more detail in Chapter V.

Perhaps the role of mitochondrial hexokinase in cell survival is much stronger than its role in metabolic regulation. This is evidenced in both the Aim I and III studies. In the metabolic rate screening experiments with clotrimazole, we observe the same average glucose consumption despite differing levels of mitochondrial hexokinase. And in the Aim III studies, we observe differing amounts of glucose consumption during batch cultures despite no changes in mitochondrial hexokinase activity. So clearly, mitochondrial hexokinase is exerting little metabolic regulation *in vitro*. And despite the effects of clotrimazole on calcium homeostasis and perhaps intracellular kinases, we do

see that hexokinase in becoming detached from the mitochondria **before** apoptotic cell death. So, these are clear reasons to believe that mitochondrial hexokinase has a stronger role in protection from apoptotic cell death rather than glycolytic control.

From this study, targeting increased mitochondrial hexokinase does still appear to be a viable option for delaying cell death in biopharmaceutical-producing cell lines despite the fact that we observe only minor changes or no change at all in the fraction bound over time in batch cultures. Presumably due to culture heterogeneity, a subpopulation is dying via this pathway, and ensuring that all VDAC sites are occupied would delay death in that particular subpopulation. Cells complete apoptosis at different rates and probably by different pathways. Learning why some cells die and other live much longer is critical in optimizing cultures for bioprocesses. Here, mitochondrial hexokinase appears to be playing no role in physiological changes during high-viability cultures, but this pathway could quickly become active just prior to cell death.

References

1. Fitzpatrick, L., H.A. Jenkins, and M. Butler, *Glucose and Glutamine-Metabolism of a Murine B-Lymphocyte Hybridoma Grown in Batch Culture.* Applied Biochemistry and Biotechnology, 1993. **43**(2): p. 93-116.

2. Parry, D.M. and P.L. Pedersen, *Intracellular-Localization and Properties of Particulate Hexokinase in the Novikoff Ascites Tumor - Evidence for an Outer Mitochondrial-Membrane Location.* Journal of Biological Chemistry, 1983. **258**(18): p. 904-912.

3. Parry, D.M. and P.L. Pedersen, *Intracellular-Localization of Rat-Kidney Hexokinase - Evidence for an Association with Low-Density Mitochondria.* Journal of Biological Chemistry, 1984. **259**(14): p. 8917-8923.

4. Nakashima, R.A., L.J. Scott, and P.L. Pedersen, *The Role of Mitochondrial Hexokinase Binding in the Abnormal Energy-Metabolism of Tumor-Cell Lines.* Annals of the New York Academy of Sciences, 1986. **488**: p. 438-450.

5. Widjojoatmodjo, M.N., A. Mancuso, and H.W. Blanch, *Mitochondrial Hexokinase-Activity in a Murine Hybridoma.* Biotechnology Letters, 1990. **12**(8): p. 551-556.

6. Fanciulli, M., et al., *Glycolysis and Growth-Rate in Normal and in Hexokinase-Transfected NIH-3T3 Cells.* Oncology Research, 1994. **6**(9): p. 405-409.

7. Vriezen, N. and J.P. van Dijken, *Subcellular localization of enzyme activities in chemostat-grown murine myeloma cells.* Journal of Biotechnology, 1998. **61**(1): p. 43-56.

8. Arora, K.K. and P.L. Pedersen, *Functional-Significance of Mitochondrial Bound Hexokinase in Tumor-Cell Metabolism - Evidence for Preferential Phosphorylation of Glucose by Intramitochondrially Generated Atp.* Journal of Biological Chemistry, 1988. **263**(33): p. 17422-17428.

9. Golshani-Hebroni, S.G. and S.P. Bessman, *Hexokinase binding to mitochondria: A basis for proliferative energy metabolism.* Journal of Bioenergetics and Biomembranes, 1997. **29**(4): p. 331-338.

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

11. Pedersen, P.L., et al., *Mitochondrial bound type II hexokinase: a key player in the growth and survival of many cancers and an ideal prospect for therapeutic intervention.* Biochimica Et Biophysica Acta-Bioenergetics, 2002. **1555**(1-3): p. 14-20.

12. Sprengers, E., A. Koenderman, and G. Stall, *Mitochondrial and Cytosolic Hexokinase from Rate Brain- One and the Same Enzyme?* Biochimica et Biophysica ACTA, 1983. **755**(1): p. 112-118.

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

14. Fogler, H.S., *Elements of Chemical Reaction Engineering*. 3rd. ed. 1999, Upper Saddle River, New Jersey: Prentice Hall.

15. Sonderhoff, S.A., D.G. Kilburn, and J.M. Piret, *Analysis of Mammalian Viable Cell Biomass Based on Cellular ATP.* Biotechnology and Bioengineering, 1992. **39**(8): p. 859-864.

16. Bergmeyer, H.U., *Methods of Enzymatic Analysis*. Methods of Enzymatic Analysis, ed. H.U. Bergmeyer. Vol. 3. 1974, New York: Academic Press, Inc.

CHAPTER V

DISCUSSION OF EXPERIMENTAL FINDINGS AND CONCLUSIONS

Summary and Discussion of Key Findings

Table 5.1 summarizes the key findings of this research project. Generally, the fraction of hexokinase bound to the mitochondria was not found to decrease over time in batch hybridoma CRL 1606 cultures. Thus, the evidence collected in this research project does not provide support for the Aim III hypothesis. The data from Aim III of this project do provide some indication that both total hexokinase and mitochondrial hexokinase activity are decreasing in batch hybridoma cultures, but no statistical differences could be established. The cause(s) of the decline in glucose consumption quantified in Chapter II and the specific pathway responsible for apoptotic death in CRL 1606 still lack clear explanation, but the results of this project eliminate one possible molecular event that may contribute to both decreasing metabolism and death during high-viability cultures. For hybridomas cultivated in spinner flasks, the decrease in total hexokinase activity is certainly commensurate with the decrease in glucose consumption, although I must claim that total hexokinase activity is remaining relatively constant throughout batch cultures in both T-flasks and spinner flasks since there were no statistical differences in the activity measurements. And even though the cells are susceptible to induced hexokinase detachment by clotrimazole, it cannot be concluded that the cells are naturally dying via this pathway in batch culture. Furthermore, although the event of induced hexokinase detachment by clotrimazole does precede cell death exhibiting apoptotic morphology and

coincides with a dose-*independent* decrease in glucose consumption, these changes in physiology cannot unquestionably be attributed to hexokinase detachment from the mitochondria.

Table 5.1. Summary of Key Research Findings. The phase of the research project is listed to the left, and the major results of each phase are listed to the right.

Project Phase	Results
Preliminary Studies	• Glucose consumption rate decreases early (<24 hours after subculture) in batch culture (statistically). • Glucose consumption rate decreases statistically prior to decreases in growth rate and apoptotic cell death.
Aim I	• Clotrimazole treatment results in cell death exhibiting apoptotic morphology. • Clotrimazole treatment results in slightly reduced, dose-*independent* glucose consumption over a 4-hour exposure time.
Aim II	• Clotrimazole treatment of hybridoma cultures causes dose-dependent reduced mitochondrial hexokinase activity prior to cell death.
Aim III	• Hybridoma cultures grown in T-flasks exhibit ~17% decrease in the fraction of total hexokinase bound to the mitochondria between 20 and 48 hours after subculture. • Hybridoma cultures grown in spinner flasks exhibit no change in the fraction of total hexokinase bound to the mitochondria between 8 and 48 hours after subculture. • Both T-flask and spinner flask data do not show statistical evidence of decreasing total hexokinase activity during batch cultures.

It is my premise that a systematic detachment is not occurring given the results and analysis of the measurements. There was some indication of a relatively slight decrease in the fraction of hexokinase bound between 20 and 48 hours after subculture in T-flask cultures, but hybridomas cultivated in spinner flasks showed absolutely no change in the fraction bound despite presumably declining glycolysis and meeting the

same apoptotic fate. And even though statistical difference could not be established, there remains evidence that mitochondrial hexokinase activity (not fraction bound) is decreasing during batch culture. Culture heterogeneity would dictate that perhaps a certain subpopulation of cells experiences this phenomenon of decreasing mitochondrial hexokinase activity, which would open VDAC sites and potentially result in apoptotic protein release, but I cannot say this is happening with any degree of certainty.

With further study, a decrease in total hexokinase activity may explain decreasing glucose consumption in spinner flasks, but the T-flask cultures exhibited no change in the total activity, despite evidence of slight re-distribution between the mitochondrial and cytosolic fractions. This suggests that perhaps the cause of declining metabolism and the pathway to apoptotic cell death is dependent on external culture conditions, particularly since spinner flask cultures differ in the amount of oxygenation. I cannot say, however, that changes in hexokinase activity are responsible for declining glycolysis. If in fact changes in either mitochondrial hexokinase activity or total hexokinase activity have any effect on glucose consumption, it is most likely working in tandem with some other event. Perhaps there are changes in the expression of cell-surface glucose transporters? This is just one of many possibilities that could be causing, or at least contributing to, declining glycolysis.

Mitochondrial hexokinase may not be controlling glycolysis per se, but the metabolic rate screening experiments of Chapter III do perhaps indicate that mitochondrial hexokinase may have some sort of influence over glucose consumption. A previous study showed dose-dependent decreases in glycolytic intermediates and ATP after 30 minutes of exposure to clotrimazole for carcinoma cells.[1] The exposure times

used in my study were much longer in order to gain significant changes in extracellular glucose concentration for specific rate calculations. As stated in the Chapter III discussion, perhaps this longer exposure time allowed for the cells to respond metabolically to the change in hexokinase location. However, if the decrease in glucose consumption was not dependent on the extent of hexokinase re-localization, this strongly suggests that mitochondrial hexokinase did not mediate the effects on glycolysis in these experiments. The intracellular calcium disruption of clotrimazole is a more likely cause for this metabolic response, but no papers have quantified metabolism of hybridoma cultures in response to calcium disruption, so I cannot compare this result to any literature results.

The results of Aims I and II do provide some indication that hexokinase detachment from the mitochondria does in fact induce apoptotic death, perhaps through the Bax-dependent pathway. Even though short-term glucose consumption was independent of clotrimazole concentration, cell viability over long times (up to 16 hours after exposure) was clearly dose-dependent. This fact suggests that death pathways can activate and execute the apoptotic mechanism with little or no influence on cellular metabolism. If hexokinase detachment from the mitochondria does in fact open VDAC sites and cause mitochondrial permeability, then it would logically follow that ATP production would be reduced, which would decrease overall glycolysis while potentially **increasing** glucose consumption (since the cells would presumably be energy starved). Hexokinase detachment by clotrimazole is clearly observed, but with little effect on glucose consumption. This could possibly mean that hexokinase binding does not affect

mitochondrial permeability, although further research must be performed to investigate this point.

Given the data in this research project, it is clear that an even more specific study is needed to more comprehensively understand the changes in mitochondrial physiology and hexokinase location during batch culture. Although the cell-free studies with isolated mitochondria cited in Chapter I indicate that mitochondrial hexokinase is needed to maintain mitochondrial integrity and prevent apoptotic protein release, we have little *in vitro* evidence of this. Are the mitochondria becoming permeable during batch culture? This is not known. Very few studies have employed the experimental methodology of measuring physiological or molecular phenomenon over time in batch culture. Although the location of hexokinase seems critical to cell survival and glycolysis, there is little or no evidence of a re-distribution during the high-viability phase of batch culture and, perhaps most importantly, in the hours just prior to apoptotic cell death.

Mitochondrial-Mediated Apoptotic Pathway in Batch Hybridoma Cultures: Active or Inactive?

Through the Aims I and II studies, we can see that the hybridoma cell line is certainly susceptible to induction of the mitochondrial-mediated apoptotic pathway, although there is little evidence to suggest that this pathway is responsible for the natural occurrence of apoptosis in batch culture. There are three predominate apoptotic pathways in which the cells can die: (i) mitochondrial-mediated, (ii) receptor-mediated, and (iii) endoplasmic reticulum (ER) stress-mediated.[2] The mitochondria are involved to some extent in all these pathways, so the mechanisms of hexokinase detachment may or may not be part of all three. Furthermore, I do have preliminary evidence of caspase-3 activity

in later stage batch cultures, although this data was not included as part of the project results since the interpretation carries a high level of uncertainty.

An assumption of this research project is that the mitochondrial-mediated pathway is the dominate mode of death in batch cultures, but the project results cast some doubt on that assumption. The fact that caspase-3 is active in later stages of cultures does not necessarily imply mitochondrial-mediated apoptosis since caspase-3 is a late apoptotic marker involved in all three pathways. However, even this research and previous studies still point to the mitochondrial-mediated pathway as the most likely pathway to apoptotic death considering that inhibition of signaling molecules or growth factors that are known to directly affect mitochondrial function induce apoptosis.[3, 4] But again, just because a pathway is identified as active through induction does not mean that this is the natural mode of death in batch cultures. Clearly, literature studies are lacking in identifying the known mechanisms of apoptosis in batch mammalian cell culture.

Table 5.2 summarizes the research into methods that are known to either delay or induce apoptotic cell death in hybridoma cultures. What can be gleaned from this conglomeration of research? It's quite difficult to say since there are clearly a host of targets which affect cell death in hybridoma cultures. Research in this area appears rather fragmented, meaning there's no clear trend as to the elucidation of active pathways in hybridoma cultures. But most notably, methodologies that affect mitochondrial physiology do exert death effects. This leads me to believe that the effects of clotrimazole on mitochondrial physiology, and more specifically the detachment of hexokinase, may also be the reason that death is induced. And I cannot be completely certain that mitochondrial-mediated apoptosis though hexokinase detachment is not predominate in

batch cultures simply because I analyzed changes during high-viability cultures only. I can only be certain that this pathway is not becoming active in the hours prior to the onset of cell death and not contributing to declining glucose consumption.

Table 5.2. Summary of Research into Apoptotic/Metabolic/Signaling Pathways and the Effects on Death in Hybridoma Cultures. This table summarizes environmental and genetic targets known to effect cell survival in hybridoma cultures.

Research	Death Effects	Intracellular Target	Reference
bcl-2 over-expression	delayed	mitochondrial permeability	Chung, 1998[5] Itoh, 1995[6]
rapamycin	delayed	mTOR	Balcarcel, 2001[7]
z-VAD-fmk	delayed	caspase inhibition	Tinto, 2002[8]
LY294002	induced	Akt/PKB inhibition	Clark, Thesis, 2005
amino acid/glucose/nutrient feeding	delayed	metabolic fluxes	Xie 1994[9]
rotenone	induced	mitochondrial electron transport	Follstad, 2000[10]
staurosporin	induced	calcium-dependent protein kinase	Follstad, 2000[10]
high MMP subpopulation selection	delayed	mitochondrial permeability	Follstad, 2000[10]
clotrimazole	induced	intracellular calcium; mitochondrial HK	Clark Thesis, 2005

Effects of Clotrimazole on CRL 1606 and ER-Mediated Apoptosis

In order to more comprehensively understand the implications of the measured effects of clotrimazole treatment and perturbation of the Bax-induced apoptotic pathway,

we must look at the exact action of clotrimazole. Clotrimazole might induce cell death through hexokinase detachment, but hexokinase detachment seemingly has little to do with natural apoptotic death in batch culture. However, clotrimazole presumably acts to detach hexokinase through disruption of calcium transport.[11] If disruption of calcium transport is in fact the root cause of the apoptotic death and decreased glucose consumption quantified in Aims I and II, then what role does calcium play in cellular homeostasis? Clearly a very large role, if this is in fact the main effect of the compound. In fact, deprivation of extracellular calcium ions, which causes a commensurate depletion of intracellular calcium ions due to equilibrium,[12] is known to induce apoptosis (by assessment of DNA laddering in gel electrophoresis) in hybridoma cell cultures, but over-expression of bcl-2 suppresses this effect.[13]

After further investigation into the function of intracellular calcium, I uncovered that it plays a role in the ER stress-induced apoptotic pathway.[2] This pathway is known to be activated by calcium depletion in pancreatic β-cells.[14] Thus, as an alternate hypothesis, clotrimazole could be inducing apoptotic death via the ER-stress mediated pathway as a result of interruption of calcium homeostasis. It doesn't appear logical that bcl-2 over-expression would counteract the apoptotic effects of calcium deprivation, but interestingly, bcl-2 protein not only binds mitochondria, but it also binds the endoplasmic reticulum in various cell types.[15-17] This might very well explain why bcl-2 over-expression successfully extends cell culture viability in hybridoma cultures—it not only binds mitochondria, but impedes ER-mediated stress pathways as well? And perhaps this is the dominate mode of death for hybridoma cultures. The fact that I witnessed caspase-3

activity in late stage cultures corroborates this hypothesis since caspase-3 is indeed part of the ER stress-mediated pathway.

Future Research

Given the results of the project, future work can be performed to gain more knowledge on the role of mitochondrial hexokinase in cellular metabolism and cell survival. Clearly, another method of hexokinase detachment would be desirable—a method that does not present other possibilities for observed changes in cellular physiology other than hexokinase detachment. There are other compounds that are known to result in decreased mitochondrial hexokinase activity, but the use of another compound would present the same challenges in the final analysis as clotrimazole. I believe the most logical continuation of this project would be to systematically identify the specific apoptotic pathways responsible for cell death in batch culture. This would involve measurement of proteins at the late stages of culture, both before and after cell death, to identify their presence and/or activity. Since there are three major apoptotic pathways, then the logical choice would be to choose three markers specific to each pathway. These would be caspase-8 (receptor-mediated), cytochrome-c (mitochondrial-mediated), and caspase-12 (stress-induced).[2]

Implications for the Biotechnology Industry

The underlying industrial application of this specific research project can be most accurately characterized as bioprocess optimization. By identifying specific molecular phenomena that occur or do not occur in batch culture which may contribute to cell death, novel opportunities for targets in optimizing cell lines to live longer and produce the

maximum amount of product are presented. Here, the intracellular movement of hexokinase during batch culture was investigated as a possible occurrence that could contribute to decreasing metabolism and eventual apoptotic cell death. Although the results seemingly disproved the hypothesis in many ways, the results do suggest that increasing mitochondrial hexokinase binding may actually be a viable option for delaying cell death. Specifically, are all the VDAC sites occupied? We don't know for sure—this study did not include VDAC protein measurements. Again, the activity of hexokinase in the mitochondrial fraction did decrease, although non-statistically. This could potentially suggest that certain sub-populations of cells in culture do possess a deficiency in mitochondrial hexokinase and are consequently susceptible to the Bax-dependent apoptotic pathway. Here, we have some evidence that this pathway is indeed active in this cell line. Cell lines could potentially be made less susceptible to death by ensuring coverage of all VDAC sites by hexokinase, which would reduce death attributed to the Bax-dependent pathway in certain subpopulations. Based on obvious inoculum variability, the most optimal methodology for delaying cell death will almost certainly involve a combination of molecular targets (both metabolic and apoptotic) based on the pathways identified to be most active.

Concluding Remarks

There is an increasing research to suggest that metabolic pathways do regulate cell survival,[18] and the fact that we see declining metabolism prior to death is certainly strong evidence to believe that metabolism may play some role in the decision for the cell to die. But given the fact that there seems to be no changes in mitochondrial hexokinase

activity throughout batch cultures, how does this change how we think about the interaction between apoptotic and metabolic pathways and their regulatory functions? The results of this research project offer a small piece of the puzzle concerning the specific molecular events that occur prior to cell death in batch hybridoma cultures. The fact that we do not see changes in the fraction of hexokinase bound to the mitochondria in spinner flask cultures despite imminent apoptotic death is an interesting result. This helps to further limit the possibilities of how mammalian cells, particularly hybridoma cells, die in batch culture. The T-flasks and spinner flask appear to exhibit different physiology concerning metabolism and fraction of hexokinase bound to the mitochondria, suggesting that perhaps the mechanisms of cell death can be variable depending on culture conditions and even within the same culture. Does mitochondrial hexokinase matter for cell survival in batch culture? And does it have effects on the rate of glycolysis? Overall, the results of this project suggest that mitochondrial hexokinase is not critical. Re-localization of hexokinase to the cytosol without interruption of calcium transport may still have an effect on metabolic and apoptotic pathways, but at present, there's no clear way to determine this *in vitro*. At any rate, there's no evidence that this is occurring naturally in batch cultures. Given these results, what is the most appropriate target to determine the cause of decreasing metabolism and the method of cell death? The most logical approach to further dissect this problem is to choose key proteins in each of the major apoptotic pathways and measure them at the end of batch culture, and in particular investigate the ER stress-mediated pathway. This would certainly elucidate the major apoptotic pathway(s) responsible for the natural occurrence of apoptosis in batch hybridoma cultures. Then, we can look for the relationship that this specific apoptotic

pathway (or pathways) may have with cellular metabolism to determine whether or not declining metabolism may signal eventual apoptotic death. Furthermore, this research focused on events that occur prior to the onset of apoptotic death in batch culture. However, the significance of changing physiological behavior during high-viability culture to eventual commitment to apoptotic cell death later in culture is still debatable. Does the physiological behavior of the cells early in the batch have any sort of influence over the outcome of apoptotic cell death? Before these questions can be answered, we must uncover the fundamental mechanisms by which the die in batch culture.

References

1. Penso, J. and R. Beitner, *Clotrimazole decreases glycolysis and the viability of lung carcinoma and colon adenocarcinoma cells.* European Journal of Pharmacology, 2002. **451**(3): p. 227-235.

2. Arden, N. and M. Betenbaugh, *Life and death in mammalian cell culture: strategies for apoptosis inhibition.* Trends in Biotechnology, 2004. **22**(4): p. 174-180.

3. Vander Heiden, M.G., et al., *Growth factors can influence cell growth and survival through effects on glucose metabolism.* Molecular and Cellular Biology, 2001. **21**(17): p. 5899-5912.

4. Gottlob, K., et al., *Inhibition of early apoptotic events by Akt/PKB is dependent on the first committed step of glycolysis and mitochondrial hexokinase.* Genes & Development, 2001. **15**(11): p. 1406-1418.

5. Chung, J.D., Sinskey,A.J., Stephanopoulos, G., *Growth Factor and Bcl-2 Mediated Survival During Abortive Proliferation of Hybridoma Cell Line.* Biotechnology and Bioengineering, 1998. **57**(2): p. 164-171.

6. Itoh, Y., H. Ueda, and E. Suzuki, *Overexpression og bcl-2, apoptosis supressing gene - prolonged viable culture period of hybridoma and enhanced antibody-production.* Biotechnology and Bioengineering, 1995. **48**(2): p. 118-122.

7. Balcarcel, R.R., G. Stephanopoulos, *Rapamycin Reduces Hybridoma Cell Death and Enhances Monoclonal Antibody Production.* Biotechnology and Bioengineering, 2001. **76**(1): p. 1-10.

8. Tinto, A., et al., *The protection of hybridoma cells from apoptosis by caspase inhibition allows culture recovery when exposed to non-inducing conditions.* Journal of Biotechnology, 2002. **95**(3): p. 205-214.

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

10. Follstad, B.D., Wang, D. I. C., Stephanopoulos, G., *Mitochondrial membrane potential differentiates cells resistant to apoptosis in hybridoma cultures.* Euro. J. Biochem., 2000. **267**(22): p. 6534-6540.

11. Penso, J. and R. Beitner, *Clotrimazole and bifonazole detach hexokinase from mitochondria of melanoma cells.* European Journal of Pharmacology, 1998. **342**(1): p. 113-117.

12. Duke, R., et al., *Cytolysis Mediated by Ionophores and Pore-Forming Agents - Role of Intracellular Calcium in Apoptosis.* FASEB Journal, 1994. **8**(2): p. 237-246.

13. Ishaque, A. and M. Al-Rubeai, *Role of Ca2+, Mg2+ and K+ ions in determining apoptosis and extent of suppression afforded by bcl-2 during hybridoma cell culture.* Apoptosis, 1999. **4**(5): p. 335-355.

14. Oyadomari, S., E. Araki, and M. Mori, *Endoplasmic reticulum stress-mediated apoptosis in pancreatic beta-cells.* Apoptosis, 2002. **7**(4): p. 335-345.

15. Rudner, J., V. Jendrossek, and C. Belka, *New insights in the role of Bcl-2 Bcl-2 and the endoplasmic reticulum.* Apoptosis, 2002. **7**(5): p. 441-447.

16. Krajewski, S., et al., *Investigation of the Subcellular-Distribution of the Bcl-2 Oncoprotein - Residence in the Nuclear-Envelop, Endoplasmic-Reticulum, and Outer Mitochondrial-Membranes.* Cancer Research, 1993. **53**(19): p. 4701-4714.

17. Bassik, M., et al., *Phosphorylation of BCL-2 regulates ER Ca2+ homeostasis and apoptosis.* EMBO Journal, 2004. **23**(5): p. 1207-1216.

18. Kuznetsov, A., et al., *Regulating cell survival by controlling cellular energy production: novel functions for ancient signaling pathways?* FEBS LETTERS, 2004. **577**(1-2): p. 1-4.

APPENDIX A

GLOSSARY OF TERMS

TERM	DEFINITION[1]
Akt/PBK	Protein Kinase B; a protein which is the downstream target of PI-3 Kinase; involved in cell survival.
ANOVA	Analysis of variance; a statistical method which determines if any pairs of means in a data set are significantly different.
AO/EB	Acridine orange/ethidium bromide; a florescent dye which differentiates among viable, early apoptotic, late apoptotic, and necrotic cell types based on the color and appearance of fragmented DNA.
apoptosis	A genetically determined process of cell self-destruction that is marked by the fragmentation of nuclear DNA, is activated either by the presence of a stimulus or by the removal of a stimulus or suppressing agent, is a normal physiological process eliminating DNA-damaged, superfluous, or unwanted cells (as immune cells targeted against the self in the development of self-tolerance or larval cells in amphibians undergoing metamorphosis), and when halted (as by genetic mutation) may result in uncontrolled cell growth and tumor formation; also called *programmed cell death.*
ATP	Adenosine triphosphate; contains high-energy phosphate bonds and is used to transport energy to cells for biochemical processes, including muscle contraction and enzymatic metabolism, through its hydrolysis to ADP.
Bax	A pro-apoptotic protein that binds to the mitochondria and facilitates the release of apoptotic proteins from the mitochondria.
biopharmaceutical	A drug created through bioengineering or biotechnological processes; example: recombinant protein drugs, recombinant vaccines, and monoclonal antibodies.
clotrimazole	$C_{22}H_{17}ClN_2$; a specific inhibitor of Ca^{2+}-activate K^+ channels.

CRL 1606	The American Type Culture Collection (ATCC) number for the hybridoma cell line used in this study.
culture	The growing of microorganisms, tissue cells, or other living matter in a specially prepared nutrient medium.
cytochrome-c	A 13 kDa protein involved in mitochondrial-mediated apoptosis. Once released from the mitochondria, it binds with caspase-9 and Apaf-1 to form the apoptosome, which activates other downstream proteins.
cytosol	The aqueous part of the cytoplasm within which various particles and organelles are suspended inside a cell.
DNA	Deoxyribonucleic acid; a nucleic acid that consists of two long chains of nucleotides twisted together into a double helix and joined by hydrogen bonds between complementary bases adenine and thymine or cytosine and guanine; it carries the cell's genetic information and hereditary characteristics via its nucleotides and their sequence and is capable of self-replication and RNA synthesis.
Dunnet's Test	A follow-up statistical test for ANOVA which compares all experimental means to the control.
enzymatic assay	A quantitative test, usually used to determine the concentration of a protein or metabolite, which utilizes enzymes.
glucose	A six-carbon sugar; the major carbon source for mammalian cell cultures.
glycolysis	The metabolic process by which glucose is converted to pyruvate and lactate; consists of a series of eleven enzymatic reactions.
hemocytometer	An instrument used to count the number of cells in a specific volume; a special type of microscope slide.
hexokinase	An enzyme catalyzing the first step of glycolysis; transfers a phosphate group from ATP to glucose to form glucose-6-phosphate.
hybridoma	A cell that is produced in the laboratory from the fusion of an antibody-producing lymphocyte and a nonantibody-producing cancer cell, usually a myeloma or lymphoma. It proliferates and produces a continuous supply of a specific monoclonal antibody.

IMDM	Iscove's Modified Dulbecco's Medium; a specific formulation of growth medium used for varying cell types.
in vitro	In an artificial environment outside a living organism.
inoculum	The culture used in an inoculation, or seeding, of another culture.
medium	A term describing the extracellular environment of a cell culture.
metabolite	Any of a number of compounds that serve as substrates for metabolic processes; example: glucose and lactate.
mitochondria	An organelle in the cytoplasm of nearly all eukaryotic cells, containing genetic material and many enzymes important for cell metabolism, including those responsible for the conversion of food to usable energy.
monoclonal antibody	Any of the highly specific antibodies produced in large quantity by the clones of a single hybrid cell formed in the laboratory by the fusion of a B cell with a tumor cell
NAD^+	$C_{21}H_{27}N_7O_{14}P_2$; nicotinamide adenine dinucleotide; an electron acceptor used in various metabolic reactions.
$NADP^+$	$C_{21}H_{28}N_7O_{17}P_3$; nicotinamide adenine dinucleotide phosphate; an electron acceptor used in various metabolic reactions.
passage	The process of maintaining a group of cells by regular feeding of fresh medium.
physiology	All the functions of any living organism and its parts.
RPMI	A specific formulation of growth medium used for varying cell types.
seed	To inoculate a culture such that it will grow.
spinner flask	A glass bottle containing a magnetic impeller; used for culturing mammalian and other cell types.
supernatant	The fluid above a precipitate, esp. in centrifugation.
T-flask	A plastic bottle used for mammalian cell and tissue culture.
trypan blue	A blue biological dye which permeates the cell membrane of dead cells.

Tukey's test	A statistical method that compares all pairs of means in a data set; usually a follow-up test for a significant model I ANOVA.
UV/vis spectrometry	A method of measurement performed by quantification of light refraction by passing wavelengths of light in the UV/visible spectrum through a sample.
VDAC	Voltage dependent anion channel; a 60 kDa protein located on the outer mitochondrial membrane; responsible for the release of apoptotic proteins into the cytosol.
well plate	A plastic plate containing small wells in which mammalian cells or tissues can be cultivated.

References

1. *The American Heritage Dictionary of the English Language*. 4th ed. 2000: Houghton Mifflin Company.