# EFFECTS OF RAPAMYCIN AND INSULIN ON THE CELL CYCLE AND APOPTOSIS OF HYBRIDOMA CELL CULTURES

by

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## ABSTRACT

Improvements in the production of recombinant therapeutic proteins from in vitro mammalian cell cultures depend on the extension of viable proliferation. The emergence of cell death within a few days of inoculation of a fed-batch process occurs for unknown reasons, and despite the optimal control of culture parameters such as temperature, pH, osmolarity, dissolved oxygen, nutrient levels, and growth factor requirements. Given that the death observed is that of genetically controlled apoptosis (a phenomenon necessary for biological systems under other circumstances), changes in cellular physiology were investigated along the emergence of apoptosis. In this regard, environmental factors such as insulin and rapamycin were studied for their effects on metabolism, cell cycle progression, and apoptosis.

The apoptotic cell death of a hybridoma cell line was studied in steady state chemostat cultures grown in reduced glutamine, serum-free, hydrolysate-free IMDM medium. Five steady states were characterized at dilution rates of 0.04 $hr^{-1}$, 0.03 $hr^{-1}$, 0.02 $hr^{-1}$, 0.01 $hr^{-1}$, and 0.04 $hr^{-1}$ again in turn. The steady states spanned a range of cultures with higher viability and faster growth rate down to lower viability and slower growth rate. The physiology of the hybridoma culture for each steady state was measured in terms of cell density and viability, growth and death rates, extent of apoptotic cell death, cell cycle distribution, metabolite uptake and production rates, and the production of MAb product. Furthermore, intracellular metabolite fluxes and ATP production were calculated based on a model for central carbon metabolism.

From this series of steady states it was observed that culture metabolism shifts towards more complete oxidation of glucose and increased TCA cycle activity for steady states under more severe nutrient limitation at lower dilution rates. Even though growth rate is inhibited and cell death emerges, the shift in metabolism is interpreted as a robust cellular response to the less optimal environmental conditions. The shift in metabolism is one that generates more intracellular energy per given nutrients, and is presumably required to meet increased maintenance energy demands resulting from environmental stresses. Upon increasing the dilution rate back to the same initial high setting, the culture whose metabolism had been shifted by exposure to leaner nutritional environment actually outperforms the high dilution rate steady state obtained initially. As a result of the increase in metabolic efficiency, the observed multiple steady state exhibited a viable cell density twice that of the first steady state. Culture physiology is thus characterized to depend not only on the imposed environment (i.e. the dilution rate setting) but also on the internal metabolism of the cells.

Despite metabolic effects, the amount of apoptotic cell death for a steady state culture still correlated inversely with dilution rate. Interestingly, even though there was a

reduction in growth rate and death emerges, cell cycle distribution did not shift towards an arrest in the G1 phase of the cell cycle (the gap following cell division and preceding DNA synthesis). Rather, cells accumulated within the S-phase (DNA synthesis). This lack of cell cycle arrest in the G1 phase for cells under nutritional limitation points to a defect in cell cycle control. In this regard, it was further shown that the hybridoma cells express high levels of *c-myc*, a proto-oncogene associated with cell cycle entry. Hence, unbridled entry to the cell cycle and the accumulation of cells in the S-phase is believed to be associated with higher levels of apoptotic cell death than would otherwise be observed under nutrient limiting conditions.

To address the apparent defect in control of cell cycle entry, the cell cycle arresting agent rapamycin was identified and employed as a medium additive to inhibit cell cycle entry of hybridoma cells during fed-batch cultures. Results demonstrate a delay in the emergence of cell death from 48 to 72 hours in the presence of rapamycin. Despite growth being mildly inhibited, the extension of viable proliferation results in higher maximum viable cell densities and greater integrated viable cell densities. Over the course of 6-day fed-batch experiments, the positive effects of rapamycin on culture physiology yield increases of monoclonal antibody titers from 0.25 g/L up to 0.55 g/L. Such an improvement of titer is due to the increase in viable cell density as well as an increase in apparent specific productivity. Rapamycin's mechanism for the above physiological changes for this hybridoma cell line, and in particular, its ability to postpone and minimize apoptotic cell death, remains an open question. It is possible that delay of progression through G1 allows for more timely production of intracellular molecular factors needed for cellular function and survival during DNA synthesis and mitosis. Yet, preliminary evidence suggests that rapamycin's effect metabolism may also play a key role in the enhanced survival of the hybridomas.

The role of insulin for protecting hybridomas against apoptosis was investigated by comparing culture physiology in the presence and absence of insulin in chemostat and batch cultures. The hybridoma's dependence on insulin for growth with high viability was demonstrated to depend on the extent of conditioning of culture medium. Under optimal conditions, insulin is not required for viable proliferation. Yet as the environment becomes more conditioned during fed-batch cultivation, insulin is then required to extend viable growth beyond 24 hours to 48 hours. The emergence of cell death with or without insulin coincides with a delay of progression through all phases of the cell cycle and a shift in metabolism. Thus, it appears that insulin protects cells more through its stimulation of metabolism and less as a growth factor for cell cycle progression. Insulin stimulation of metabolism may confer protection against apoptosis by bolstering the metabolic shift needed to counter environmental stresses that promote cell death.

Overall, rapamycin and insulin protect cells against apoptosis indirectly through their effects on cell cycle and metabolism. Rapamycin, a newly discovered protective factor for the hybridomas, protects cells against apoptosis through its inhibition of cell cycle entry. Such an effect by rapamycin yields significant improvements to fed-batch productivity. Further improvements will stem from engineering ways to alleviate the metabolic impediment that is also associated with apoptosis.

Thesis Supervisor: Gregory N. Stephanopoulos
Title: Professor of Chemical Engineering

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it more apparent that that Brian/Robert guy was sure doing a lot of work. And I think I still owe Brian a week of work as he once maintained both chemostats by himself as I took a pre-arranged vacation. All in all, roughly two-thirds of my experimental work was conducted through teamwork. Such collaboration has been a blessing greater than I could have anticipated or intentionally designed beforehand.

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# 1 INTRODUCTION

## 1.1 THE PRODUCTION OF RECOMBINANT THERAPEUTIC PROTEINS

The overall aim for this thesis was to increase the final titer of recombinant therapeutic proteins produced from mammalian cell culture during fed-batch processes. Recombinant DNA technology has allowed us to use cells to produce many kinds of proteins: enzymes, antibodies, growth factors, hormones, blood factors, neuropeptides, etc. Today, approximately 20 years after the production and sale of the first recombinant therapeutic protein (Genentech's recombinant insulin from bacterial culture), the market for such products is well established and continues growing. As of 1992, the estimated worldwide sales for 14 recombinant therapeutic proteins was 4.4 billion dollars (Klausner 1993). A more recent report optimistically expresses growth for the future, from which I excerpt a portion of its abstract:

> "During the past ten years, the biopharmaceutical landscape has changed significantly, from 5 drugs on the market in 1987 with combined sales of less than $1 billion to more than 30 drugs for as many as 50 indications, with sales close to $13 billion. In 1996, for the first time in history, sales of two biopharmaceutical products-Amgen's epoetin alfa (EPO, Epogen) and granulocyte colony stimulating factor (G CSF, Neupogen)-achieved more than $1 billion in worldwide sales. In 1997, we anticipate that Johnson & Johnson's Procrit, also an epoetin alfa, will join the ranks of billion dollar pharmaceuticals. We expect worldwide sales of biopharmaceutical products to reach $12.8 billion in 1997, an 8.6% increase over 1996 sales. Although worldwide biopharmaceutical sales make up just 5% of world prescription drug sales, they have grown tremendously over the past decade."
>
> **Biopharmaceutical Sales and Forecasts to 2002**
> Samantha S. Cheng
> Pharmaceutical Industry Dynamics
> Issue #159, 12/9/97

The use of recombinant bacterial cultures for the production of therapeutic proteins is the superior method to produce titers as high as possible during a fermentation. The achievable product titer is a key parameter that determines whether a given process is

economically feasible. Yet, the limitation of bacterial systems for the production of therapeutic proteins is the inability of the bacterium to perform post-translational modifications to the protein of interest. Producing a backbone amino acid structure by mRNA translation is a necessary step for the formation of a protein, but, depending on the protein of interest, it is not always sufficient for producing a more complex protein with the desired bioactivity. Many proteins are modified post-translationally. Types of modifications include glycosylation, sulfide bridge formation, cleavages, and assembly of subunit proteins. Such modifications determine the actual properties of the protein, above and beyond just the amino acid sequence.

Mammalian cells are able to perform the variety of post-translation modifications that are needed for proteins of a more complex nature. This ability of mammalian cells makes them an attractive system in which to produce therapeutic proteins that are secreted in active form. As not all mammalian cell types necessarily have the same post-translational capacities, part of the decision regarding which type of mammalian cell to use for a particular protein depends on the nature of the post-translational modifications required.

The downsides of using mammalian cells are the relatively lower secretion rates for the recombinant protein, the dramatically slower growth rate of the cells, and the relatively lower achievable maximum cell density. All these factors severely impact the economic viability of a therapeutic protein production process. As the product titer coming from the primary fermentation vessel is dramatically reduced, not only is the gross amount of product less, but the costs associated with downstream processing and purification are much higher. Unfortunately, given today's current technology and economic climate, many drugs produced by cell culture technology are very expensive or cannot even be produced at all.

Yet despite these obstacles, mammalian cell technology has emerged as the best way to produce a variety of therapeutic proteins. It is possible today to culture a multitude of different types of cells from a variety of different organisms, including humans. Indeed, the cultivation of mammalian cells has gone far beyond just using the cells as catalysts to produce a therapeutic protein. In vitro cultivation has become a means of cultivating

cells for the purpose of studying human illnesses and diseases, regenerating cells taken from a patient in need of tissue repair, or to produce cellular stocks and artificial organs for implantation.

## 1.2 PROGRAMMED CELL DEATH

The in vitro cultivation of cells has in many ways been satisfactorily reduced to practice. A researcher today can fairly simply choose from a multitude of cell lines in a given cell bank, test a few well-established media, setup flasks or bioreactors, and culture cells for his or her particular needs. Many of the engineering and technological challenges have been addressed. There is plenty of information concerning the overall effects of and methods to control many culture parameters, such as temperature, pH, osmolarity, nutrient concentrations, serum and growth factors, and dissolved oxygen and carbon dioxide levels. Likewise there are many alternate ways to grow cells, such as in batch, or fed-batch, or continuous mode, or on a surface or microcarriers or within a matrix for perfusion or within polymer bead capsules. All of these advances have given researchers the means in which to cultivate a sufficient quantity of cells for analysis and experiments.

The caveat to the above statements concerning the progress that has been made thus far is that we have yet to learn how to cultivate cells at "ultra-high" cell densities. The ultra-high cell density we desire would make the production of nearly any therapeutic protein economically feasible. It is also of interest for the production of artificial tissues. One of the main obstacles we encounter in growing cells at higher densities is cell death. Cell death by necrosis occurs due to shear damage, sparging damage, and accumulation of toxic by-products such as lactate, ammonia, and $CO_2$. Such type of necrotic cell death has been adequately addressed and minimized by a combination of engineering and biological remedies. Yet, another type of cell death, called programmed cell death or apoptosis, also plagues cultures depending on the mode of operation of the process and the environment.

The detrimental effect of apoptosis on the performance of a culture has been identified as a primary limitation in a variety of animal cell culture systems over the past several

years. Since the overall productivity of a culture process is strongly dependent on the viable cell density integrated over the life span of the culture, the emergence of cell death directly detracts from the overall productivity. During the investigation of apoptosis, many stimuli of apoptosis have been identified and studied. It is known that the emergence of apoptosis is affected by nutrient and growth factor availability, as well as by the presence of genetic and molecular factors related to apoptosis and cell cycle progression.

Despite the variety of advances in cell culture engineering and our better understanding of apoptosis, apoptotic cell death still persists in continuing to limit cell culture processes. Even despite the control of all the possible environmental parameters that we know can stimulate apoptosis and that we can manipulate, apoptosis emerges within the first several days of a fed-batch cultivation. The ultimate cause for apoptosis remains unknown. The complexity of the phenomenon of such cell death is that it is associated with several other global cellular phenomena, such as growth, cell cycle progression, and metabolism.

## 1.3 THESIS ORGANIZATION

The motivation and development of my thesis objectives is first presented in Chapter 2. Thereafter, my own investigations into the relationship between metabolism, cell cycle progression, and apoptotic cell death are provided in Chapter 3, 4, and 5, where I look at the effects of nutrients, rapamycin, and insulin in turn. Chapter 6 integrates my findings in a discussion of their implication to the field of cell culture engineering. In Chapter 7, I conclude by summarizing the major findings.

# 2 BACKGROUND AND MOTIVATION

## 2.1 CELL CULTURE ENGINEERING TECHNOLOGY

### 2.1.1 Production of Recombinant Therapeutic Proteins

The ability to grow cells in vitro is a prerequisite for biochemical, biological, and biomedical research and engineering. Today, the in vitro cultivation of cells is common practice for a wide variety of applications. From making therapeutic proteins for human beings, to cultivating human tissue cells for tissue repair, to investigating cellular function, and to testing genetic therapies, a cell culture is a powerful platform in which to investigate, elucidate, manipulate, emulate, and recreate biological phenomena for the production of biological materials.

The accumulated experience of worldwide research has yielded a technology that allows researchers today grow cells, handle them in a aseptic fashion, cryogenically freeze them for preservation, partially manipulate their behavior, and use genetic techniques to alter or enhance or clone them. With these tools comes the knowledge of how best to grow cells in vitro. Depending on the particular application, experimental and process objectives can be most readily achieved through the understanding and exploitation or manipulation of cellular behavior.

One particular application of cell culture engineering is the production of recombinant therapeutic proteins for use as human pharmaceuticals. The first such commercial cell culture process was initiated in the 1970's (by Genentech and produced by Lilly in 1982) for recombinant insulin using bacterial cells. The advent of recombinant technology and its debut in the commercial production of recombinant insulin set the stage for the myriad of other therapeutic protein drugs on the market today, 20 years later.

As the technology evolved, the desire to accommodate the production of more complex proteins led to recombinant technology in conjunction with mammalian cells, which can perform additional post-translation protein modifications. The benefits of using mammalian cells to produce therapeutic proteins do not come without additional

technical challenges. The major drawback for mammalian cell culture processes is the lower cell densities that are obtainable and the smaller specific production rates for the product. These drawbacks lead to processes that yield much lower product titers relative to their bacterial counterparts for less complicated proteins. For this reason, the production of a therapeutic protein is even more tightly dependent on overall process economics. Unfortunately, with current technology, it is not possible to produce just any therapeutic protein.

Many technical challenges were overcome in the development of the first cell culture process and since then. This chapter, which serves as background for my own contributions to the field, describes a cumulative body of work regarding the culturing of mammalian cells in vitro, with the principle aim of increasing production capacity of cell culture systems for recombinant therapeutic proteins. As will be evident in this review and in my own contributions, cell death by apoptosis remains the ultimate limitation for many cell culture processes. For this reason, the advances and challenges that I describe for the fed-batch cultivation of cells for the production of therapeutic proteins are cast in the light of how they impact culture longevity and cell death.

### 2.1.2 Proposed Milestone for Fed-batch Production

In considering the advances of cell culture towards producing therapeutic proteins from animal cells, it is important to be aware of the best possible outcome for a proposed process. When I began my work, the ultimate (unofficial) objective was to develop a cell culture process that is capable of producing 10 g/L of a desired therapeutic product within a couple weeks of fed-batch cultivation. Despite advances in the field, and taking a step back in production potential by switching to serum-free medium, this milestone in product titer of 10 g/L has yet to be achieved.

The following example shows that this objective would be attainable, even despite the slower growth of animal cells and their lower specific productivity, if cell death could be prevented altogether. Let us imagine a cell line with a constant growth rate of 0.03 $hr^{-1}$ and a constant specific productivity of 1.5 x $10^{-9}$ mg/cell/hr, and that the cell line does not succumb to cell death during the course of a batch. To reach 10 g/L in such a system, the cells would need to grow from 2e5 cell/ml at inoculation up to 2e8 cells/ml

over a 10-day period. The corresponding integrated viable cell density for such a batch run would be 6.7e9 cell-hr/ml. In this scenario, the ten-day fed-batch would yield the desired 10 g/L of product. The final cell density achieved for such a hypothetical culture is reasonable in regard to a calculated maximum cell density for close-packed cells of 2e9 cell/ml (assuming a cell diameter of 15 μm and no void space). The final cell density, though quite high, is only one-tenth that of the close-packed limit.

I use this scenario for the theoretical objective to compare improvements to fed-batch cultures. Below I plot the cell densities, viabilities, and product titers for the objective along with two real processes below (Figure 2.1 a b). The real processes shown are those for hybridoma cells grown in serum-based medium (Xie and Wang 1996) and hybridoma cells grown in serum-free medium (my own data). For the former data, I show the first 10 days of the 23-day run (that yielded 2.4 g/L overall). For the latter data, I extend the six-day run to ten days by calculating total and viable cell densities using growth and death rates from the sixth day. It is obvious that the actual process data pale in comparison to the best case for the desired objective. Maximum viable cell densities for the actual processes are 40 to 100 times lower than that of the scenario. Product titers (0.70 g/L and 0.35 g/L for serum and serum-free) are 15 to 30 times smaller than the objective (Figure 2.2). From the cell density and viability plots, it is clear that the emergence of cell death has a severe negative impact on culture productivity.

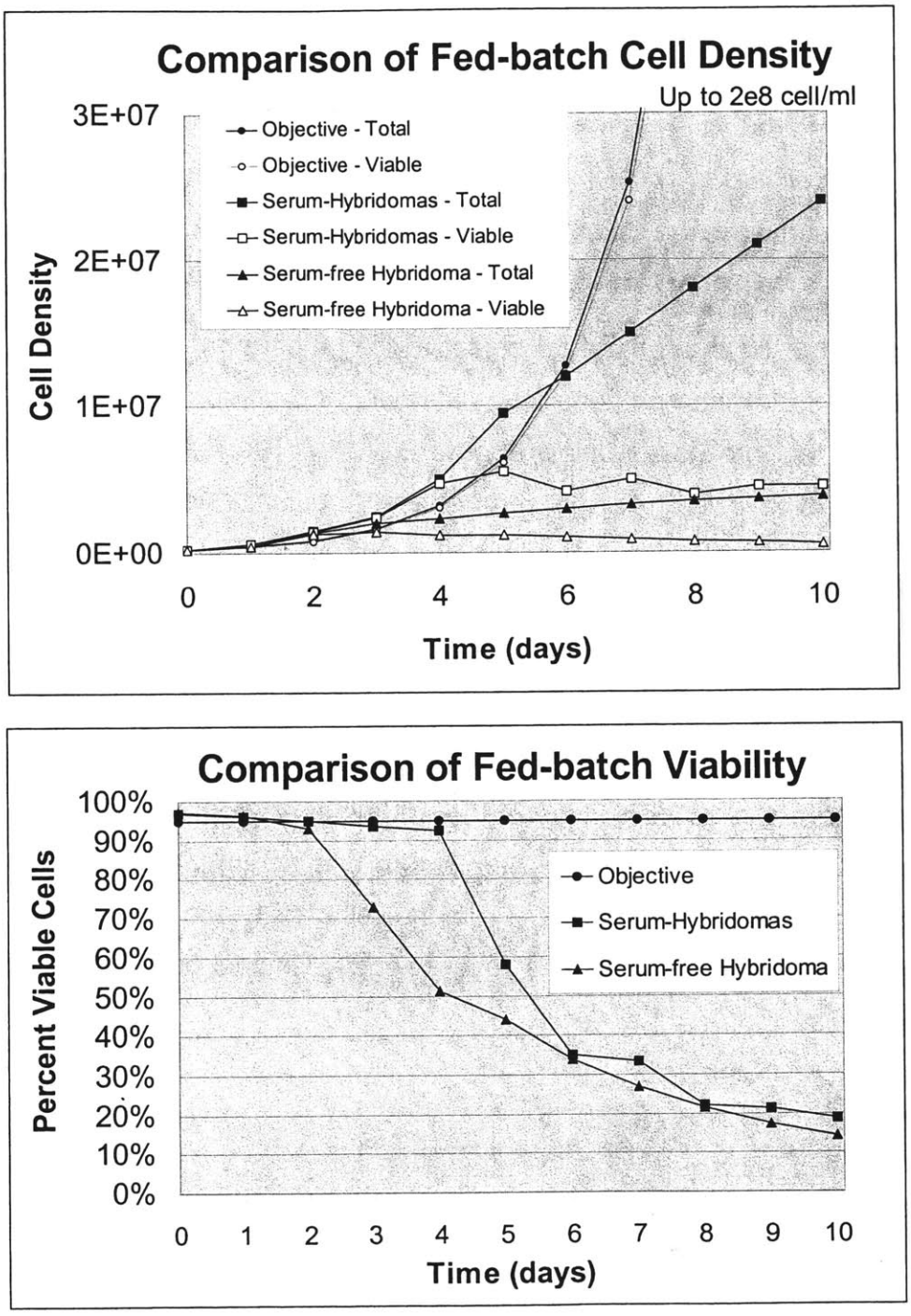



**Figure 2.1 a,b. Comparison of cell density and viability for serum- and serum-free hybridoma fed-batch cultivations vs. a theoretical death-free culture.**

Data for the serum-hybridoma truncated to 10 days from a 23-day run performed by Xie and Wang (Xie and Wang 1996). Data for serum-free hybridomas extended from data for a 6-day run, using growth rate and death rate of 0.011 and 0.018 $hr^{-1}$, respectively. Theoretical data is calculated using a constant growth rate of 0.03 hr-1 and constant viability of 95%.

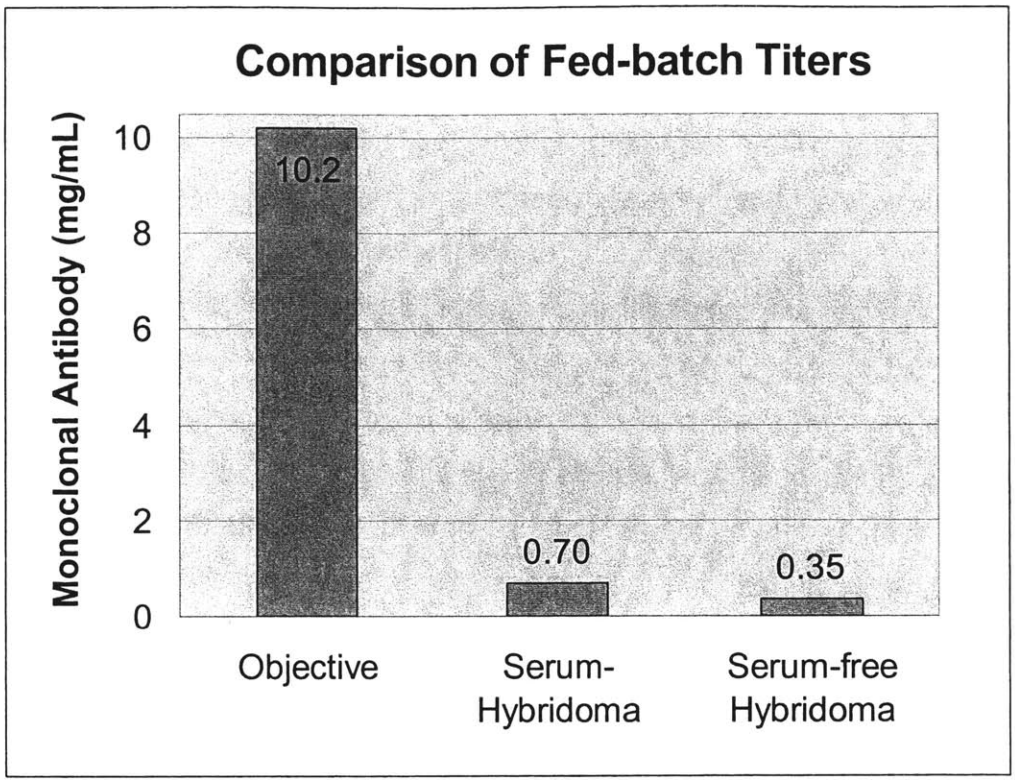



**Figure 2.2 Comparison of 10-day fed-batch titers from serum- and serum-free hybridoma cultivations vs. a theoretical death-free culture.**

Titer for the serum-hybridoma is that for the 23-day run performed by Xie and Wang at day 10 (Xie and Wang 1996). 10-day titer for serum-free hybridomas is based on the 6-day titer of 0.25 g/L, extended using the viable cell density from Fig. 2.1 and a specific productivity of 1.5e-9 mg/cell/hr. Theoretical data is calculated using viable cell density from Fig. 2.1 and a specific productivity of 1.5e-9 mg/cell/ml.

There remains ample room for further improvement of today's fed-batch processes. Even for the data shown above it is evident that the use of serum has a beneficial effect on culture performance by supporting viable proliferation up through four days. The switch to serum-free medium actually pushed progress backwards – cells grow viably for just two days and yield half the product titer. Nonetheless, the improvement of serum-free processes still depends on understanding and defeating cell death. As will be discussed in Chapter 4, the extension of viable growth of a serum-free hybridoma culture by one day and the increase of apparent specific productivity (by using a cell cycle arresting agent rapamycin) results in a doubling of product titer during a six-day serum-free cultivation of hybridomas. On the same 10-day basis described above, such rapamycin treated cultures can rival the serum-based counterparts by yielding an estimated titer of 0.95 g/L.

Thus, the ability to make vast improvements on culture production rests on learning how to extend viability by reducing cell death. Two types of death can plague a cell culture process: necrosis and apoptosis. The following sections discuss relatively recent works that have helped minimize or understand the emergence of these types of cell death during culture processes. The works and concepts presented comprise the knowledge base on which I developed my own hypothesis, experiments, and results.

## 2.2 ADVANCES AND OPPORTUNITIES IN FED-BATCH CULTIVATION

The following topics in cell culture engineering pertain to advances in the culturing of cells in batches and fed-batches. The topics and references chosen place the focus of the discussion on continuous cell lines, such as hybridoma and Chinese hamster ovary cells. The scope is further narrowed to focus on process limitations that may possibly be implicated in the emergence of apoptosis.

Today the field of cell culture engineering is "maturing" to a new level of analysis, objectives, and engineering goals. The figure below represents my view of the hierarchy for research and analysis of cells in vitro.

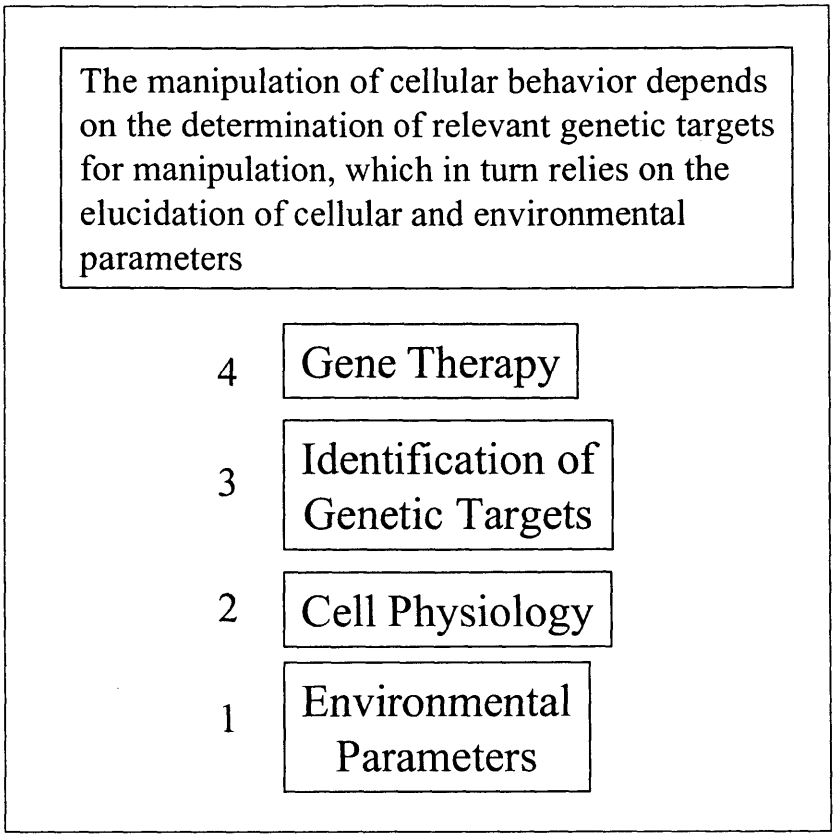



**Figure 2.3 Hierarchy for the investigation and manipulation of cellular behavior of mammalian cells.**

Initially, cell culture research involves the study of the many various culture parameters that affect growth, productivity, and/or survival of cells in vitro. For the culturing of continuous cell lines for use in fed-batch processes, such investigation has been exhausted for the current achievable cell densities. Yet, in the cultivation of new types of cells (such as hematopoeitic and bone marrow cells) researchers find themselves testing the effects of pH, shear, dissolved oxygen, nutrients, and growth factors on culture physiology all over again. Establishment of the optimal culture parameters for a given cell line is usually a necessary first step. Today, researchers finding themselves with a new cell line or cell type to cultivate, can readily characterize it using the knowledge base methods established over the previous 20 years.

Implicit in determining the optimal environment for the cultivation of a cell line is the description of the overall physiology of a cell culture that is observed and that which is

ultimately desired. Having designed the best possible environment, the next endeavor may be to characterize cellular phenomena such as growth, metabolism, cell cycle control and progression, specific productivity, and/or programmed cell death. These five phenomena are used as a basis to characterize cellular behavior. Their variation is observed to be related to one another in vitro and can be affected by the choice of culture parameters. Furthermore, all the phenomena are genetically controlled. The observed relationships in these cellular phenomena can be used to determine the genetic constraints for cellular behavior and identify potential opportunities to manipulate it for our particular needs. The work described in this thesis falls within this scope of determining and manipulating cellular physiology.

The determination of targets for genetic manipulation relies on information pertaining to the cellular phenomenon of interest as well as the relevant environmental parameters that must be maintained in vitro. Today, the choice of possible genetic targets relies on observations of physiology coupled with established biological models from the literature. A more rational and quantitative approach is the use of metabolic engineering, a proven methodology for the analysis of biochemical pathways and their control. Metabolic flux analysis is used to quantify intracellular fluxes and determine potential ramifications of altering the flux network. Besides metabolic pathways, the methodology is now finding its way to be used for analysis of signal transduction pathways and the supply and fate of precursors for post-translation modification of proteins. Yet without the genetic map for the cells or a full complement of tools with which to study genetic modifications for mammalian cells, the identification of the best targets remains a challenging endeavor.

The emerging fields of genomics and proteonomics promise to develop the means by which to implement the genetic changes that are warranted from the study of cellular physiology and proposed by metabolic engineering analysis. Manipulation of animal cell culture behavior and productivity through genetic means has yet to be reduced to practice. The insertion or knockout of key genes in mammalian cells is still a formidable endeavor. Furthermore, the choice of critical genes that could have desired impacts is not available. Advances in genomics and proteonomics will enable cell culture researchers to more fully probe and understand the genetic "circuits" and protein

networks that ultimately control cellular behavior. This direction of evolution for cell culture engineering technology will ally itself with developments in gene therapy techniques for inserting and manipulating mammalian genes. Together, the genetic revolution, which is being fueled by biomedical applications, will exploit the in vitro cultivation of mammalian cells in its quest to cure human illnesses. Cell culture researchers, however, will also be able to make use of such techniques to "cure" their own cultures of the limitations of low specific productivity and susceptibility to apoptotic cell death during batch cultivation. These are indeed exciting times to capitalize on the synergy between the genetic manipulations proposed by gene therapy for humans, and the genetic manipulations needed to maximize cell culture processes. In both cases, we seek to use genetic modifications to achieve a desire phenotype of cellular behavior.

## 2.2.1 Environmental Culture Parameters

### 2.2.1.1 Temperature

For many fed-batch processes, temperature is simply fixed near or at 37°C throughout the cultivation. Yet, as has been recently reported (Chuppa, Tsai et al. 1997), the choice of temperature setpoint, or the variation of its setpoint during a culture process, may have beneficial effects on viability and productivity. As reported, the lowering of the temperature setpoint down to 34° C during perfusion culture was associated with a variety of significant physiological changes for a recombinant hamster cell line. At temperatures of 37°, 35.5°, and 34°, cell density and viability remained constant at 20 x $10^6$ cell/ml and 90%, where as growth rate decreased 20%, glucose uptake decreased 50%, lactate production decreased 30%, glutamine uptake decreased 15%, ammonia production decreased 20%, oxygen and carbon dioxide rates decreased by 25%, and production of the therapeutic protein was mildly enhanced and glycosylation was comparable. Thus, it appears that the downshift in metabolism follows the downshift in growth rate, without detriment to cell density being that it is a perfusion process.

On hindsight for the work of this thesis, the changes in metabolism and cell cycle progression associated with temperature could be of interest in the extension of viable proliferation of hybridoma fed-batch cultures. As is demonstrated in later chapters, the

inhibition of G1 progression by rapamycin greatly reduces apoptotic cell death, cell death is associated with changes in metabolic efficiency, and productivity is a function of cell cycle progression, metabolism, and cell death. Thus, it could be of interest to compare rapamycin's positive effects on viable proliferation to those that are reported for a decrease in fermentor temperature. Nonetheless, given the ease of controlling temperature, neither disturbances in culture temperature nor the choice of setpoint at 37°C is implicated in the emergence of death in fed-batches.

#### 2.2.1.2 Extracellular pH

Optimal extracellular pH for viable hybridoma cell density has been measured to be 7.1 to 7.4 (Miller, Wilke et al. 1987). Some reports for hybridomas quote the larger range of 6.8 to 7.6 where effects on growth are minor (McQueen and Bailey 1990), while other reports indicate that there is pronounced growth inhibition for hybridomas as extracellular pH is lowered from 7.0 to 6.7 (Cherlet and Marc 1998). Freshney quotes a range of 7.0 to 7.4 cells for transformed cell lines as determined by Eagle (Freshney 1994).

Within a $CO_2$ incubator and using buffered medium or within a properly controlled bioreactor with acid/$CO_2$ and base addition, suboptimal pH is not a likely suspect for the growth arrest and cell death that occurs in fed-batches. In my own work, chemostat cultures were maintained within a pH range from 7.15 and 7.25. For T-flask cultures kept in incubators, pH begins at 7.2 and is observed to fall below 7.0 (6.8-7.0 measured by blood gas analyzer) as batches progress, but this drop coincides with or follows after the emergence of cell death. Furthermore, bioreactor batches suffer the same fate of cell death despite pH control. Thus, I am confident that pH is not the limiting factor for today's fed-batches.

On hindsight to my observations concerning metabolic changes associated with cell death, it is interesting that the metabolic symptoms associated with an extracellular pH that is too low (around 6.7) are mild to severe growth arrest, decreases in specific glucose consumption (higher cellular yield), and increases in specific glutamine consumption (lower cellular yield) (McQueen and Bailey 1990; Cherlet and Marc 1998). These metabolic shifts are attributed to higher maintenance requirements as the

differential between intracellular and extracellular pH increases. My own observations reveal a similar shift towards increased energy production as death emerges, but as the result of unknown causes rather than low pH. The similarity in metabolic responses for pH-induced stress and that of growth arrest and death in fed-batches suggests that the unknown factor(s) that plague fed-batch processes may exercise its negative impact by raising maintenance requirements by some as of yet undetermined mechanism.

#### 2.2.1.3 Dissolved Oxygen

Supply of oxygen is indispensable for the cultivation of aerobic mammalian cells. This issue is further intimately tied to the method and efficiency of stirring and sparging, which can be detrimental to cell integrity (Gardner, Gainer et al. 1990). During agitation, the presence of a vortex and the resultant bubble entrainment can be detrimental to cell density. In the absence of a vortex, agitation as high as 700 rpm is not harmful (Kunas and Papoutsakis 1990). Thus, even though the mammalian cells are more sensitive to shear damage than bacterial cells, agitation by itself is not implicated in causing cell death and cultures can be thoroughly agitated to ensure a well-mixed environment.

Many engineering configurations are available for the supply of oxygen. Headspace aeration only allows for a limited cell density. Sparging can be used with ease and successfully with the presence of pluronic F-68 and other surfactants that help minimize cell disruption (Meier 1998). Other techniques include the use of a coiled silicone tubes, perfluorocarbon, or an external aerator (Aunins, Croughan et al. 1986; Mano, Kimura et al. 1990).

In addition to supplying oxygen, the dissolved oxygen concentration chosen for setpoint can also affect culture growth and metabolism, though the optimal setpoint varies from one report to another (Ozturk and Palsson 1990). An optimum in viability for hybridomas was observed at the very low steady state dissolved oxygen level of 0.5% of air saturation and is attributed to a reduction in oxidative stress (Miller, Wilke et al. 1987). This finding is supported by the more recent study showing the ill effects of hyperoxic conditions on viability, taken to be due to oxidative stress since anti-oxidant enzymes are induced many-fold (Jan, Petch et al. 1997). Furthermore, the reports indicate that DO levels between 1% and 10% are not limiting to mitochondrial respiration. Thus, for

the fed-batch cultivation of cells, a DO setpoint which can be feasibly and reliably implemented in the transient process must be chosen which would eliminate the possibility of oxygen depletion without causing undue oxidative stress. As a general guideline, a setpoint of 5-10% saturation is a safe initial range. Such a reduction in setpoint from the typically used 40-60% DO would also allow a near doubling in oxygenation capacity for a constant mass transfer coefficient, kLa.

My own investigations have been carried out with a setpoint of 40-50% in bioreactor cultures and dissolved oxygen concentrations anywhere from 10-100% of air saturation in incubator cultures. Depletion of oxygen is therefore not considered to be the cause for cell death in fed-batches. Furthermore, the emergence of cell death that is exhibited within my own fed-batch processes does not appear to have the same metabolic symptoms as those reported for oxygen depletion or for hyperoxic (high oxidative stress) conditions.

#### 2.2.1.4 Osmolarity

Osmolarity is an important culture parameter to monitor and to maintain within a reasonable range that does not impede cell proliferation or increase death rate (Ozturk and Palsson 1991). Osmolarity of fresh culture medium is 260-320 mOsm. The effect of higher starting osmolarity for batch processes reveals that culture growth is inhibited, that cell death is increased (even during exponential growth phase), and that metabolic rates are increased (i.e. cell yield is decreased, implying higher maintenance). Furthermore, once the death phase commences higher osmolarity also leads to greater death rates than controls. Thus, higher osmolarity is viewed as an environmental stress that should be avoided in the design and control of an optimal fed-batch process.

The global symptoms of growth arrest and exacerbated cell death are similar to the ones we observe for our current fed-batch processes, even though osmolarity is maintained within a healthy range (Bibila, Ranucci et al. 1994; Robinson, Seamans et al. 1994; Xie and Wang 1994). For my own work, osmolarity was infrequently measured and assumed to be within a healthful range since were using Xie and Wang's feeding strategy for fed-batches or running chemostats. The interesting question remains as to what is the mechanism by which a higher osmolarity leads to slower growth and cell

death. Investigation of this mechanism may reveal other cellular phenomena that are possibly related to the emergence of cell death in today's fed-batches. For now, suboptimal osmolarity is not considered to be the cause of death in fed-batches.

#### 2.2.1.5 Toxicity of Waste Metabolites: Lactate and Ammonia

Two well-known culprits that cause cessation of culture growth and cell death are the waste metabolites lactate and ammonia (Hassell, Gleave et al. 1991). These compounds arise from the normal catabolism of the two major substrates glucose and glutamine. Ammonia also further accumulates as the result of non-biological degradation of glutamine within culture medium. Toxic lactate concentrations are reported to range from 25 mM to 80 mM and toxic ammonia concentrations are reported to range between 2 mM and 4 mM. Furthermore, the toxicity effects are more severe from an initial spike of exogenous ammonia or lactate than in the gradual accumulation throughout culture (Xie 1997).

Though the sensitivities for such substances are cell-line dependent, the response of a culture once inhibited is growth arrest and cell death. This occurs even in the absence of changes in pH. Furthermore, a decrease in pH from 7.8 to 6.8 reduces the effect of ammonia inhibition in a hybridoma cell line, since it is ammonia rather than ammonium that is the toxic species (Doyle and Butler 1990). For this reason, the presence of lactate (hence lowering the pH) can somewhat minimize the effect of ammonia (Hassell, Gleave et al. 1991). At the biochemical level, ammonium has been reported to elevate the amount of intracellular UDP-GNAc, whose concentration correlates with the level of growth inhibition in mammalian cell lines (Ryll, Valley et al. 1994). The effect of ammonium ion has also been modeled for its effect on intracellular pH of hybridoma cells (McQueen and Bailey 1990). Lactate toxicity on the other hand is believed to be toxic through its effects on osmolarity and extracellular pH (Xie 1997).

Ammonia addition to continuous hybridoma cultures resulted in lower steady state cell density due to increases in cell death, but did not affect the steady state specific growth kinetics. Higher ammonia concentrations also were observed along with lower cell yields for glucose, glutamine, and oxygen, hence symptoms of a loss in metabolic efficiency (Newland, Kamal et al. 1994). The effect of a step up in ammonia

concentration was followed by a period of adjustment in cellular metabolism. It would have been interesting to see if the shift in metabolism would have yielded a multiple steady state upon a downshift in ammonia concentration (which was not apparently performed by Newland, et. al.). As will be shown in Chapter 3 for nutrient limitation, the metabolic adaptation of hybridomas is involved in the occurrence of steady state multiplicity.

Strategies to minimize lactate and ammonia production include proper medium design and feeding strategies (Glacken, Fleischaker et al. 1986; Xie 1997) as well as removal by electrical or chemical means (Chang 1994). The adjustment of metabolism through nutrient control to minimize production of toxic by-products is the preferred method to minimize lactate and ammonia since it can be applied at all scales.

For the hybridoma used in this study, limits for the accumulation of lactate and ammonia were taken to be 40 mM and 4 mM, respectively. Their concentrations were measured and shown to be below such limits during fed-batch experiments. For the moment, lactate and ammonia concentrations can be kept just below inhibitory levels for the viable cell densities that can presently be achieved. As advances are made towards achieving the super-high cell density fed-batches needed to produce 10 g/L titers, ammonia and lactate inhibition will resurface as a threat to such productivity.

#### 2.2.1.6 Nutritional Supply - the Essence of the Fed-Batch Process

The most significant improvements in the extension of culture span in batch processes have stemmed from the development of proper feeding strategies, and hence the implementation of the "fed-batch" process. Culture life span has been extended well beyond the point of depletion of nutrients from the initial batch medium by the feeding of additional nutrients to the growing culture. Several strategies have been reported to yield significantly increased titers over an increased culture life span (Bibila, Ranucci et al. 1994; Bushell, Bell et al. 1994; Robinson, Seamans et al. 1994; Xie 1997). Strategies are based on varying frameworks according to research or process development needs, using 10 X concentrates to specifically tailored stoichiometrically-designed supplemental media. Feeding strategies have also been coupled to on-line measurements with more sophisticated protocols and multiple nutrient feeds with much

success (Zhou and Hu 1994; Bibila and Robinson 1995). Thus, the supply of nutrients has been very successful in augmenting production capacity for therapeutic proteins. As of the early 1990s, such accomplishments in fed-batch technology had reduced the finished price of therapeutic MAbs by 10-fold ($10k to $1k per gram) (Bibila and Robinson 1995).

The proper feeding of fed-batch cultures can be said to have successfully eliminated apoptotic cell death as the result of nutrient limitations. Nonetheless, apoptosis emerges shortly (within 4 days of inoculation) after commencing a fed-batch and plagues the culture throughout its run. The emergence of cell death not only detracts from productivity due to the loss of viable cells, but also possibly due to physiological changes associated with the emergence of cell death, such as shift in metabolism, which may or may not influence specific productivity. As fed-batch development solves one major cause for cell death in bioreactors (nutrient limitation), it leaves open the identification of other causes for cell death. In my own work, I employ the feeding strategy of Xie and Wang, which allows me to focus on elucidating other causes for apoptosis besides nutrient depletion.

#### 2.2.1.7 Serum and Serum-free Medium

The shift to serum-free medium follows from several process, research, and regulatory pressures. For the full-scale production of therapeutic proteins, the addition of high levels of additional proteins to the culture, though useful for cell growth, only complicates the final isolation of the desired product. Since a large fraction of the overall process costs are associated with downstream processing, separations, and quality control, a lower protein burden downstream is economically desired. The savings in raw material are roughly balanced by the cost of new supplements. From the research perspective, the analysis of culture physiology is best achieved under as well-defined conditions as possible. The seasonable variation in serum-products, and the unknown identities and roles of serum components, can lead to differences in culture behavior that are not necessarily occurring by experimental design (the same issue exists for the use of conditioned medium, as is discussed in Chapters 5 and 6). Lastly, from the perspective of validating a process for FDA approval, the use of serum as a raw

material adds another uncertainty to the overall production process. For these reasons and more, the use of animal products is becoming increasingly undesirable.

While the step-down in serum concentration initially inhibits cell growth, the long-term cultivation of cells in low- and non-serum media results in faster growth and less dependence on serum (Ozturk and Palsson 1991). Such adaptation is reported not to alter culture metabolism, yet there may be a negative influence on specific productivity that is cell line dependent. The role of serum on the emergence of cell death has been characterized more recently (Chung, Sinskey et al. 1998). The snap-shift of cells from 5% serum to serum-free is associated with the earlier (at between 24 to 48 hours) emergence of apoptotic cell death, in addition to a slower growth rate. Such death can be postponed by 24 hours by the addition of insulin (as well as by the over-production of Bcl-2, an anti-apoptotic protein.) The message of such studies is the role that environmental factors can have on both the stimulation of growth and the emergence of apoptosis. Serum apparently contains factors that support growth (such as insulin), but also factors that prevent apoptosis beyond the 48 hours that insulin alone cannot.

The adaptation to serum-free media for my own hybridoma cell line was accompanied by even lesser productivity for my fed-batch process. The emergence of cell death (in the presence of insulin supplement) now emerged soon after 48 hours rather than at 72-96 hours with serum, resulting in lower cell density and product titer. One of the themes which runs throughout my work is the role of environmental factors which protect cells against apoptosis. To this end, insulin's effects on metabolism and cell cycle are further investigated. Furthermore, rapamycin is identified as another environmental factor that further delays apoptosis. Finally, new evidence is presented to support the hypothesis that hybridoma cells produce different factors that may support viability or may stimulate cell death.

### 2.2.2 Genetic and Molecular Phenomena

The analysis of cell culture at the genetic and molecular level became necessary when it was realized that the emergence of cell death still occurred even when environmental culture parameters were controlled to the best of our knowledge and ability. The genetic control of cell death was an obvious choice to investigate at that time. Furthermore,

there was emerging evidence to suggest the role of other cellular phenomena (genetic and molecular) in the emergence of cell death. The following sections briefly describe the works that served as the basis for my own investigations.

#### 2.2.2.1 Apoptosis

Following the increasing interests and discoveries of the biological community regarding programmed cell death in the late 80's and early 90's, by 1994 the cell culture engineering community identified the phenomenon of apoptosis as a significant mode of cell death in bioreactor cultures of continuous cell lines (Franek, Vomastek et al. 1992; Mercille and Massie 1994; Singh, Al-Rubeai et al. 1994). This form of genetically controlled cell death is a natural and necessary cellular function for homeostasis, development of organisms, and the prevention of cancerous growth. Yet in the setting of a bioreactor, such a phenomenon is essentially "genetic baggage" which amounts to a very detrimental nuisance. Though apoptosis is related to the culture parameters and cellular physiology, it stands alone in its genetic nature as a phenomenon worth targeting directly for improvements in viable cell density.

Cell death in vivo or in a bioreactor can result from necrosis or apoptosis. Necrotic cell death is that resulting from severe physical or chemical trauma and leading to the ultimate disruption of the cell. Necrotic cell death is not involved in development and does not require synthesis of new proteins. In vivo, the release of cellular contents as the result of necrotic death often leads to an inflammatory response and damaging secondary effects (Franek, Vomastek et al. 1992; Schwartz and Osborne 1993). From my own experience, I know that necrosis results from undue variations of pH and osmolarity, excessive shear, damage due to sparging, and possibly the toxic effects of ammonia and lactate.

Apoptotic cell death is an active cellular phenomenon requiring metabolic energy and continuing protein synthesis (Fesus, Davies et al. 1991; Franek, Vomastek et al. 1992). Apoptotic cell death is accompanied by visible morphological features such as cell shrinkage, membrane blebbing, and chromatin condensation as the result of digestion of DNA by an endogenous nuclease (Fesus, Davies et al. 1991; Schwartz and Osborne 1993). Apoptosis may be induced as a programmed event in developmental processes

by biological agents, via cell surface receptors and secondary messenger molecules or by interaction with nuclear receptors that control the expression of related genes directly (Fesus, Davies et al. 1991). Conversely there are numerous extracellular factors that act as survival factors by preventing apoptosis (Fesus, Davies et al. 1991). The triggering or suppression of apoptosis by extracellular factors is dependent on signal transduction through distinct signaling pathways or through pathways that are not solely involved in apoptosis (Schwartz and Osborne 1993). Apoptosis also results from suboptimal culture parameters, such as nutrient or serum limitation and oxygen depletion. Deprivation of any amino acid can trigger apoptosis in hybridoma cells (Simpson, Singh et al. 1998). Thus, with the many possible stimuli, both environmental and genetic in nature, research in prevention of apoptotic cell death in bioreactors has pushed the study of cells further onto the cellular and molecular level of analysis.

Research into the mechanism of apoptosis has revealed several genes and proteins that are involved in the stimulation or prevention of apoptosis. In *Drosophila*, the reaper gene is found to play a central role in the control of the emergence of apoptosis (White, Grether et al. 1994), and its deletion prevents apoptosis under a variety of conditions. In C. elegans, proteins CED-3 and CED-4 have been found necessary for cell death to occur, whereas CED-9 is shown to protect cells against death (Chinnaiyan, O'Rourke et al. 1997; Golstein 1997; Wu, Wallen et al. 1997). In mammalian cells, CED-3 are homologous to the family of proteins called caspases, CED-9 corresponds to the Bcl-2 family of proteins, and the CED-4 equivalent remains unknown (Chinnaiyan, O'Rourke et al. 1997; Golstein 1997; Wu, Wallen et al. 1997). Bcl-2 appears to be the more robust anti-apoptotic agent since it blocks the release of cytochrome C from the mitochondrion into the cytosol, which can activate caspases and the apoptotic pathway (Kluck, Bossy-Wetzel et al. 1997; Yang, Xuesong et al. 1997). Overall, I believe the role of mitochondrial proteins in apoptosis may provide a molecular mechanism with which to better understand the possible role of metabolism and cellular energetics in the initiation of cell death.

These findings have been applied to help minimize apoptosis in batch and fed-batch cultures (Terada, Itoh et al. 1997; Chung, Sinskey et al. 1998; Goswami 1998; Simpson, Singh et al. 1998; Goswami, Sinskey et al. 1999; Sanfeliu and Stephanopoulos 1999;

Sanfeliu, Chung et al. 1999 in press). The overexpression of Bcl-2 leads to an extension of viability under both optimal and suboptimal conditions as compared to control cultures. The over-production of Bcl-2 is without effect on culture growth rate, yet the impact on other parameters of culture physiology (such as metabolism and product quality) remains unidentified. Thus, improvements to fed-batch cultures result from the maintenance of cultures with higher viable cell density, which continue producing the desired recombinant protein product. Titer improvements of 75% correlate well with the increase in integrated-viable-cell-density (IVCD) for suspension CHO cells producing gamma-interferon (Goswami 1998). Treatment of cultures with caspase inhibitors (zVAD-fmk) has shown only mild improvements in culture viability (Goswami 1998; Goswami, Sinskey et al. 1999). This is presumably the result that caspase activation by cytochrome C is further downstream in the apoptotic pathway.

While the search for genetic means to block the cascade of events that occur during apoptosis is justifiable, it is still ultimately important to ascertain the principle cause for this detrimental cellular behavior. It is not clear whether the blocking of apoptotic cell death will alone necessarily yield the optimal cellular behavior. The underlying causes for cellular "unrest" leading to cellular "suicide" must be addressed as part of the total solution.

#### 2.2.2.2 Metabolism of Cell Culture

Metabolism of cell cultures has been widely studied for the principle aim of designing supplemental feed media (Glacken, Huang et al. 1989; Bibila, Ranucci et al. 1994; Bushell, Bell et al. 1994; Robinson, Seamans et al. 1994; Xie and Wang 1994; Xie and Wang 1994; Bibila and Robinson 1995; Zhou, Rehm et al. 1995; Mollborn 1996; Xie 1997). Simply put, knowledge of cellular nutritional needs is a prerequisite for the development of optimal feeding strategies that will supply the right mix of nutrients and other factors to avoid limitation without over-feeding. Metabolic data is obtained from variations of batch, fed-batch, and chemostat experiments. In each case, attention to the effects of limiting factors on growth and death (which can influence metabolism) is necessary to translate metabolic data to a culture intended not to have any such limitations. Determining the optimal feeding strategy is further complicated since cell

yields on glucose and glutamine depend on their concentrations in culture and metabolism has also been shown to be different for different phase of the cell cycle (Miller, Wilke et al. 1987; Miller, Wilke et al. 1989; Miller, Wilke et al. 1989; Ramirez and Mutharasan 1990; Hiller, Aeschlimann et al. 1991; Hiller, Clark et al. 1994; Linz, Zeng et al. 1997; Xie 1997).

In terms of a possible relationship between the emergence of apoptotic cell death and changes in metabolism, both phenomena have been reported to occur simultaneously during fed-batch cultures (Xie 1997; Goswami 1998). During fed-batches of hybridomas, it was observed that metabolic rates for glucose, lactate, alanine, and glutamine change dramatically during a fed-batch. Lactate was even reported to be consumed rather than produced during the stationary phase. Such changes occurred even when the nutritional environment was kept relatively constant by a proper feeding strategy. Material and energy balances revealed that TCA cycle was more active, generating more energy from TCA activity for cultures with lower glucose and glutamine concentrations. For CHO fed-batch cultures, the glucose uptake rate is measured to decline rapidly just before reaching the stationary phase. This phenomenon was investigated in terms of other coincident changes in cell cycle progression, p53 expression and cyclin E expression, and the possibility that insulin is depleted by degradation.

Shifts in culture metabolism have also been investigated in the course of studying other cellular functions, such as productivity and glycosylation of recombinant proteins (Hayter, Curling et al. 1993; Nyberg, Balcarcel et al. 1999). In glucose-limited CHO continuous culture, Nyberg and co-workers observed a shift in metabolism at different dilution rates. Analysis of intracellular fluxes for central carbon metabolism revealed that metabolism shifted from glycolysis towards TCA cycle activity and oxidative phosphorylation as the dilution rate was decreased. This metabolic shift occurred as a result of, in conjunction with, or despite the emergence of cell death. Thus, the evidence regarding metabolic shifts in both the hybridoma cell line and the CHO cell line were instrumental in the development of my thesis objectives.

Another finding, occurring in parallel to my own, provided further supporting evidence to the relationship between metabolism, cell cycle, and apoptosis (Sanfeliu and

Stephanopoulos 1999). Sanfeliu demonstrated that growing serum-dependent anchorage CHO cells under glutamine-limitation could minimize apoptosis following insulin stimulation in serum-free medium. Glutamine-limited growth was accomplished in glutamine-free medium by the addition of glutamate and the reliance of glutamine formation on glutamine synthetase activity, which she discovered could be induced in this cell line. The cells grown in serum-free medium (insulin(+), glutamine(+)) grew more quickly, yet exhibited growth arrest and cell death within several days. Conversely, the cells grown without glutamine grew more slowly and did not exhibit cell death throughout the 12-day experiment. The growth of the glutamine-limited culture was presumed to be limited by the rate at which glutamate was converted to glutamine by glutamine synthetase. Thus cellular robustness against apoptosis (in the absence of protective factors in serum) is observed to be enhanced by constraining metabolic consumption of glutamine and the resulting slower growth rate.

A link between metabolism and apoptosis is also supported by the report that overexpression of Bcl-2, an anti-apoptosis protein, reduces specific nutrient consumption (Simpson, Singh et al. 1998). Such a relationship is of interest in further elaborating Bcl-2's mechanism of protection. However, the interpretation of the effects of Bcl-2 on cellular physiology is open to debate. Though, it is accepted today that apoptosis, cell cycle, and metabolism are intimately related phenomena, it is very challenging to determine the direct role of Bcl-2 on one or more of the phenomena because its effect on one phenomenon also affects the others. Though genetic alterations of the Bcl-2 protein can be made to alter Bcl-2's "cell cycle" function or "apoptotic" function (Huang, Oreilly et al. 1997), I believe it still remains unclear exactly how Bcl-2 is affecting one, both, or all of the phenomena. In my own work, I came across the similar difficulty in trying to understand how rapamycin and insulin may affect cell cycle, apoptosis, and/or metabolism.

In this thesis, changes in metabolism are placed in the same framework as the emergence of cell death. The quantification of shifts in metabolism is coupled with the emergence of cell death in the hope of determining metabolic effects that either promote or are symptomatic of cell death. The basis for the relationship between cell death and metabolism is provided in Chapter 3, which I further build on in the discussion of the

effects of rapamycin and insulin on viability in Chapters 4 and 5. Brian Follstad has also pursued the relationship to investigate the role of mitochondrial potential on culture metabolism and to design feeding strategies that result in cultures of greater metabolic capacity and higher viable cell density (Follstad, Ph.D. Thesis, in progress).

#### 2.2.2.3 Culture Growth and Cell Cycle

The cell cycle is a model for the progression of cells through the various phases of division as they grow. Newborn cells, or recently induced cells, begin the cell cycle in the G1 phase of the cell cycle, with one copy of DNA. Under the right conditions, cells can enter the next phase, called the S-phase, where they duplicate their DNA. Thereafter cells proceed through mitosis (M-phase) following a short preparative gap, called G2-phase. Progression through the phases is tightly regulated by environmental and genetic factors. Environmental factors such as nutrients and growth factors are required for entry to the S-phase and successful completion of mitosis. Genetic factors (in combination with environmental cues) provide checkpoints prior to cell cycle entry, within the S-phase, and before mitosis. The checkpoints serve to control proliferation and differentiation and prevent the propagation of errors in DNA synthesis and chromosome separation during the cell division cycle. The checkpoints are regulated by proteins that oscillate in concentration throughout the cell cycle (cyclins), which at peak concentrations bind to cyclin-dependent kinases, complexes that trigger successive molecular events necessary for cycle progression (Lodish, Baltimore et al. 1995).

Cell cycle distribution and progression can be measured and calculated for cell culture applications in conjunction with other culture parameters and phenomena, such as growth rate and productivity (Kromenaker and Srienc 1991; Martens, Gooijer et al. 1993). The incorporation of cell cycle phenomena is useful to better interpret culture data. To this end, models for cell cycle progression have been combined with those for metabolism and cell death to interpret data of hybridoma cell cultures (Suzuki and Ollis 1989; Linardos, Kalogerakis et al. 1992; Martens, Gooijer et al. 1996).

Of value for the improvement of fed-batch processes is knowledge pertaining to the interaction between cell cycle function and apoptosis. Previous models and observations claim that continuous cell lines such as hybridomas and CHO cells exhibit

increased apoptosis in conjunction with an arrest in G0/G1 and a decrease in S-phase residence (Suzuki and Ollis 1989; Linardos, Kalogerakis et al. 1992; Martens, Gooijer et al. 1993; Goswami 1998). Thus the inhibition of cell cycle progression (or dysfunction in general) has been implicated in triggering apoptosis. Such phenomena served as one of my motivations for measuring cell cycle distribution in my own experiments.

In a very thorough review, both cell cycle and apoptosis are explained as highly regulated, genetically controlled processes which are intimately linked (Fussenegger and Bailey 1998). The elucidation of key environmental and genetic factors continues to yield opportunities for manipulation of either or both phenomena. In some cases, advances can be implemented by the alteration of single genes, such as for the moderation of c-Jun, a gene related to cell cycle entry, to viably arrest F-MEL cells in G0-G1 or to grow them at a slower growth rate without apoptosis emerging (Kim, Iida et al. 1998). In other cases, the insertion of multiple genes is needed to achieve the desired cellular behavior (Fussenegger, Mazur et al. 1998). Furthermore, some genes may possibly affect both cell cycle and apoptosis simultaneously, as has been proposed for the anti-apoptotic protein Bcl-2.

In this thesis, cell cycle phenomena are investigated to help explain the decrease in growth rate and the emergence of apoptosis that occur during fed-batch cultivation of a hybridoma cell line. The effects of three distinct types of environmental factors are used to probe the relationship between cell cycle progression and cell death, as well as metabolism: glutamine limitation, insulin withdrawal, and rapamycin inhibition.

#### 2.2.2.4 Insulin Stimulation and Protection

Insulin is a very widely studied protein that is associated with a variety of cellular phenomena, from in vitro stimulation of cells to the regulation of glucose metabolism in human beings. For the cultivation of cells in vitro, insulin is a commonly used supplement for serum-free medium formulations. Its positive effects on the stimulation of proliferation are mediated by signal transduction through the map kinase cascade, the endpoints of which are numerous.

Insulin also plays a role in stimulating and supporting metabolic function. It has been reported to directly support metabolism by increasing the expression of the transport

genes, such as *glut-1* for glucose (Goswami 1998), and activating phosphatase activity of enzymes which regulate pyruvate dehydrogenase (PDH) activity (Gottschalk 1990). Insulin also supports metabolic function by enhancing mitochondrial Kreb's cycle activity up to 30% (Bessman and Mohan 1997). The evidence that this metabolic support is independent of insulin receptor tyrosine kinase activity will later lend support to my findings that insulin's protective role is likely to be more related to metabolism than to cell cycle.

Insulin also plays a key role in regulating progression through the cell cycle. It stimulates (via the map kinase cascade) the entry of quiescent cells into the cell cycle by inducing the expression of *c-myc*, a proto-oncogene that acts early within the G1-phase. Yet it appears that *c-myc* induction alone is not sufficient to ensure survival of cells as they progress through the cell cycle. The emergence of apoptosis has also been reported to be associated with *c-myc* expression. c-Myc protein "can act as a bivalent regulator, determining either proliferation or apoptosis, depending on whether the free movement around the cell cycle is supported (by growth factors) or is limited by growth factor deprivation or treatment with other cycle-blocking agents. In vivo, *c-myc* expression may be associated with high-turnover state in which cell proliferation and apoptosis co-exist." (Wyllie 1993)

For anchorage dependent, serum-arrested CHO cells, *c-myc* was confirmed to be inducible by insulin, reaching a peak intracellular concentration four hours after stimulation and leading to cell cycle entry and proliferation (Sanfeliu, Chung et al. 1999 in press). Yet the expression of c-Myc and cell cycle entry that follow insulin stimulation are associated with the emergence of cell death after several days of fast proliferation. Presence of survival factors from serum or the over-expression of Bcl-2 can minimize the emergence of cell death resulting from c-Myc expression. From a different perspective, overexpression of c-Myc in the hybridoma cell (which was accurately hypothesized by Chung, and later confirmed herein) is observed to require insulin for fast and viable proliferation up to 48-72 hours following a snap-shift to serum-free medium (Chung, Sinskey et al. 1998). Without insulin, the cells grow slower and cell death occurs between 24 to 48 hours. In this cell line, it appears that insulin plays more of a role as a survival factor, given that *c-myc* is already induced anyway. Taking these

findings together, it would appear that cells require additional protection against apoptosis when proliferating (when *c-myc* is induced). Insulin, though also the stimulus for *c-myc* expression in the CHO cell line, probably also confers some protection for viable growth to the CHOs, but further protection is also needed from other survival factors just as for the hybridoma cell line.

The role of insulin on metabolism and cell cycle progression is further studied in this thesis, with the aim of better understanding its protective role for hybridoma cells, and as a tool to probe the cause for the emergence of apoptotic cell death that it protects against.

#### 2.2.2.5 Specific Productivity

Specific productivity of monoclonal antibodies (and recombinant therapeutic proteins in general), as well as product quality, can depend on many of the above mentioned culture parameters and cellular phenomena (Al-Rubeai and Emery 1990; Ramirez and Mutharasan 1990; Bibila and Flickinger 1991; Bushell, Bell et al. 1993; Cain and Chau 1998). Sometimes optimal conditions for productivity differ from those for culture growth, for instance, as for the optimal osmolarity for MAb production and growth rate being 350 and 300 mOsm, respectively (Robinson, Seamans et al. 1994). Nevertheless, although it may be beneficial on a short-term basis to sacrifice viability for productivity, the long-term goal for improving fed-batch processes remains the increasing and maintaining of viable cell densities, since it is the viable cells that ultimately produce the product. After achieving optimal performance of the process in terms of density, further improvements in productivity can be attempted by considering trade-offs between density and titer.

Antibody productivity has been reported to be greater for cells within the G0-G1-phase of the cell cycle compared to other phases (Ramirez and Mutharasan 1990). In continuous culture, specific productivity has been observed to be a function of dilution rate, with non-growth associated behavior prevailing for hybridoma cultures (Miller, Wilke et al. 1987; Martens, Gooijer et al. 1993). Instability of productivity has also been documented, showing that productivity may decrease progressively over long-term experiments (Hiller, Aeschlimann et al. 1991; Martens, Gooijer et al. 1993).

The relatively constant specific productivity that is measured for my hybridoma cell line for batches and fed-batches is investigated herein within chemostat cultures, along with metabolic, cell cycle, and cell death phenomena. The preposition that higher specific productivity is associated with environmental and nutritional stress (Miller, Wilke et al. 1987) is re-addressed here with regard to the changes in metabolism, cell cycle, and cell death that accompany such stresses.

## 2.3 THESIS OBJECTIVES

The objectives of my own work in trying to understand and minimize apoptotic cell death are listed below, and correspond to Chapters 3, 4, and 5, respectively.

1. The first objective was to characterize the physiology of a hybridoma cell line in glutamine-limited chemostats. The physiology of cultures at high, intermediate, and low dilution rates is analyzed for insights regarding the relationship between cell cycle progression, metabolism, and cell death.
2. Based on the cell cycle phenomenon observed in chemostats, I hypothesize that inhibiting entry to the cell cycle may help minimize apoptotic cell death. Thus, the second objective was to investigate the use of cell cycle arresting agents to minimize apoptosis.
3. The third objective was to investigate insulin's apparent protective role against apoptotic cell death for hybridoma cells. Due to the connection of insulin to both cell cycle progression and metabolism, I compared hybridoma physiology in the presence and absence of insulin in order to gain further insight to the primary causes for the emergence of cell death.

As is presented within the following pages, the analysis of hybridoma physiology in chemostats provided quite a wealth of findings, on which my partner Brian Follstad and I have built the rest of our theses. From the cell cycle hypothesis developed from the chemostat runs, I crafted a significant new hypothesis for cell death of hybridomas and was successful in using rapamycin to minimize cell death. From the observed metabolic

steady state multiplicity in the chemostats, Brian Follstad pursued metabolic differences between cells in culture and the role of mitochondrial potential to show that the initial metabolic state of cells at the beginning of a batch can influence the growth and death behavior of cultures. Finally, Brian and I, in the pursuit of additional physiological information concerning the relationship between insulin protection, cell cycle progression, metabolism, and apoptosis, discovered that insulin's protective role depends on the nature and extent of conditioning of the culture environment.

# 3 THE PHYSIOLOGY OF HYBRIDOMA CELLS IN STEADY STATE CHEMOSTAT CULTURES

## 3.1 ABSTRACT

Apoptotic cell death of a hybridoma cell line is studied in steady state chemostat cultures grown in reduced glutamine, serum-free, hydrolysate-free IMDM medium. Four steady states are characterized from a single chemostat experiment in which the dilution rate was initially set at 0.04 $hr^{-1}$, then decreased to 0.03 $hr^{-1}$, 0.02 $hr^{-1}$ and 0.01 $hr^{-1}$ in turn for each steady state. The steady states span a range of cultures exhibiting physiologies from high viability and faster growth rate down to low viability and slower growth rate. The physiology of the hybridoma culture for each steady state is described in terms of cell density and viability, growth and death rates, extent of apoptotic cell death, cell cycle distribution, metabolite uptake and production rates, oxygen uptake and carbon dioxide evolution rates, and production of MAb product. Furthermore, intracellular metabolite fluxes and ATP production are calculated based on a model for central carbon metabolism.

Culture metabolism exhibits a robust response to increasingly lower nutrient levels at the lower dilution rates. Measured metabolic uptake and production rates and calculated intracellular metabolite fluxes show that central carbon metabolism shifts towards more complete oxidation of glucose and increasing TCA cycle activity. Nonetheless, the amount of death for a steady state culture correlates inversely with the dilution rate. Interestingly, even though there is a reduction in growth rate and death emerges, cell cycle distribution does not shift towards an arrest in the G1 phase of the cell cycle. This lack of cell cycle arrest in the G1 phase for cells under nutritional limitation points to a defect in cell cycle control. In this regard, it was further shown that the hybridoma cells express high levels of *c-myc*, a proto-oncogene associated with cell cycle entry. Hence, unbridled entry to the cell cycle is believed to be associated with higher levels of apoptotic cell death than would otherwise be observed under nutrient limiting conditions. The lack of G1 arrest also provides insight as to why the apparent specific production of

MAb does not increase at lower growth rates, as has been reported for hybridomas elsewhere.

## 3.2 INTRODUCTION

The detrimental effect of apoptosis (programmed cell death) on the performance of a culture has been firmly established in a variety of cell culture systems over the past several years (Franek, Vomastek et al. 1992; Mercille and Massie 1994; Singh, Al-Rubeai et al. 1994). Since the overall productivity of a culture process is strongly dependent on the viable cell density integrated over the life span of the culture, the emergence of cell death directly detracts from the overall productivity of a culture. Thus, a common objective for research in this field is to better understand the phenomenon of programmed cell death in the hope of being able to minimize it or avoid it altogether during batch and fed-batch processes.

Many stimuli of apoptosis have been studied. The emergence and extent of apoptosis is affected by the availability of nutrients, growth factors, and genetic factors, including so-called anti-apoptosis genes. Yet despite characterizing the effects of known inducers or inhibitors of apoptosis, the emergence of apoptosis continues to negatively impact fed-batch processes, even in seemingly well-maintained culture processes. Fed-batch processes, in which nutrients and growth factors are kept in adequate supply, are still plagued by apoptosis.

Aiming to identify the cellular and environmental components that trigger apoptotic cell death, we turn to characterizing the "symptoms" of death that are evident during a culture process. The emergence of apoptotic cell death during batch and fed-batch processes occurs simultaneously with changes in culture growth rate, metabolism, cell cycle distribution, and product synthesis. The question is which of the measured phenomena are causes for or effects of the underlying emergence of cell death. Whatever the cause for the physiological changes during batch cultivation, such changes are associated with an increase in the death rate. Given the genetically programmed nature of apoptosis, it is fitting to include this cellular phenomenon along with the others for the description of the physiological profile for cultures. Just as growth

and metabolism are genetically determined and have been characterized under different conditions, so must cell death be characterized under a variety of conditions and in conjunction with other major cellular phenomena.

Shifts in culture metabolism appear to be implicated in the cause for apoptosis. As a fed-batch culture slows in its growth rate and death emerges, the metabolism of the culture also changes (Xie 1997). It is reasonable that some metabolite uptake or production rates decrease when growth slows and death emerges. Yet of more interest is that some of the rates increase or even reverse their direction (a production becomes an uptake, for instance). The shifts in metabolism during changes in growth and/or death rates may be symptomatic of the underlying requirements for energy and mass production in the culture. The cell must maintain an adequate supply of energy (ATP and NAD(P)H) to continue carrying out cellular functions, such as DNA synthesis, mitosis, nutrient transport, and protein production. Shifts in metabolism may therefore provide a clue as to the underlying cause(s) that lead to culture growth arrest and apoptotic cell death.

Cell cycle phenomena are also often implicated in the emergence of cell death (Fussenegger and Bailey 1998; Goswami 1998; Sanfeliu, Chung et al. 1999 in press). Data from Goswami shows a typical arrestment in the G1-phase for suspension Chinese Hamster Ovary cells as growth declines and death emerges. Such an inability of cells to passage through the cell cycle may be related to or indicative of the causes for other physiological changes, including the coinciding emergence of cell death. On the other hand, Sanfeliu shows that stimulating entry to the cell cycle in the absence of environmental or genetic survival factors (by replacing serum with just insulin) leads to growth accompanied by apoptosis (Sanfeliu, Chung et al. 1999 in press). Cell cycle entry is seen as a necessary but not sufficient criterion for viable proliferation. Viable proliferation also depends on appropriate environmental and internal genetic/protein factors. The growth behavior for the hybridoma cell line used in this study demonstrates a phenomenon similar to that observed for inappropriate cell cycle entry (Chung, Sinskey et al. 1998). My own cell cycle data during batch cultivation reveal that the majority of hybridoma cells are found within the cell cycle (in the S-phaseG2/M-phase) as a batch progresses. Despite known limitations that can be placed on the culture

(such as nutrient or growth factor removal), the cells continue to enter the cell cycle without arrestment in G1 even as culture growth rate declines and death emerges. For this cell line, it is the lack of a specific arrest within the cell cycle that may be another symptom of the emergence of cell death. Thus, the changes in the behavior of a culture in terms of growth, death, and (lack of) cell cycle function appear to be related.

Striving to make sense of the many changes which occur as cells die, we choose continuous culture experiments as a tool to dissect the phenomena which are associated with cell death. Continuous culture (chemostat) experiments are superior to batch and fed-batch experiments in that cell physiology can be determined within a steady state environment. Furthermore, by changing the dilution rate (or other system parameters) different culture physiologies can be achieved. In the following study, we vary the dilution rate in order to characterize cultures at the extremes of faster growth and high viability versus lower growth and low viability. The different measured steady states may be representative of the physiological states observed as a fed-batch progresses. The fed-batch counterpart for fast viable growth in a high dilution rate chemostat is the period of exponential growth at the beginning of the fed-batch, whereas the fed-batch counterpart for slow growth with apoptosis in a low dilution rate chemostat is the period of growth arrest and cell death at later times in a fed-batch. However, the comparison of such between physiological states in chemostats and fed-batches states is not entirely fair. The death resulting in chemostats is due to nutrient limitation as well as unknown causes, whereas the death resulting in fed-batches, where nutrients are kept in adequate supply, is strictly, by design, due to the unknown causes. Nonetheless, the physiology of cell death obtained in chemostats provides very interesting and valuable insights into the relationship between growth, death, metabolism, and cell cycle progression.

Thus, the objectives of this chapter are to present the physiological data for a variety of steady state conditions acquired at different dilution rates. The determination of cell cycle progression and culture metabolism in the presence and absence of apoptosis provide the evidence with which two novel hypotheses concerning cell physiology and cell death have been formulated. One hypothesis, developed and pursued by Brian Follstad and is the subject of a related paper and his own thesis, concerns metabolic

steady state multiplicity and the role of mitochondrial potential on viable cell density, apoptosis, and overall culture performance. The second hypothesis, which I developed, applies knowledge gained concerning the dysfunction of cell cycle entry in the hybridoma towards a strategy to inhibit cell cycle entry and minimize apoptosis using cell cycle arresting agents (Chapter 4).

## 3.3 MATERIALS AND METHODS

### 3.3.1 Cell Line and Culture Medium

The cell line used is a murine hybridoma (ATCC CRL-1606) which produces and secretes an immunoglubulin IgG against human fibronectin. The hybridomas (previously adapted to serum-free medium) are grown in a glutamine-free IMDM basal medium (Specialty Media, Inc., Lavallette, NJ), supplemented with 10 mg/L insulin (USB), 5 mg/L transferrin (USB), 2.44 μL/L 2-aminoethanol, 3.5 μL/L 2-mercaptoethanol, 7.5 mg/L protease free bovine serum albumin (Sigma Chemical Co.), and 1 U/ml penicillin – 1 μg/ml streptomycin (Sigma). Glutamine is added at a concentration of 4.0 mM for thawing and passaging inocula or at 0.8 mM for the chemostat feed medium. No serum or hydrolysates are added. Insulin and transferrin stock solution are prepared at 2 g/L following the method of Jenkins (Jenkins 1991) and stored at -20°C.

The inoculum culture was prepared from a frozen stock of CRL 1606 hybridomas and grown in T-flasks and spinner flasks (Bellco, Vineland, NJ) placed within a 37°C humidified incubator (95% relative humidity) with 10% $CO_2$ atmosphere. The inoculum was passaged every two days by subculturing the approximately 1.5e6 cell/ml culture down to 2.0e5 cell/ml.

### 3.3.2 Bioreactor Operation

Steady state chemostat cultures were obtained using a 2-liter Applikon reactor (Foster City, CA) at a working volume of 1.2 liters. A New Brunswick Scientific (Edison, NJ) ML 4100 controller was used to monitor the bioreactor temperature, monitor and control the pH and dissolved oxygen, and control mixing of nitrogen, air, and oxygen for the feed

gas via a solenoid control assembly. A heated water jacket maintained the temperature at 37 °C and agitation was set at 200 rpm. The pH was controlled at 7.20 +/- 0.05 by addition of 320 mM HCl or a solution of 6.2 g/l NaOH and 0.4 g/l KOH as needed. The dissolved oxygen concentration was maintained at 40-50% of air saturation by 600 mL/min (0.5 VVM) of headspace aeration. No carbon dioxide gas was fed to the reactor to facilitate the measurement of the carbon dioxide evolution rate. Process data (T, pH, DO, control outputs, and $\%O_2$ and PPM $CO_2$ for gas effluent) were logged through the ML-4100 controller to a computer using the AFS software from New Brunswick Scientific (Edison, NJ).

In a sterile manner throughout, continuous fluid flow was established using Pharmed tubing and Alitea peristaltic pumps. Feed medium in a 5.0 L refrigerated reservoir bottle was first pumped to a 250 mL premixer vessel (a customized water-jacketed Bellco Spinner Flask), to equilibrate the feed with 12% $CO_2$ at 37°C. From the premixer, the medium was pumped through a vertical, in-line 2 mL glass volumetric tube on its way to the bioreactor, where it was delivered as a drop-wise stream from the headplate fitting down into the culture broth. The feed flowrate was measured periodically (or in replicate during steady states) by evacuating the contents of the in-line tube and timing the fill-up of the tube. Such flow measurements were used to maintain the flowrate setpoint, which over many days could drift due to tubing wear, by making minor adjustment to pump settings. During the measurement of a steady state, the dilution rate was calculated from duplicate or triplicate in-line flow determinations. The volume of the culture in the reactor was maintained constant by the removal of culture broth through an Applikon "chemostat tube" assembly. This assembly consists of two concentric tubes. The inner tube sets the level for the reactor culture, while the outer tube serves to draw culture medium from an intermediate culture depth, so that the effluent is drawn from the bulk rather than the surface of the culture. The effluent flowrate is set greater than the feed rate to ensure constant level. (The faster flow is also necessary to minimize clogging of the downstream lines by cell debris, a situation which can lead to reactor fill up, and possibly termination of the experiment.) The effluent is collected in 5.0 L waste reservoirs.

Over the course of a two to three month continuous culture, feed reservoir and waste reservoirs are replenished and exchanged in a sterile fashion by "ethanolizing" and "flaming" steel quick-connect fittings. Both the feed line and the waste line have a "T" with two quick-connect outlets so that fluid flow need not be interrupted by reservoir maintenance. Alitea pump cartridge tubing, which lasts for only about one month, is "replaced" by switching to a fresh cartridge tubing that was connected in the feed line in parallel and clamped off until needed. When the in-line flow measurements show accelerated drifting, the old cartridge tubing is clamped off and the new cartridge tubing is then calibrated in-line and used thereafter.

Culture samples, for the analysis of cells or medium, were obtained through a sampling tube which is distinct from the effluent "chemostat" tube. The sample tube (here just a single tube) is placed to draw culture medium from the center of the reactor. The sampling assembly consists of the sampling tube, connected to a three-way valve, which connects to a disposable syringe as well as a 50 ml waste reservoir, vented with a 0.22 μm air filter. To avoid contamination and to ensure a fresh sample, the first 4 mL of sample are discarded to the waste reservoir (the sample line volume is about ~ 3 mL), after which the sample (anywhere from 1 to 100 mL) can be collected in the syringe. The syringe is removed and immediately replaced with a fresh, sterile one, while the sample line is clamped and the three-way is set away from the syringe port. This method of sample proved to be very safe against contamination, as well as very easy to use.

The reactor was inoculated with approximately 200 ml of an inoculum culture of approximately 1.5 x $10^6$ cell/ml (including the conditioned medium) to give a starting density of 2.0 x $10^5$ cell/ml. After 48 hours, the feed and waste flows were initiated at an intermediate dilution rate, and over a period of days the feed rate was gradually adjusted to the first desired dilution rate of 0.04 $hr^{-1}$. After five residence times, and when process variables such as cell density, viability, oxygen demand, residual glucose concentration, and lactate concentration were steady within a 10% range for 48 hours, the system was taken to be at steady state and the physiology of the culture was measured (Steady State A). Following the measurement of the first steady state, the

dilution rate was reduced stepwise over three days to 0.03 $hr^{-1}$. After waiting 5 residence and verifying that culture parameters were steady, the second steady state (B) was measured. Thereafter, in a similar fashion, steady states C and D were obtained at dilution rates of 0.02 $hr^{-1}$ and 0.01 $hr^{-1}$. A set of physiological measurements were made once for the first steady state (on one day), in triplicate for the second steady state (on three consecutive days), and in duplicate for the third and fourth steady states (on two consecutive days for each). A single measurement of a fifth steady state (E) was made following the gradual increase of dilution rate back up to 0.04 $hr^{-1}$ and waiting an additional 5 residence times.

## 3.3.3 Analytical Methods

### 3.3.3.1 Cell Number, Viability, and Apoptosis

Cell number and viability were determined using cell counts on a hemacytometer with trypan blue dye exclusion. A minimum of three slides was counted for each steady state data set.

The extent of apoptosis was determined by cell counts on a hemacytometer using an acridine orange / ethidium bromide (AO/EB) assay (Mercille and Massie 1994). Apoptosis cell counts consisted of counting cells for 4 different hemacytometer chambers for a total of 600 to 1400 cells altogether. Such cell counts were used to calculate the observed percentage of cells that were viable, apoptotic (early and late), or necrotic.

### 3.3.3.2 Metabolite Concentrations

Steady state samples taken from the reactor were centrifuged at 200*g for 8 minutes, and the supernatant was removed and stored at -20°C for future analysis. To deproteinate the thawed samples, 100 μL of 20% m/v trichloroacetic acid was added to 340 μL of sample. After microcentrifugation at 7000*g for 10 minutes, 200 mL of the deproteinated sample was neutralized with 50 μL of 25% m/v potassium bicarbonate. The deproteinated samples were used in performing the glucose (Sigma protocol 16-UV), lactate (Sigma protocol 826-UV), and ammonia (Sigma protocol 171-UV) assays, with a Becton-Dickonsen uv/vis spectrophotometer.

Samples for amino acid analysis were thawed and diluted between 1.5 and 3.5 times to ensure that the maximum expected amino acid concentration was less than 1 mM (the upper calibration limit). The internal standards norvaline and sarcosine were added during the dilution, so that they were both present at a final concentration of 0.5 mM. Samples were then deproteinated using Ultrafree-MC 5,000 MW cutoff ulrafilters microcentrifuged at 4°C for 70 minutes at 4000*g (Millipore, Bedford, MA). The amino acids were analyzed using the AminoQuant protocol on an HP 1090 HPLC (Hewlett Packard, Palo Alto, CA). This protocol uses pre-column derivitization with ortho-phthalaldehyde (OPA) for detection of primary amino acids and 9-fluorenylmethyl chloroformate (FMOC) for detection of secondary amino acids (AminoQuant Series II Operator's Handbook, Hewlett Packard part #01090-90025). Separation was achieved on a C18 reverse-phase column (Hewlett Packard, Palo Alto, CA).

#### 3.3.3.3 MAb Antibody Concentration

Antibody concentrations were determined using a standard ELISA procedure. First, 100 μL of a solution containing the capture antibody (Sigma M8770) diluted to 5 μg/mL in a capture antibody solution (Sigma C3041) containing 0.1% (w/v) sodium azide was added to each well on a 96 well plate and incubated overnight at 4°C. Then, 200 μL of a blocking solution (SuperBlock™, Pierce 37515) was added and removed from each well three times in succession. Next, 100 μL of either samples or standards (Sigma M9269), diluted 400-900 times in a solution of 1 part blocking solution to 9 parts washing solution (PBS with 0.05% (w/v) Tween 20), was added and incubated for 1 hour at room temperature. Then, 100 μL of a detection antibody solution (diluent solution with 40 μg/ml detection antibody, Sigma A2304) was added and incubated for 1 hour at room temperature. After each of the previous steps, the plate was washed three times in washing solution. Finally, 200 μL of a substrate solution (SigmaFAST™ OPD, Sigma P9187) was added and incubated for 30 minutes in the dark. The reaction was stopped with 3 M HCl and the absorbance was read at 492 nm on a microplate reader (Molecular Devices, Palo Alto, CA). MAb antibody concentrations for the unknowns were calculated from the linear portion of an OD vs. Standard concentration plot.

### 3.3.3.4 Calculation of Growth and Death Rate and Specific Uptake and Production Rates

The culture growth rate, $\mu$, and death rate, $k_d$, were determined from total and viable densities, $n_t$ and $n_v$, obtained by Trypan blue counts, using cell balances around the bioreactor:

$$\frac{dn_T}{dt} = \mu n_V - Dn_T \qquad (3\text{-}1)$$

$$\frac{dn_V}{dt} = \mu n_V - k_d n_V - Dn_V \qquad (3\text{-}2)$$

where $D$ is the dilution rate and $t$ is the time. At steady state, these balances yield the following expressions for culture growth and death rates:

$$\mu = D\frac{n_T}{n_V} \qquad (3\text{-}3)$$

$$k_d = \mu - D \qquad (3\text{-}4)$$

Specific metabolite production or uptake rates, $q$, were determined by a species balance over the liquid phase of the reactor:

$$\frac{dC_R}{dt} = DC_F - DC_R - qn_V \qquad (3\text{-}5)$$

where $C_R$ and $C_F$ are the concentration of a particular metabolite within the reactor or the feed, respectively. At steady state, a specific rate is thus given as:

$$q = \frac{D(C_F - C_R)}{n_V} \qquad (3\text{-}6)$$

### 3.3.3.5 Oxygen Uptake Rate

The oxygen uptake rate (OUR) was obtained from the mass balance for oxygen in the liquid phase of the reactor at steady state:

$$\text{OUR} = k_L a(C^* - C_R) + D(C_F - C_R) \qquad (3\text{-}7)$$

where $k_La$ ≡ liquid phase mass transfer coefficient for oxygen; $C^*$ ≡ liquid phase concentration of oxygen at equilibrium with the gas phase; $C_R$ ≡ concentration of oxygen

in the liquid phase of the reactor; $D \equiv$ dilution rate; and $C_F \equiv$ concentration of oxygen in the liquid feed.

The first term on the right-hand side represents oxygen transport across the gas-liquid interface. The second term accounts for the net inflow of oxygen with the liquid streams. Liquid concentrations of oxygen in the reactor and the feed were measured with a blood gas analyzer (Ciba Corning, Medfield, MA). The equilibrium concentration, $C^*$, was calculated based on the gas-phase oxygen concentration of the effluent reactor gas stream, which was determined with a gas analyzer containing a paramagnetic oxygen sensor (Columbus Instruments, Columbus, OH). The partial pressures of oxygen, obtained from either the blood gas analyzer or the gas analyzer, were converted to liquid concentrations using Henry's Law ($C_{O2} = P_{O2}/H_{O2}$). The Henry's constant for our serum-free medium at 37°C was estimated as $H_{O2} = 9.99 \times 10^7$ Pa*L/mol based on correlations from literature (Schumpe, Quicker et al. 1982), which allow for the correction of gas solubilities to account for medium composition.

### 3.3.3.6 Carbon Dioxide Evolution Rate

The carbon dioxide evolution rate (CER) was determined from a mass balancing approach similar to that of Bonarius (Bonarius, Gooijer et al. 1995), except that liquid-phase carbon dioxide levels were measured rather than calculated. A mass balance for carbon dioxide over the entire reactor at steady state yields:

$$\mathrm{CER} = \frac{\dot{n}_g}{V_R}(y^R_{CO_2} - y^F_{CO_2}) + D(C^R_A - C^F_A) \qquad (3\text{-}8)$$

Where $\dot{n}_g \equiv$ molar gas flowrate to the reactor; $V_R \equiv$ liquid volume of the reactor; $y^R_{CO_s} \equiv$ mole fraction of $CO_2$ in the reactor headspace; $y^F_{CO_s} \equiv$ mole fraction of $CO_2$ in the feed gas; D $\equiv$ dilution rate; $C^R_A \equiv$ concentration of $CO_2$ and bicarbonate in the liquid phase of the reactor; $C^F_A \equiv$ concentration of $CO_2$ and bicarbonate in the liquid feed.

The first term on the right hand-side represents the net inflow of $CO_2$ between the entering and exiting gas streams. The mole fractions of $CO_2$ in gas streams were measured with a gas analyzer containing an IR $CO_2$ sensor with a maximum span of

1% (10,000 PPM) for $CO_2$ (Columbus Instruments, Columbus, OH). To allow for more accurate determination of $y^R_{CO_s}$, $CO_2$ was omitted from the feed gas ($y^F_{CO_s}$ < 10 ppm).

The second term on the right-hand side of equation (3-8) represents the net inflow of $CO_2$ with the liquid streams. A 250-mL pre-mixing vessel was used to equilibrate the feed liquid with air containing 12% $CO_2$ in order to eliminate variation in the feed $CO_2$ levels due to loss of $CO_2$ from the feed bottle. The amount of $CO_2$ in a particular liquid stream, present mainly as dissolved $CO_2$ and bicarbonate, was determined by measuring the partial pressure of $CO_2$ ($P_{CO2}$) and the pH using a blood gas analyzer (Ciba Corning Diagnostics, Medfield, MA). The partial pressure of $CO_2$ is related to the dissolved $CO_2$ concentration through Henry's Law. The bicarbonate concentration can then be determined by assuming the simplified equilibrium relationship,

$$CO_2 + H_2O \Leftrightarrow HCO_3^- + H^+$$

which has an apparent equilibrium constant $K' = \frac{C_{HCO_3} a_H}{C_{CO_2}}$ (Putnam and Roos 1991).

Thus measuring the partial pressure of $CO_2$ and pH allows the total dissolved carbon dioxide plus bicarbonate concentration to be calculated from

$$C_A = C_{CO_2} + C_{HCO_3} = \frac{P_{CO_2}}{H_{CO_2}}\left(1 + \frac{K'}{10^{-pH}}\right) \qquad (3\text{-}9)$$

Based upon correlations in Schumpe (Schumpe, Quicker et al. 1982), the Henry's law constant for our serum-free medium at 37°C was estimated as $H_{CO2}$ = 4.20 x $10^6$ Pa*L/mol, which is very close to the value (4.19 x $10^6$ Pa*L/mol) for cerebrospinal fluid at 37°C and pH = 7.20 (Severinghaus 1965). Because both the Henry's law and equilibrium constants are functions of ionic strength, one would expect aqueous solutions with similar Henry's constants to have similar equilibrium constants. Therefore, we estimated the apparent equilibrium constant as pK' = -log(K') = 6.138, which is the measured value for cerebrospinal fluid at 37°C and pH = 7.20 (Severinghaus 1965).

### 3.3.3.7 Calculation of Metabolic Fluxes

Metabolic fluxes were calculated by metabolite balancing using the central carbon metabolism network shown in Figure 3-1. Serial reactions were lumped into single reactions. In addition, metabolite requirements for biomass and product synthesis were accounted for as described in Zupke and Stephanopoulos (Zupke and Stephanopoulos 1995) using the cell composition determined for CRL 1606 by Xie and Wang (Xie and Wang 1994). The pentose phosphate pathway was analyzed by considering only the biosynthetic demand for ribose carbon skeletons. The production of NADPH can not be used to determine the pentose phosphate pathway recycle due to the uncertainty of transhydrogenase activity in this cell line, as well as the fact that the malic enzyme may use either $NAD^+$ of $NADP^+$ (Eigenbrodt, Fister et al. 1985). Therefore, reducing power was lumped in an NAD(P)H pool. This may introduce some error in distinguishing $CO_2$ released from the pentose phosphate pathway and the TCA cycle.

A matrix of stoichiometric coefficients (A) was constructed using the reaction network shown in Figure 3-1 and used to calculate the metabolic fluxes (x). The material balance on the metabolites in the reaction network can be represented as

$$\mathbf{x} = (\mathbf{A}^{\mathrm{T}}\Psi^{-1}\mathbf{A})^{-1}\mathbf{A}^{\mathrm{T}}\Psi^{-1}\mathbf{r} \qquad (3\text{-}10)$$

and is explained more thoroughly in Zupke and Stephanopoulos (Zupke and Stephanopoulos 1995)(1995) and Nyberg, et al. (Nyberg, Balcarcel et al. 1999).

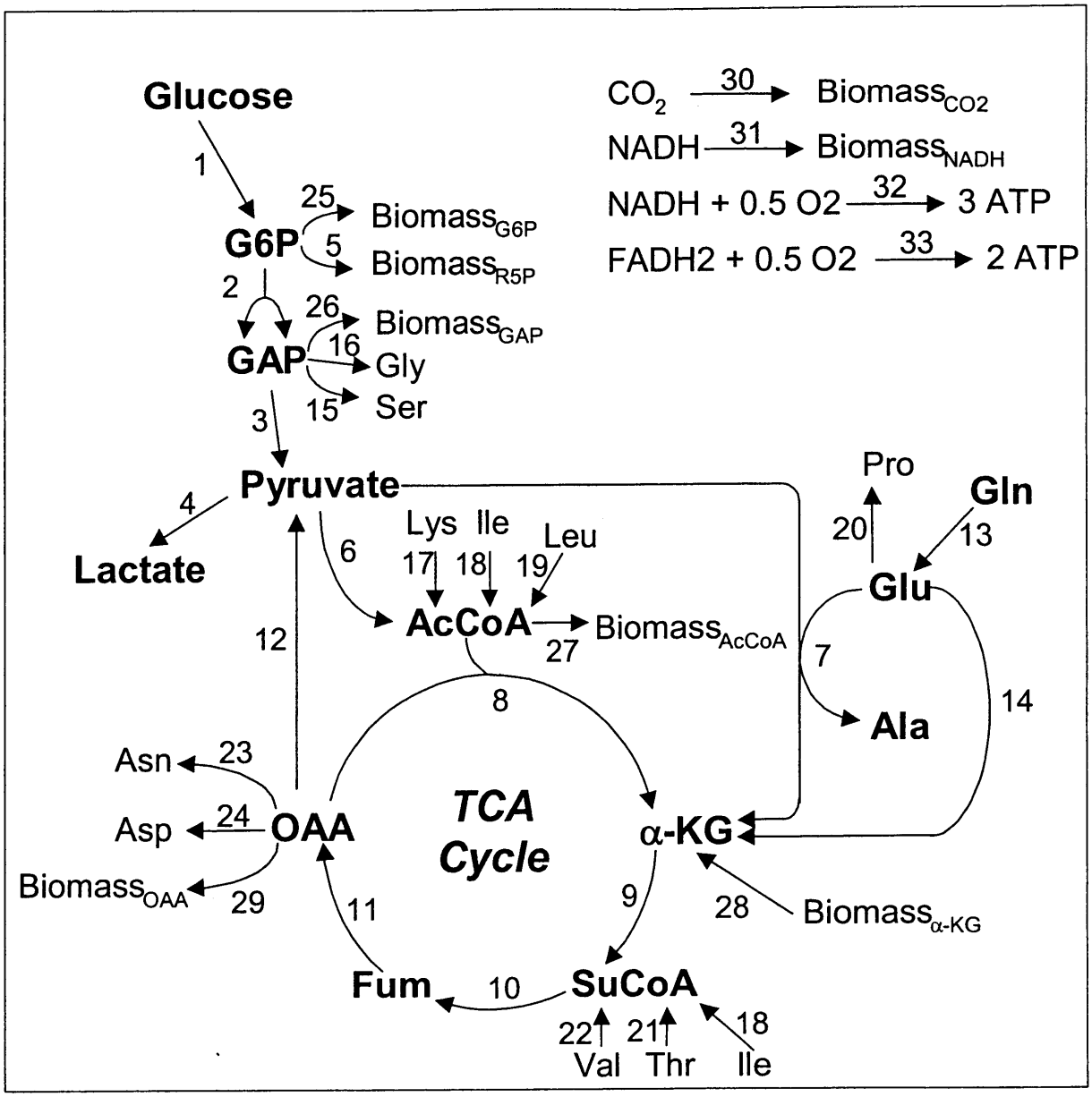



**Figure 3-1. Assumed Reaction Network for Central Carbon Metabolism of Hybridoma Cells.**

### 3.3.3.8 Estimation of ATP Production and ATP demand for Biosynthesis

Specific ATP production for the viable cells at a steady state was calculated using the calculated metabolic fluxes and the associated stoichiometry for ATP, NADH, and $FADH_2$ for each reaction included in the metabolic network (Table 3-1). The total ATP produced is determined by summing the product of each flux with the stoichiometry of each of the 33 reactions. A confirmation of the consistency of the network is that NADH and FADH pools sum very nearly to zero. The net ATP produced is used for a variety of cellular processes, including biomass synthesis. The portion of ATP produced from glycolysis is determined from reactions 1 through 4. Any leftover NADH not spent to make lactate is assumed to yield only 2 additional ATP since it is generated in the cytosol (to be used for oxidative phosphorylation, it would be converted to $FADH_2$ by the glycerol phosphate shuttle as it entered the mitochondrion, Stryer, p. 417). The amount of ATP made from the TCA cycle is determined from the fluxes and stoichiometry of reactions 6 through 24 and 31. As another internal check, the ATP produced by oxidative phosphorylation of NADH and $FADH_2$ from TCA cycle reactions (6 through 24, minus 31 (NADH to biomass)) should agree well with the ATP produced from the reduction of oxygen in reactions 32 and 33.

Some of the ATP produced by the cells is used in the synthesis of biomass. The ATP demand for biomass is calculated from the stoichiometric coefficients for synthesis of cellular and product protein and synthesis of non-protein cellular components. The coefficients are determined using biochemistry for the known cellular composition of this cell line (Xie and Wang 1994). The rate of ATP demand for biosynthesis is simply the sum of the coefficients times the growth rate. The ATP demand for other cellular functions are not estimated here.

**Table 3-1. Stoichiometry for ATP, NADH, and $FADH_2$ for the central carbon metabolic network.**

| | | Reaction | ATP | NADH | $FADH_2$ |
|---|---|---|---|---|---|
| Glycolysis | 1 | Glc → G6P | -1 | | |
| | 2 | G6P → 2 GAP | -1 | | |
| | 3 | GAP → Pyr | +2 | +1 | |
| | 4 | Pyr → Lac | | -1 | |
| TCA Cycle | 6 | Pyr → AcCoA + $CO_2$ | | +1 | |
| | 7 | Pyr + Glu → α-KG + Ala | | | |
| | 8 | OAA + AcCoA → α-KG + $CO_2$ | | +1 | |
| | 9 | α-KG → SuCoA + $CO_2$ | | +1 | |
| | 10 | SuCoA → Fum | +1 (GTP) | | +1 |
| | 11 | Fum → OAA | | +1 | |
| | 12 | OAA → Pyr + $CO_2$ | | | |
| | 13 | Gln → Glu | | | |
| | 14 | Glu → α-KG | | +1 | |
| | 15 | GAP + Glu → Ser + α-KG | +1 | +2 | |
| | 16 | GAP + Glu → Gly + α-KG | +1 | +2 | |
| | 17 | Lys → 2 AcCoA + 2 $CO_2$ | | +5 | +1 |
| | 18 | Ile → AcCoA + SuCoA | -1 | +3 | +1 |
| | 19 | Leu → 3 AcCoA | -2 | +2 | +1 |
| | 20 | Glu → Pro | -1 | -2 | |
| | 21 | Thr → SuCoA | -1 | +1 | |
| | 22 | Val → SuCoA + $CO_2$ | -1 | +4 | +1 |
| | 23 | OAA +Gln → Asn + α-KG | | | |
| | 24 | OAA + Glu → Asp + α-KG | | | |
| | 31 | NADH → $Biomass_{NADH}$ | | -1 | |
| | 32 | 0.5 $O_2$ + 3 ADP + NADH → 3 ATP + $NAD^+$ | +3 | -1 | |
| | 33 | 0.5 $O_2$ + 2 ADP + $FADH_2$ → 2 ATP + FAD | +2 | | -1 |

### 3.3.3.9 Cell Cycle Distribution

2.0 x $10^6$ cells from a reactor sample were spun down and resuspended in 1.0 ml of stain solution (10 ml: 0.3g PEG 6000, 0.5 ml propidium iodide (50 μg/ml in PBS), 0.5 ml RNAase (180 U/ml in PBS), 0.1 Triton X (10% in PBA), 8.9 ml sodium citrate 4mM pH 7.8; final pH adjusted to 7.2), and incubated at 37°C for 20 minutes. After incubation, 1.0 ml of salt solution (10 ml: 0.3g PEG 6000, 0.5 ml propidium iodide (50 μg/ml in PBS), 0.1 Triton X (10% in PBA), 9.4 ml NaCl 0.4 M; final pH adjusted to 7.2) was added, and the sample was stored in the dark at 4°C for 24 to 48 hours. Flow cytometry analysis was performed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). 30,000 events per sample were measured for their fluorescence of 300nm uv-light. The raw data were processed with ModFit Lt v.2 analysis software (Verity Software House, Topsham, ME) to determine the cell cycle distributions. Sampling and analysis was done in duplicate for every data set, with the exception of steady state A, for which only one sample was analyzed.

Cell cycle data obtained from the above analysis are for the fraction of cells that were measured to be in either G0- or G1-phase (one copy of DNA), G2/M-phase (two copies of DNA), or S-phase (intermediate amount of DNA). The fractions ($f_{G1}$, $f_S$, and $f_{G2/M}$) are used to numerically calculate the progression time for each of the three phases ($t_{G1}$, $t_S$, and $t_{G2/M}$) using the following equations from Susuki and Ollis (Suzuki and Ollis 1989)

$$t_C = \ln(2)/\mu = t_{G1} + t_S + t_{G2/M} \qquad (3\text{-}11)$$

$\overline{X}$

$$f_{G1} = \frac{\int_0^{t_{G1}} 2^{(1-t/t_C)}\,dt}{t_C/\ln(2)} \qquad (3\text{-}12)$$

$$f_S = \frac{\int_{t_{G1}}^{t_{G1}+t_S} 2^{(1-t/t_C)}\,dt}{t_C/\ln(2)} \qquad (3\text{-}13)$$

$$f_{G2/M} = \frac{\int_{t_{G1}+t_S}^{t_C} 2^{(1-t_{G1}/t_C)}\,dt}{t_C/\ln(2)} \qquad (3\text{-}14)$$

I adopted the equations assuming that there does not exist an arrested, non-cycling population of cells (which implies that the growth rate is a function of the total cycle time, $t_C$) and by lumping the equations for G2 and M phases together. The assumption agrees with the observed cell cycle distributions for this hybridoma cell line.

### 3.3.3.10 Determination of Intracellular c-Myc Protein Concentration

c-Myc western blots were performed using cells taken from two additional continuous culture experiments, conducted separately from the five other steady states. One of the cultures was at a steady state with a dilution rate of 0.02 $hr^{-1}$ and with 10 mg/L insulin present in the feed (the regular amount of insulin). The other culture was at a steady state with D = 0.03 $hr^{-1}$, yet with insulin levels in the reactor diluted out to below the detectable limit of 1 mU/L (< 0.1 μg/ml insulin), following 15 residence times after switching to an insulin-free feed (insulin concentration measured by ELISA kit (ALPCO #10-1113-01, NH). Both cultures exhibited the similar physiologies as their counterpart steady state from the initial data set.

Cell extracts for each culture were obtained from $1.25x10^7$ cells and washed twice in ice-cold PBS. The pellet was then resuspended in 0.5 ml lysis buffer (50 mM Tris-HCL pH 8, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 100 μg/ml PMSF, 1 μg/ml aprotinin, 1% Nonidet P-40, and 0.5% sodium deoxycholate) and incubated at 4°C for 30 minutes. Next cellular debris was pelleted out by centrifugation at 12000g for 10 minutes at 4°C. Supernatant was stored at -80°C.

The amount of total protein in the cell extracts was determined using the Lowry method (Bio-Rad). 37 to 50 μg of protein from cell extracts in 20 μl sample buffer (50 mM Tris-HCL pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) were electrophoresed on a 10% SDS-polyacrylamide gel. Separated proteins were transferred to a nitrocellulose membrane (0.45 μm pore size). The nitrocellulose membrane was blocked overnight at 4°C with 5% (w/v) nonfat dry milk in TBS (8.76 g/L NaCl, 10 mM Tris-HCl pH 7.4) After rinsing with TBST (TBS plus 0.05% Tween 20), the membrane was incubated for 1h at room temperature with rabbit polyclonal IgG anti-c-Myc (Santa Cruz Biotechnologies), diluted 1:500 in 5% (w/v) nonfat dry milk in TBST. Blots were again rinsed in TBST and further incubated for 1h at room temperature with

anti-rabbit IgG peroxidase linked antibody (Amersham), diluted 1:1000 in 5% (w/v) nonfat dry mild in TBST. After a final wash in TBST followed by TBS, c-Myc bands were visualized by the ECL system (Amersham). For positive control, an amino terminal domain of c-Myc corresponding to amino acids 1-262 was used (Santa Cruz Biotechnologies). The hybridoma bands were compared to bands for anchorage-dependent CHO cell lysates, corresponding to time points following the stimulation of the serum-arrested, synchronized CHO culture with insulin.

## 3.4 RESULTS

The physiology of hybridoma cells in glutamine-limited chemostat steady states was determined in terms of total and viable cell densities, growth and death rates, extent of apoptosis, cell cycle distribution, metabolic uptake and production rates, the net intracellular fluxes into the pyruvate node, ATP production by central carbon metabolism, ATP requirement for biomass and product synthesis, and production rate for the MAb product. The figures below depict these quantities versus the dilution rate (for densities, growth and death rates, and apoptosis) or versus culture growth rate (for other quantities) for each of the four steady states, called A, B, C, and D for dilution rates 0.04, 0.03, 0.02, and 0.01 $hr^{-1}$, respectively. When distinct from steady state A, data for steady state E (D=0.04 $hr^{-1}$) is also shown.

### 3.4.1 Cell Death

Steady state cell counts (Figure 3-2) reveal increasing total cell densities at the two lowest dilution rates. The total cell density at D=0.03 $hr^{-1}$ (steady state B) is slightly suppressed, presumably the result of the glutamine limitation in the absence of a shift in metabolism (see metabolism below). Viable cell densities were also greater at the two lowest dilution rates, yet to a lesser degree than the total density due to cell death. The viabilities for the measured steady states, determined by Trypan Blue exclusion, span a range from 95% down to 40% at the slowest dilution rate. The cell density for steady state E (a steady state at D = 0.04 $hr^{-1}$, after returning from D = 0.01 $hr^{-1}$) is approximately twice that of the density at the first steady state at 0.04 hr-1 (A). This

multiplicity of steady states is the result of sustained metabolic efficiency even upon re-supplying ample nutrients (Follstad, Balcarcel et al. 1999).

The growth and death rates (Figure 3-3) depict a reduction in growth rate and an increase in death rate as the dilution rate is decreased. Interestingly, the reduction in dilution rate from 0.02 hr-1 to 0.01 hr-1 (C → D) results in only a modest decrease in growth rate, yet a dramatic increase in death rate. The cell death that occurs at lower dilution rates is indeed the result of apoptosis, as measured by the acridine orange / ethidium bromide assay (Figure 3-4).

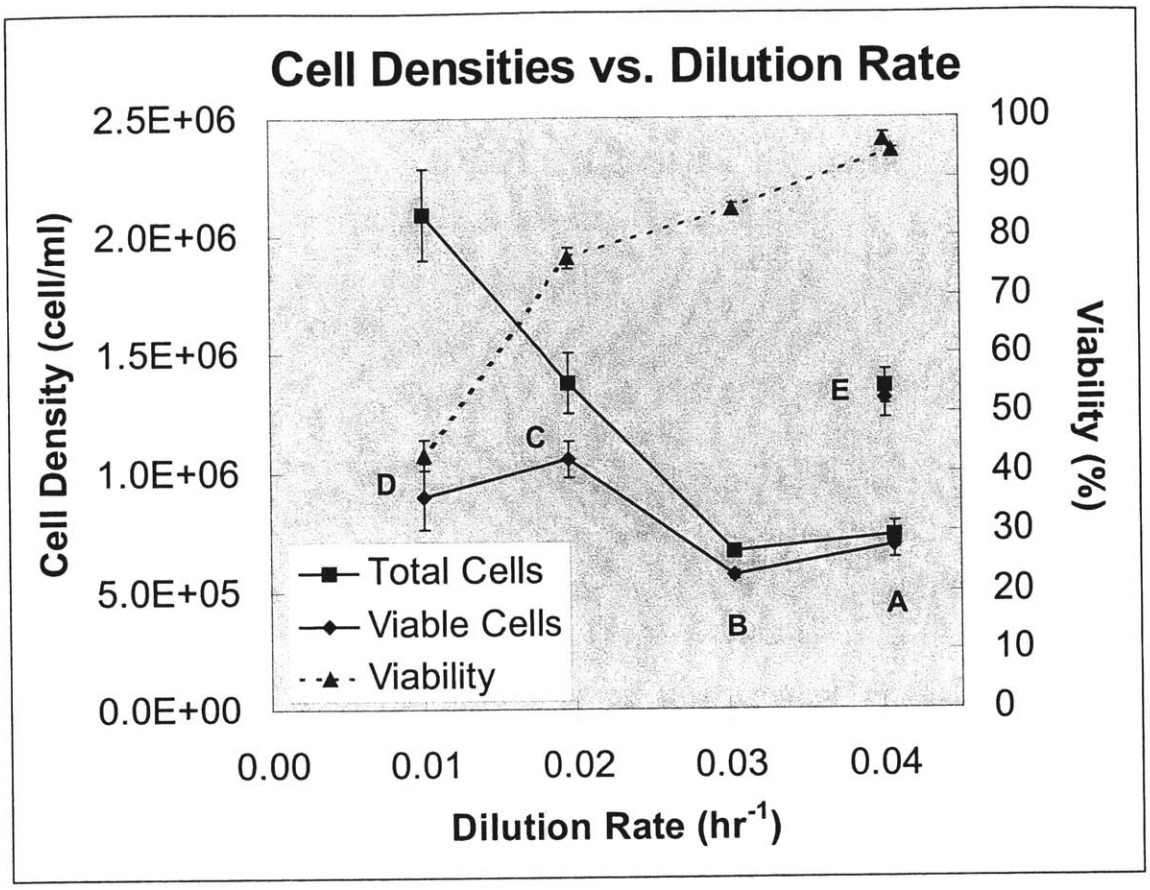



**Figure 3-2. Total and viable cell density and viability for five glutamine-limited hybridoma steady states**

Error bars represent 95%-confidence limits for the measurement of A and E, or the 95%-confidence interval for data taken on two consecutive days (C and D) and three consecutive days (B).

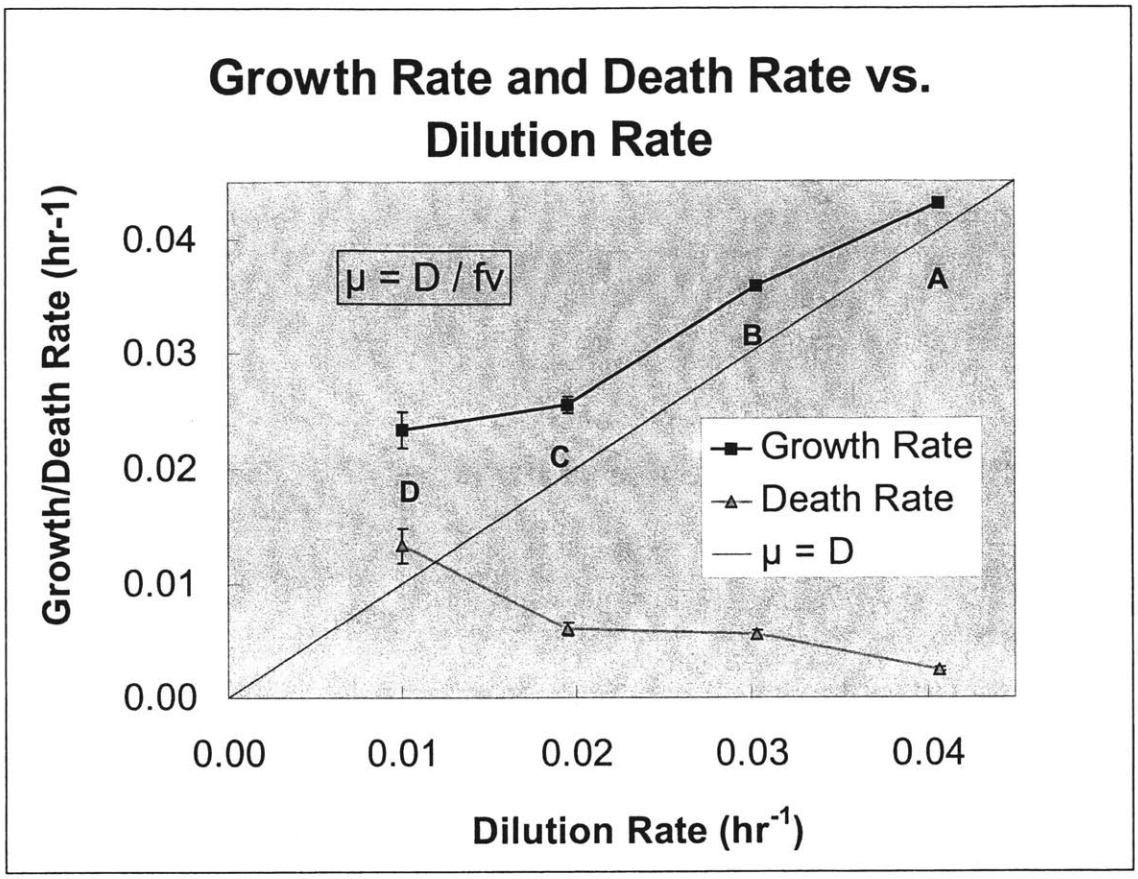



**Figure 3-3. Steady state growth and death rates as a function of chemostat dilution rate.**

Error bars represent 95%-confidence limits for the measurement of A, or the 95%-confidence interval for data taken on two consecutive days (C and D) and three consecutive days (B).

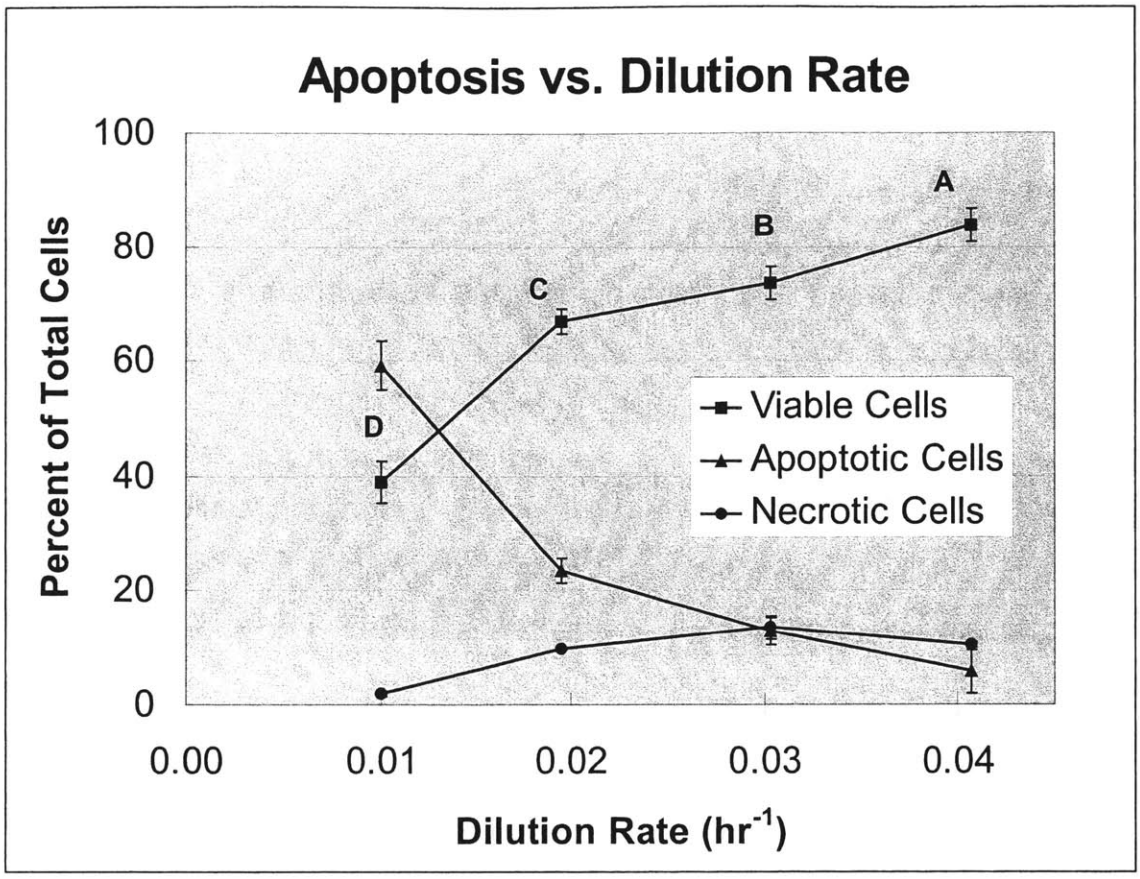



**Figure 3-4. Viable, Apoptotic, and Necrotic cell populations for steady state chemostats as a function of dilution rate.**

Error bars represent 95%-confidence limits for the measurement of A, or the 95%-confidence interval for data taken on two consecutive days (C and D) and three consecutive days (B).

### 3.4.2 Metabolism

The metabolic uptake and production rates (Figure 3-5) reveal distinct differences in the metabolism for the steady state cultures. Cells at steady state A and B have similar metabolic rates for glucose and glutamine consumption. They also have high lactate to glucose ratios (1.90 and 1.99, respectively) and relatively lower oxygen-to-glucose ratios (0.76 and 0.94). Cells at steady state C and D however show a marked shift in metabolic activity. Glucose and glutamine rates have dropped off severely. Lactate production has also severely declined, yielding lactate-to-glucose ratios for C and D of 1.49 and 1.35. Oxygen consumption rate actually increased, resulting in oxygen-to-glucose ratios of 2.17 and 2.94, respectively. Steady state E shows similar glucose uptake compared to Steady State A, but lesser lactate production and glutamine consumption and greater oxygen consumption.

The analysis of intracellular metabolite fluxes by Follstad, et. al. (Follstad, Balcarcel et al. 1999) reveals that the net surplus of carbon at the pyruvate node coming from glycolysis and TCA recycle (reactions 3 and 12 in the metabolic network) and leaving to the TCA cycle (reaction 6) is higher for steady states A and B, yet lower for steady state C and D (Figure 3-6). The shift in metabolism can also be visualized in the ratio of carbon fluxes entering the pyruvate node (reactions 3 + 12) to the carbon flux going to TCA cycle (reaction 6). This ratio decreases with growth rate, indicating that the cells under glutamine limitation are utilizing more of the carbon at the pyruvate node for the TCA cycle rather than for lactate production. Data for steady state E reveal the sustained efficiency of carbon utilization as seen for steady states C and D, which is dramatically different than that for steady state A.

The intracellular fluxes are used to calculate the ATP that is produced by glycolysis and TCA cycle activity coupled with oxidative phosphorylation. The level of ATP generated from glycolysis decreases with growth rate, while the level of ATP generated from TCA activity increases (Figure 3-7a). Thus, the percent of total ATP generated from glycolysis drops from 29% to 19% as the growth rate decreases and death rate increases. The ATP requirement for biomass (Figure 3-7b) reveals that the majority of the ATP is being utilized for other purposes (maintenance). Assuming the ATP

production balances the requirement, it is evident that more ATP is needed for the other requirements at the lower growth rates. ATP is generated to a greater degree from glycolysis for steady state E (36%) versus steady state A (29%).

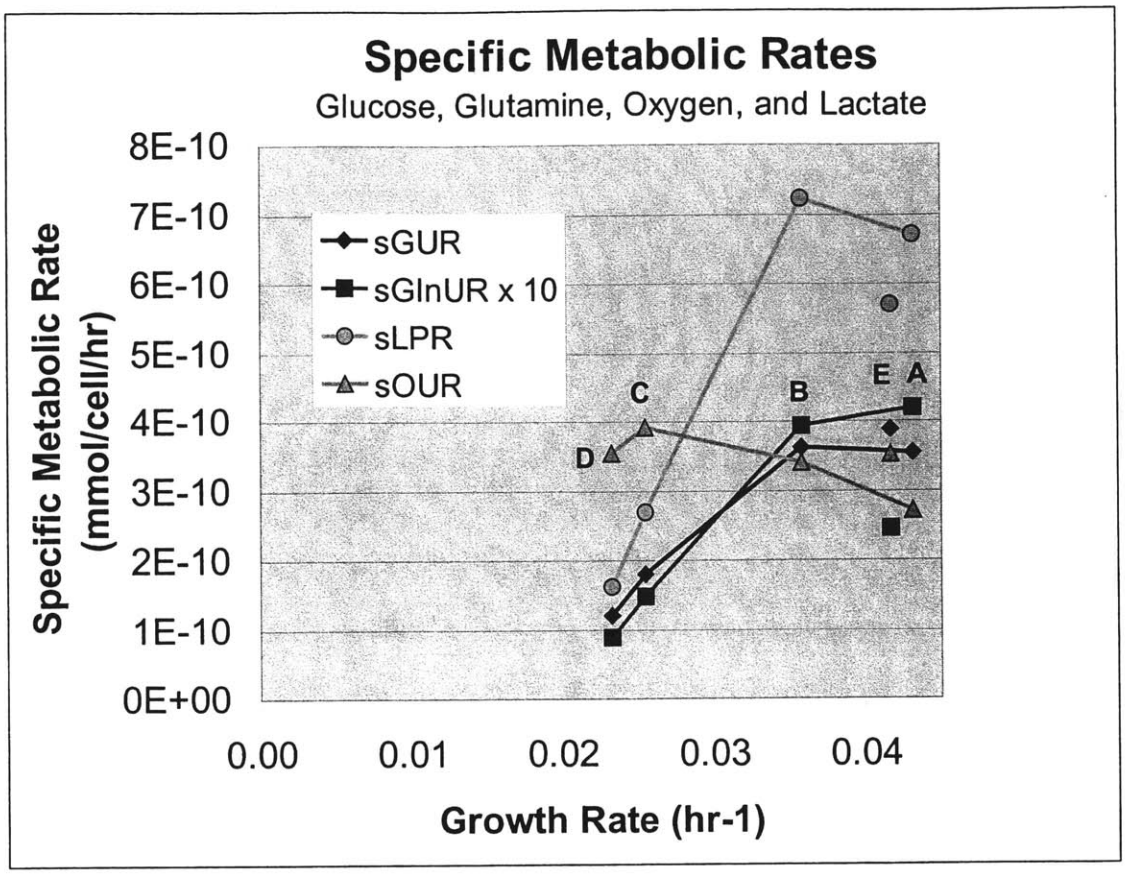



**Figure 3-5. Specific metabolism for steady state chemostat cultures.**
Unattached data points correspond to steady state E.

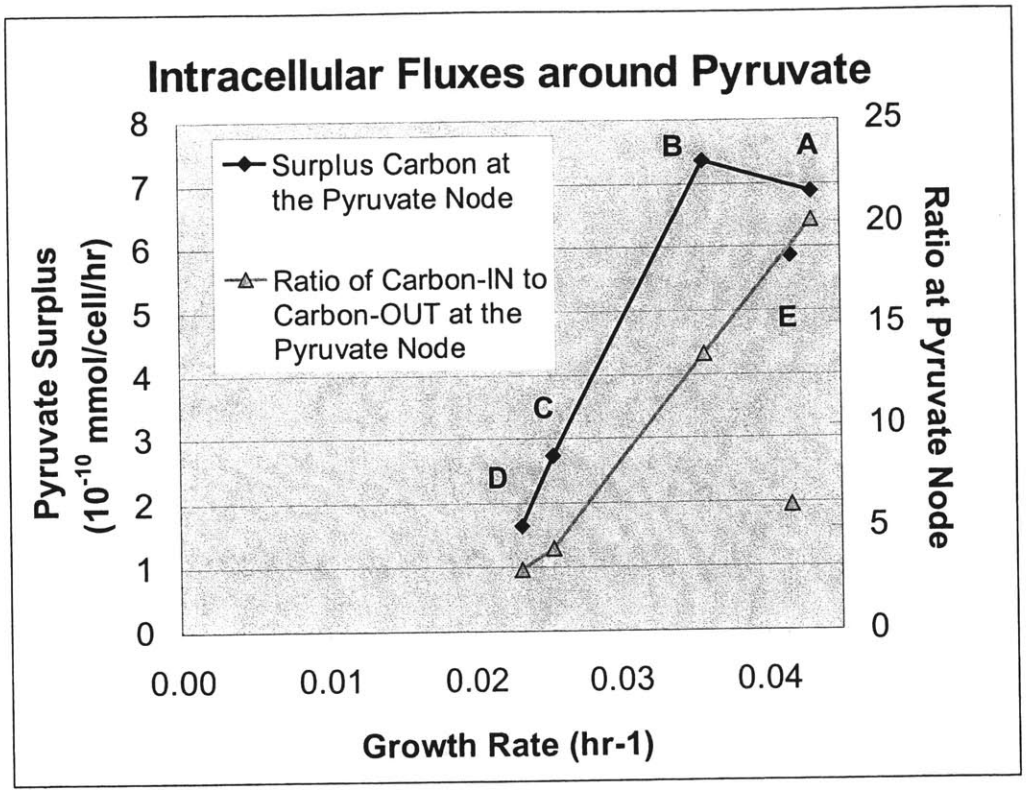
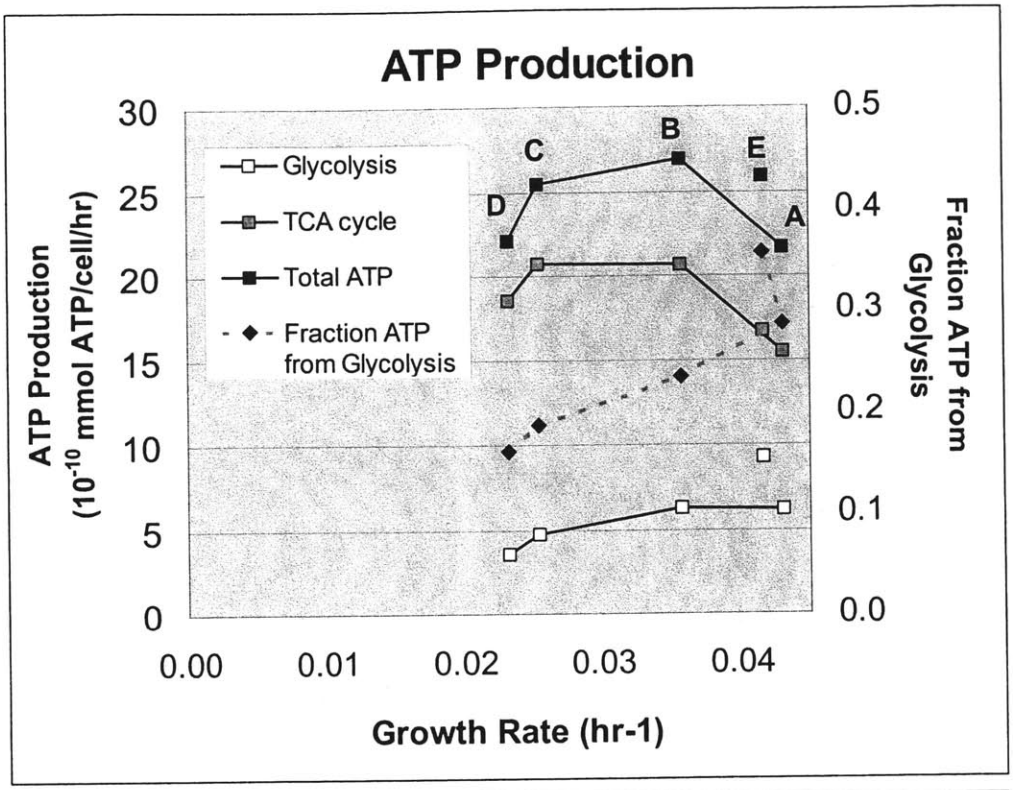



**Figure 3-6. Intracellular fluxes around the pyruvate node for steady state chemostat cultures.**

The surplus is equal to the sum of reactions 3 and 12 minus 6 from the metabolic network. The ration is equal to the quotient of reactions 3 and 12 over reaction 6. Data for metabolic fluxes calculated by Follstad, et. al., (Follstad, Balcarcel et al. 1999).



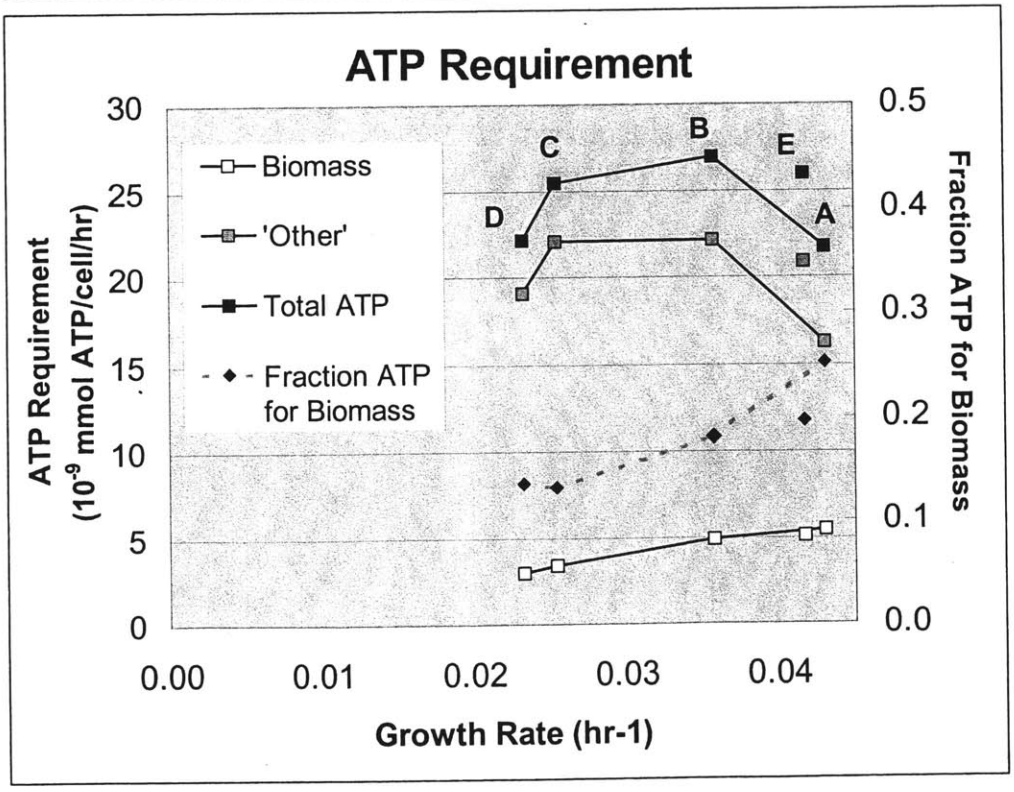




**Figure 3-7 a-b. ATP production and requirement for steady state chemostat cultures.**

### 3.4.3 Cell Cycle

The measurement of cell cycle distribution (Figure 3-8a) for our hybridoma cell line reveals that the culture growth rate does not correlate with the percentage of cells that are within the cycle (S, G2, and M phases). In all cases, about 70-80% of the culture is cycling. Furthermore, the trend in percent of cells in G1 is one that decreases from about 30% for fast growth down to 20% for slower growth. When the cell cycle data is plotted as progression times for each phase (where the total progression time for the cycle is the doubling time), it is apparent that it is the increase in time for S-phase progression that is the primary cause for increasing the total progression time.

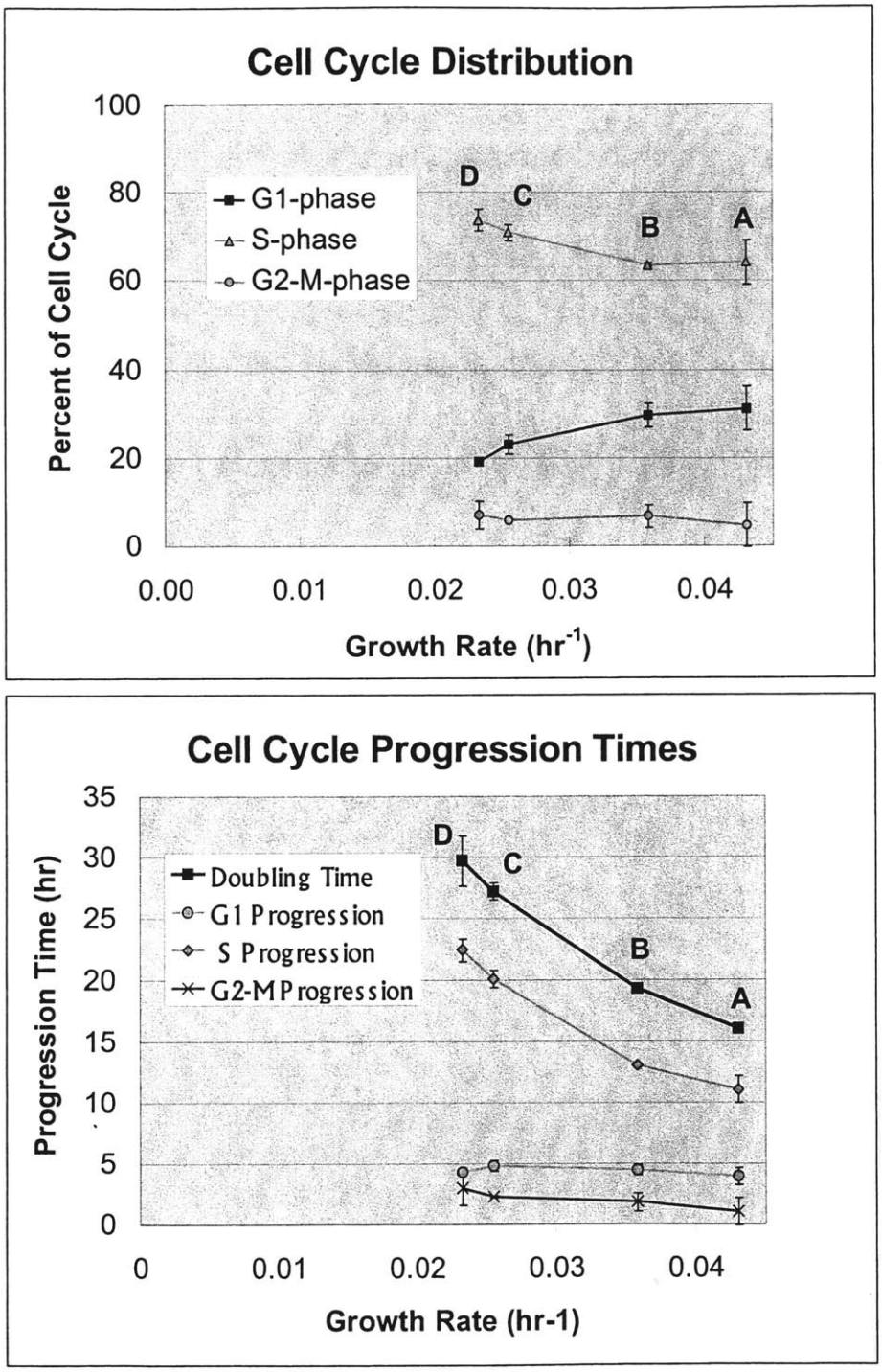



**Figure 3-8 a-b. Cell cycle distribution and cell cycle phase progression times for steady state chemostat cultures.**

Error bars for distributions represent 95%-confidence limits for the measurement of A, or the 95%-confidence interval for data taken on two consecutive days (C and D) and three consecutive days (B). Errors for progression times are the propagated from the 95% confidence limits for the growth rate and the distribution.

### 3.4.4 Monoclonal Antibody Production

Monoclonal antibody production was measured and is shown as specific mass production per viable cell per hour (Figure 3-9). The specific productivities span a greater than 2-fold range. A maximum in productivity occurs at the intermediate growth rate of 0.036 $hr^{-1}$ (Dilution rate = 0.03 $hr^{-1}$ for steady state B). Lower productivity for steady state E compared to steady state A (33% lower) is presumed to be due primarily to the differences in metabolism since cell death and cell cycle are very similar for both steady states.

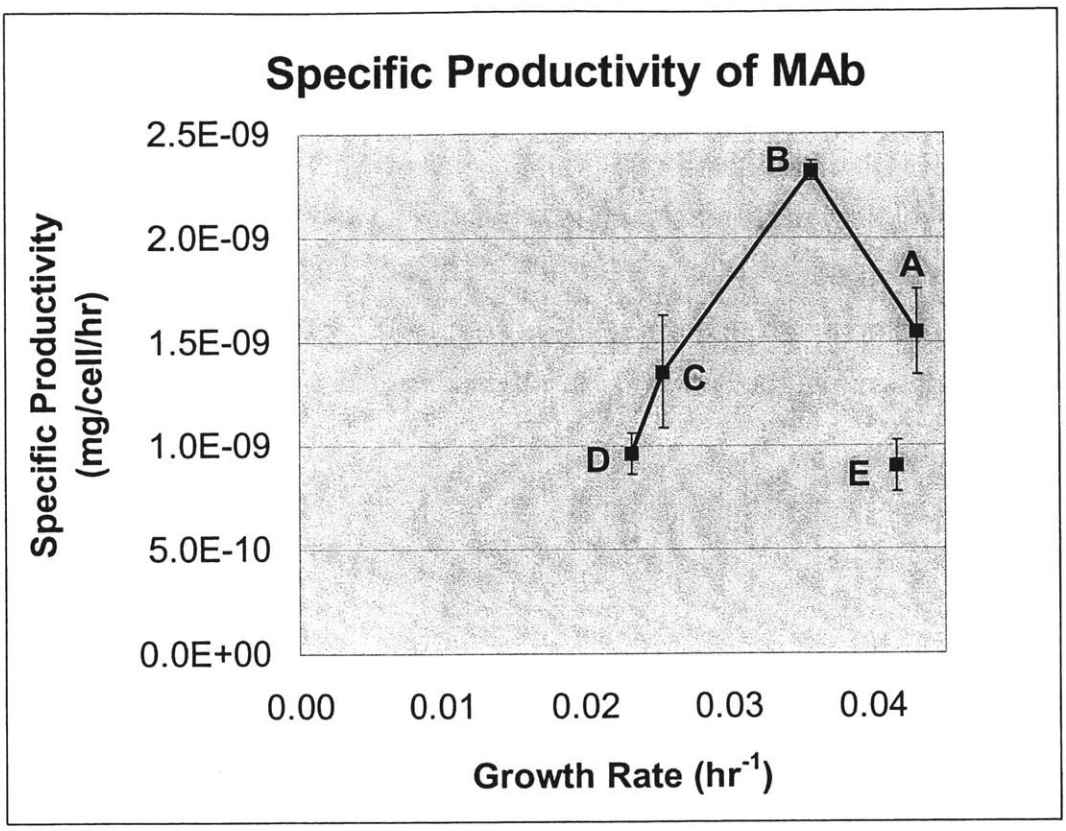



**Figure 3-9. Specific production of monoclonal antibody for steady state chemostat cultures.**

Errors bars for A and E are those propagated from the 95% confidence level for MAb measurement, viable cell density, and dilution rate. Errors bars for B, C, and D are the 95% confident intervals for the average value taken from multiple days.

## 3.5 DISCUSSION

### 3.5.1 Emergence of Apoptotic Cell Death at Steady State

The nature of the equilibrium between growth and death that is achieved in a steady state culture is not one that is easy to grasp. At the simplest level, the viable cell density is the highest one allowable given the environmental and genetic constraints. Yet the difficulty in understanding the steady state emergence of cell death lies in trying to imagine which cell from a particular mitotic division will survive and which will die. For a steady state with a viability of only 40% (such as for steady state D), only about one in two (4 in 10) of the initially viable daughter cells from a successful division will survive. One could try to argue that the parent cell, which was successful in viable proliferation, conferred its advantages to both of its daughters, and that both of the daughters should live, where as daughter cells from an inferior cell would both die. This very well may occur occasionally, but eventually those daughters or their descendants must die, otherwise we would observe only high viability steady state cultures as dead cells washed out.

Thus, some sort of selection process occurs among all daughter cells to determine which will live or die in such a steady state. This competition among the viable cells ensues for 5 complete residence times (21 days), at the dilution rate of 0.01 $hr^{-1}$, with cells growing at 0.023 $hr^{-1}$, for a total of about 17 generations. After an initial quick decline in viability as the dilution rate was decreased from 0.02 $hr^{-1}$, the cell density and viability remained steady throughout the wait for a fluid dynamical steady state. At the steady state, the viable cell density of the culture hovers around the residual nutritional levels that remain after the growth of cells. Any burst in viable cell growth by an advantaged subpopulation would be checked by the tightening of nutritional requirements, which would stimulate more death. Thus, for the nutrient limited steady state, the equilibrium viable cell density hovers around the residual nutrient levels.

The competition for nutrients that is hypothesized above would result in the survival of the fittest daughter cells that exhibit the most robust physiology for the given environmental situation. Without direct measurements and accepting the fact that the

culture is at a steady state, the cells that are proliferating in a viable fashion cannot be part of a subpopulation of the viable cells. Any such subpopulation would overtake the culture and reach a new equilibrium. During the transient, this is what may have happened actually, rather than assuming that all the viable cells adapted uniformly. Possible microscale differences in metabolic potential or other cellular characteristic may have paved the way for the viable culture of cells with a dramatically different metabolic behavior. Yet once at steady state again, the population of viable cells must be homogeneous and death must occur as a stochastic process based on nutrient availability and possibly random fluctuations in intracellular events.

The data from this series of chemostats reveals that cell death emerges as a function of nutritional environment. Furthermore, as will be discussed below, the impediment to growth appears to be progression through the S-phase. The arrest of cells in S-phase as the result of glutamine-limitation is understandable given that glutamine is a building block for all five nucleotides (Xie and Wang 1994). Thus, the stochastic selection of cells that live or die can be imagined to occur within the S-phase. As cells arrest in S-phase waiting for available glutamine, they eventually die by apoptosis (presumably by p53 activity) or just barely make it to the G2/M phase. Death of some cells in the S-phase frees up glutamine for the others. The role of metabolism during the S-phase arrest is unclear. As shown below, metabolism is shifted towards higher efficiency, indicating an increase in demand for cellular ATP. Such a metabolic shift may or may not be related to the S-phase arrest and/or the emergence of apoptotic cell death.

### 3.5.2 Shift in Metabolism

Shifts in metabolism and cell yield have been documented by several other researchers in the past (Miller, Wilke et al. 1987; Miller, Wilke et al. 1989; Miller, Wilke et al. 1989; Hiller, Aeschlimann et al. 1991; Hayter, Curling et al. 1993; Hiller, Clark et al. 1994; Bonarius, Hatzimanikatis et al. 1996; Linz, Zeng et al. 1997; Vriezen and Dijken 1998), including by myself and Nyberg et. al. for a suspension CHO cell line (Nyberg, Balcarcel et al. 1999). In this particular study, the metabolic analysis reveals that the viable cells respond to the nutrient limitation by shifting their metabolism from glycolysis toward TCA cycle and oxidative phosphorylation, utilizing glucose carbon more efficiently and

consuming more oxygen. Looking at the four steady states together, it appears that there are two dominant profiles for metabolism. One, for fast growing cells of high viability with plentiful nutrient supply, utilizes the majority of its glucose for ATP production via glycolysis resulting in high lactate production. Another profile, that for slower growing, lower viability cultures under glutamine limitation, utilizes noticeably more glucose and more oxygen for ATP production via the TCA cycle, resulting in less lactate formation. The fifth steady state culture (E) shows a mix between these two profiles, with both high glucose consumption and high oxygen consumption.

Overall, the shift in metabolism most probably reflects a robust response for the hybridoma cells as they become more and more constrained by the reduction of the dilution rate. Though under severe nutrient limitation (which is probably triggering death by impeding passage through the S-phase), it appears that the viable cells are capable of shifting their metabolism to generate sufficient ATP for survival. The ATP production that was calculated and plotted above assumes that the P/O ratio remains constant at a value of 3. Under this assumption, the shift in metabolism is one that maintains adequate ATP levels for the survival of some cells. Without the shift in metabolism, ATP production would decrease as a function of growth rate. That the shift is one towards better efficiency is evident in the physiology of the fifth steady state culture (E) that was obtained after steady state D (D=0.01 hr-1) by increasing the dilution rate back up to 0.04 $hr^{-1}$. This fifth steady state, rather than flexibly reverting back to higher lactate production and inefficiency, maintained the metabolic efficiency seen at the lower dilution rates. The metabolic efficiency, coupled with a more favorable nutrient environment, resulted in an equally high viability as seen for the first steady state at 0.04 $hr^{-1}$, yet one that had roughly twice the cell density. Thus, the shift in metabolism following nutrient limitation and its effect on viable cell density for steady states at the same dilution rate, implicate culture metabolism as a parameter which can dramatically influence viable cell density and the extent of death in a culture. Brian Follstad has followed up the findings in this work concerning culture metabolism in fed-batch cultures and in regard to mitochondrial potential.

Yet, the higher ATP production at lower growth rates (or the lack of relationship between ATP and growth rate) reveals that the cell is either spending more ATP for

maintenance or possibly that the P/O ratio has decreased from 3 and the cell is not actually making the amount ATP than I calculate assuming P/O = 3. In either case, the metabolic shift can therefore also be hypothesized as being the result of accumulated maintenance for cellular functions and/or the impediment of metabolic capacity. One possible sink for maintenance ATP is the energy required to protect cells within an S-phase arrest, or apoptosis may result from ATP shortages. Nonetheless, the cell with higher maintenance requirements and/or reduced metabolic capacity would also respond by shifting its metabolism and even increasing its metabolic capacity to provide more ATP under the limiting conditions. Although this second possible explanation for metabolic shifts is probably overshadowed by the effect of nutrient limitation, it is a hypothesis that is necessary to describe the metabolic shifts and cell death observed for fed-batch cultures in the absence of nutrient limitations.

Thus, for these chemostat steady states, the shift in metabolism in taken to be a robust response to the increasing glutamine-limitation, and possibly as well as a metabolic impediment. The relationship between apoptosis and metabolism is unclear. Evidence that metabolism is not the primary cause for apoptosis is inferred from the doubling of cell density that occurred when the dilution rate was increased again.

### 3.5.3 Defect in Cell Cycle Control

The trend observed in cell cycle distribution for these four steady states is quite contrary to what is expected of a normal cell cycle function (Lodish, Baltimore et al. 1995) or what has already been modeled or documented in other hybridoma cell lines (Suzuki and Ollis 1989; Martens, Gooijer et al. 1993; Goswami 1998). As described in a cell biology text, the cell cycle contains two checkpoints that prevent passage through the cycle in the absence of necessary factors, such as nutrients and growth factors. The data obtained for this hybridoma reveal that the first checkpoint (at the transition form G1 to S) is not operating correctly. Instead of exhibiting reduced entry to the cell cycle as the dilution rate is decreased and the nutritional environment becomes leaner, the culture actually shows a moderate increase in entry to the cell cycle (G1 residence drops from 30% to 20%). In terms of progression time through each cycle phase, the cells maintain a constant residence time in G1 of approximately 5 hours. The result of

sustained entry to the cell cycle is the accumulation of cells in the S-phase. S-phase progression time doubles as the dilution rate is decreased. In fact, of all the measured parameters, the S-phase progression time is the only one that matches the trend for the emergence of apoptotic cell death. G2/M progression remains constant at around 2 hours. Thus, it appears that the cell cycle for the hybridomas is defective in controlling entry to the cell cycle, and cells may be inferred to be dying within the S-phase. This finding explains the previously described phenomenon of "abortive proliferation" that this hybridoma is known to exhibit under suboptimal culture conditions (Chung, Sinskey et al. 1998).

Further evidence for this cell cycle defect was obtained by measuring expression of *c-myc* (a proto-oncogene involved in entry to the cell cycle) at different dilution rates and with or without insulin. c-Myc protein was measured as a possible cause for defective cell cycle control following the hypothesis made by Chung, et. al. for this cell line (Chung, Sinskey et al. 1998). In contrast to an anchorage dependent CHO cell line (Sanfeliu, Chung et al. 1999 in press), *c-myc* expression did not depend on the presence of insulin and was not noticeably different at the two different dilution rates investigated. Furthermore, c-Myc in the hybridoma was expressed well above that of the anchorage CHO cell line in all cases. The relatively high levels of hybridoma c-Myc are even more impressive given that the CHO cells were synchronized (c-Myc normally shows peak expression in early G1), whereas the hybridoma cells were cycling, with only about 30% of the cells in G1 as usual. Thus, c-Myc for the hybridoma is either expressed constitutively throughout the cycle or is expressed at extremely high levels during early G1. In either case, this measurement of increased c-Myc levels for our hybridoma cell line supports the observed deregulation of cell cycle entry. c-Myc is taken to be at least one of the possibly several factors which obviate the first checkpoint in the cell cycle.

1 2 3 4 5 6 7 8

**1.** Positive control

**2-6.** C-myc expression from anchorage-dependent **CHO** cells stimulated with insulin following arrest by serum withdrawal. Time points for the synchronized culture at 0, 2, 4, 6, and 8 hours following insulin addition. (load = 50 µg-protein)

**7.** C-myc for **Hybridomas** form an insulin(**-**) steady state culture at D = 0.03 $hr^{-1}$. (load = 50 µg-protein)

**8.** C-myc for **Hybridomas** form an insulin(**+**), steady state culture at D = 0.02 $hr^{-1}$. (load = 38 µg-protein)

**Figure 3-10. Western blot for c-Myc protein.**

In contrast to batch and fed-batch phenomena (Chapter 4), death in these chemostats is not associated with increases in the progression times for all the phases of the cell cycle (Chapter 4). Whereas cells in fed-batches are given an optimal nutrient supply, chemostat cells are under glutamine-limitation. It is not unreasonable to believe that a nutrient limitation in the chemostat results in a slowing of S-phase progression and may possibly mask a potential arrest in G2/M. Conversely, fed-batch cultures would not necessarily exhibit slower S-phase progression with adequate nutrients available, and so an arrest in G2/M would be more evident. The critical difference between the chemostat and fed-batch cell cycle phenomena is that the G1-phase progression time remains constant for the chemostats, whereas the G1-phase progression time for fed-batch increases. As will be discussed in Chapter 4, the increase of progression times for all the phases during fed-batches points to another underlying phenomenon as the cause for slower growth and cell death.

Overall, this defect in the cell cycle confers the ability for the hybridoma cells to grow very fast, with doubling times as low as 14 hours. The following figure (Figure 3-11) contrasts the "textbook" model for cell cycle control vs. one that better describes the CRL 1606 hybridoma. As denoted in the figure, apoptotic cell death is known to be reduced by the presence of additional protective environmental and genetic factors that help the cells complete the cycle as they enter it constitutively. Another approach, which is the subject of Chapter 4, is to slow down the entry to the cycle using an arresting agent, such as rapamycin.

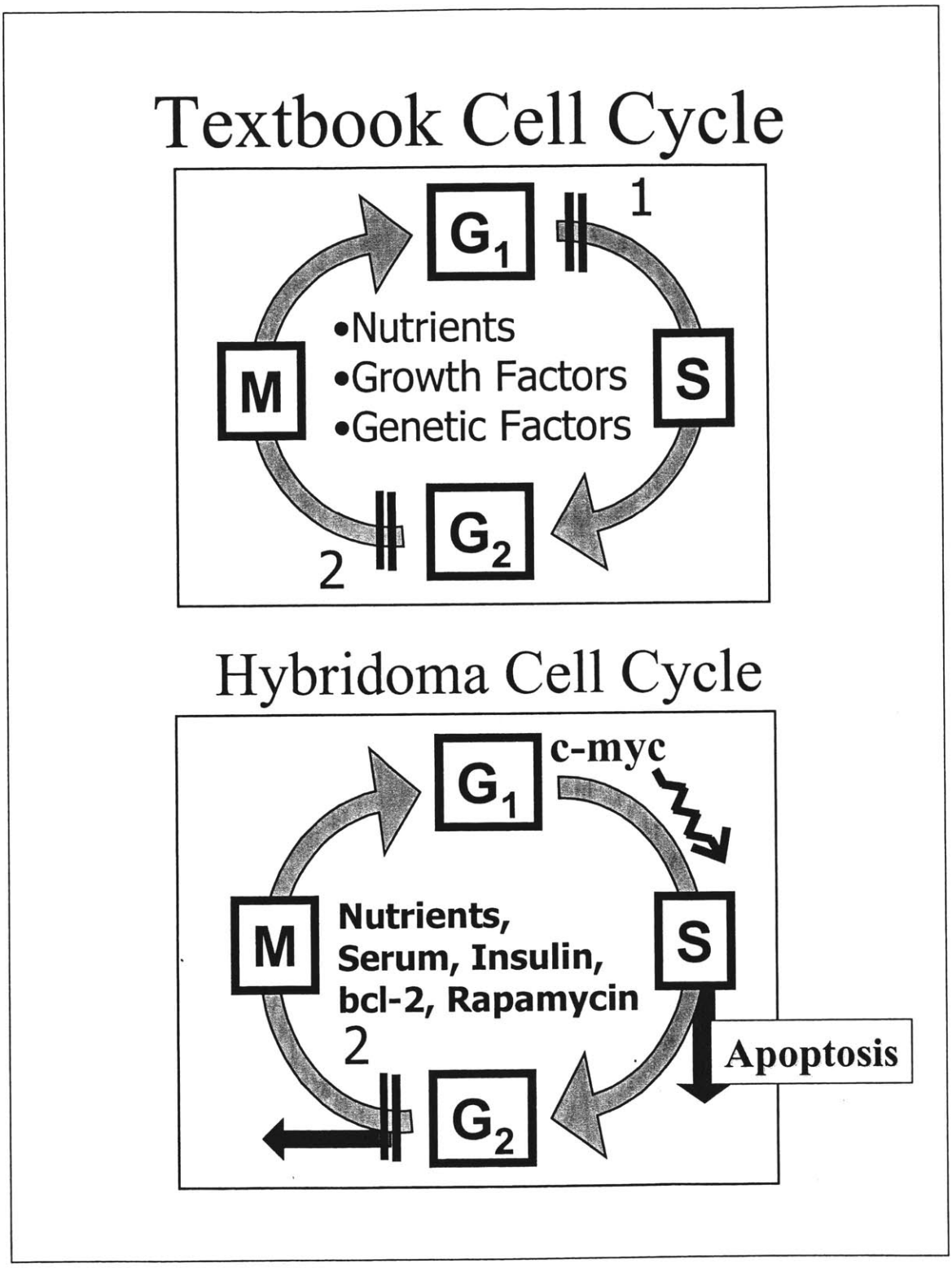



**Figure 3-11. Cell cycle diagram for "textbook" cell cycle and hybridoma cell cycle.**

### 3.5.4 Specific Production of Monoclonal Antibody

The specific production of MAb does not appear to be a simple function of growth rate. Previous studies have shown that hybridoma cultures which are growing slower may exhibit higher specific production rates (Suzuki and Ollis 1989; Al-Rubeai and Emery 1990; Martens, Gooijer et al. 1993). This phenomenon is related to the nature of production of antibodies and its better efficiency in the G1 phase of the cell cycle. Thus, for cells that can arrest in G1 as the dilution rate is decreased, it is observed that there is a higher specific MAb production rate. However, in my hybridoma cell line that does not arrest in G1, there is no observed increase in specific production rate. In fact, the reduction in specific productivity at dilution rates of 0.02 and 0.01 $hr^{-1}$ could be due in part to the reduction in percent of cells in G1 phase.

Another consideration for the observed trend in specific productivity is the emergence of apoptosis. In addition to having fewer cells in G1 at the lower dilution rates, the viable cells of a culture that is suffering from high levels of apoptosis could reasonably be imagined not to be the most productive ones. The high death rates and the delay in S-phase reveal that their metabolic shift, though helpful, cannot entirely compensate for the shortage of glutamine.

In addition to cell cycle and cell death effects, it appears that metabolic differences between the steady states may also play a role in specific productivity. The maximum productivity observed for steady state B coincides with a metabolic state that has an increased production of ATP from the TCA cycle in addition to sustained glycolysis and lactate production. Perhaps the observed optimum in specific productivity for MAb's is due to the balance of biosynthetic precursors and ATP production, which ultimately depend on the metabolic state of the cells.

Evidence for the role of metabolism on specific productivity is found in the MAb production rate for the multiple steady culture that was achieved at D = 0.04 $hr^{-1}$ (E) following the D=0.01 hr-1 steady state. The superior density steady state that was observed upon returning to the initial dilution rate of 0.04 $hr^{-1}$ had a specific production rate that was nearly half that of the initial 0.04 $hr^{-1}$ culture. Here, with growth rate and cell cycle distribution the same in each case, and also with very little apoptosis in both

cases, it clearly appears that MAb productivity is affected by the metabolism of the hybridoma cell. A shift in metabolism away from glycolysis towards TCA cycle apparently negatively impacts MAb production. Perhaps MAb precursors are derived from glycolytic biosynthetic precursors, and hence MAb production is negatively impacted upon a metabolic shift towards TCA cycle.

These observations shed some light on the observed variable nature of specific MAb production seen between different batch experiments. Just as different inocula of the same cell type have variable growth and death behavior, different inocula can also have different specific MAb production rates. Interestingly, both the growth and MAb production phenomena intersect with culture metabolism, which as presented in this investigation, can be very different depending on their environment and cultivation history.

## 3.6 CONCLUSIONS

Hybridomas were observed at a wide range of dilution rates, spanning a full range of culture physiologies for fast growing cultures with high viability down to slow growing ones with very low viability. It was observed that cell death correlates with the aberrant and persistent entry of cells to the cell cycle. The culture growth rate is apparently not being controlled by the first check point to the cell cycle, which would have resulted in an arrest of cells in the G1 phase of the cycle. Rather, it is evident that the cells entered the cycle and were delayed within the S-phase as a result of nutrient limitation at lower dilution rates. Thus, the emergence of cell death for this hybridoma under suboptimal conditions is exacerbated by the defect in cell cycle entry.

Under conditions of higher levels of apoptotic cell death, the viable population of cells shows a shift in metabolism towards TCA activity and oxidative phosphorylation. The shift is due to glutamine-limitation, and presumably implies that cellular energetics may be compromised as a result of or in conjunction with the emergence of cell death. The shift in metabolism is a response that enables cells to produce ATP more efficiently as resources become scarcer at lower dilution rates. The enhanced metabolic efficiency is demonstrated to yield higher viable cell densities following the lifting of nutritional constraints.

Specific productivity of monoclonal antibody is observed to be a complex function of cell death, metabolic state, and cell cycle distribution. Cultures with increased cell death, metabolism shifted towards TCA cycle, and a lower percent of cells in G1-phase exhibit lesser specific productivities. Of two steady state cultures with similarly high viability and comparable cell cycle distributions, the one with higher lactate production and lesser TCA cycle activity exhibits the higher specific productivity. The highest specific productivity is observed for the culture with an intermediate metabolic state, modest apoptosis, and average G1-phase residence.

## 3.7 ACKNOWLEDGEMENTS

I acknowledge the National Science Foundation for financial support through Cooperative Agreement 9843342 to the MIT Biotechnology Process Engineering Center and the National Institutes of Health Biotechnology Training Grant #5T32GM08334-10. I thank Brian Follstad for his partnership during the chemostat experiments and for his determination of the metabolite uptake and production rates and the metabolic fluxes and related analysis. I also thank Glen Paradis of the Cancer Center for his assistance with the flow cytometric analysis and Dr. Anna Sanfeliu for her assistance with the western blot analysis.

# 4 ENHANCEMENT OF CULTURE VIABILITY AND PRODUCTIVITY USING THE CELL CYCLE ARRESTING AGENT RAPAMYCIN

## 4.1 ABSTRACT

The cell cycle arresting agent rapamycin is used as a medium additive to inhibit cell cycle entry of hybridoma cells. Though rapamycin's most notable effect is growth arrest within the G1-phase of the cell cycle, in this study, rapamycin is used as an anti-apoptosis factor for a cell line with defective control of cell cycle entry, a defect which has been implicated in exacerbating apoptotic cell death.

Results demonstrate the 24-hour delay in the emergence of cell death for the hybridoma cell line in the presence of rapamycin. Despite growth being inhibited mildly, the extension of viable proliferation results in higher maximum viable cell densities and greater integrated viable cell densities. Over the course of 6-day fed-batch experiments, the positive effects of rapamycin on culture physiology result in a doubling of monoclonal antibody titer, due to the increase in viable cell densities as well as an increase in apparent specific productivity.

Rapamycin's mechanism for the above physiological changes in this hybridoma cell, and in particular, it ability to postpone and minimize apoptotic cell death, remains an open question. Among rapamycin's physiological effects is the arrestment of cells in the G1-phase of the cell cycle. Such an effect is observed for this hybridoma cell line. The typical 30% fraction of total cells found in G1 is increased to 40-45% with rapamycin present. This corresponds to an increase of 30-50% in G1 residence time. It is possible that additional time in G1 allows for more timely production of intracellular genetic factors needed for cellular function and survival during DNA synthesis and mitosis. Yet, preliminary data showing that rapamycin also affects metabolism indicates that a shift in metabolism might also possibly play an important role in the enhanced survival of the hybridomas.

## 4.2 INTRODUCTION

The initial interest in identifying arresting agents for the hybridoma cell line lay in having controls for the observations of cell cycle distribution that were to be obtained during chemostat studies. Cell cycle progression was a possible physiological parameter that was expected to be associated with the emergence of apoptotic cell death in this cell line. It was my intention to use phase-specific cell cycle arresting agents to verify any findings regarding cell cycle phenomena from chemostats in follow-up batch studies. My initial hypothesis during the spring of 1997 was that cell death was associated with or perhaps caused by an arrestment in a particular phase of the cell cycle. Thus, as a side project and with the aid of an undergraduate student, Ryan Gilbert, I went forward during the summer of 1997 to find and test various arresting agents that would trigger apoptotic cell death by arresting the hybridoma cell line.

From the literature, Ryan and I chose four agents (alleged to arrest in a particular phase for other cell lines) with which to begin our studies: (1) sodium butyrate (S-phase) (2) olomoucine (G1 and/or G2/M), (3) n-butyl 5-chloro-2-pyrimidoxyacetate (G2/M-phase) and (4) rapamycin (G1-phase) (Crosby and Bethold 1960; Gacek, K et al. 1979; Benneche, Stande et al. 1993; Aagaard-Tellery and Jelinek 1994; Glab, Labidi et al. 1994; Joensuu and Mester 1994; Vesely, Havlicek et al. 1994; Misteli and Warren 1995). Batch experiments were performed at various concentration of each arresting agent to see if any of the substances had any effect on cell growth, cell death, and cell cycle distribution for our hybridoma cell line. By the end of the summer, we had observed that sodium butyrate, olomoucine, and rapamycin all had G1-phase arresting activity, whereas 5-butyl n-cholo-2-pyrimidoxyacetate did not have any observable activity. The remarkable observation at that time was that the arrestment did not necessarily result in more apoptotic cell death. Depending on the concentration of the arresting agent, each of the three active agents actually delayed apoptosis, with rapamycin showing the best overall effect.

The full impact of such a result would only come to be appreciated after the completion of the chemostat study described in Chapter 3 (Fall 1997). From the chemostat experiments I learned that the nature of the hybridoma cell obviates nutrient limitation

as a means to regulate the passage of cells from the G1-phase of the cell cycle into the S-phase. The apparent constitutive entry to the cell cycle confers the ability of the cells to grow very quickly under optimal conditions, with doubling times as low as 14 hours. Yet at the same time, the unbridled entry to the cell cycle could possibly be leading to apoptotic cell death as a result of nutrient, growth factor, or genetic factor limitations. Overall, I was forced to reverse my initial hypothesis concerning cell cycle arrestment and apoptosis. For my hybridoma cell, it is actually a lack of arrest that can be detrimental to the culture. Eventually (by the summer of 1998), the combination of results from the chemostats and cell cycle arresting agents would serve as the basis for a new hypothesis: *slowing down entry to the cell cycle may lead to less apoptosis, especially for suboptimal environmental conditions.* During the summer of 1998, and with the help of another undergraduate student, Jose Alemán, I investigated rapamycin as a medium additive to enhance viability and show that an inhibition of entry to the cell cycle can be beneficial. With rapamycin's positive effects confirmed and applied successfully to a variety of batch and fed-batch cultures, and with the consent and urging of my thesis committee, the rapamycin story was upgraded from the status of a side-project to a primary focus of my thesis. During the fall of 1998 I finally turned to look deeper into the literature and to additional experiments to try to ascertain how rapamycin was protecting the cells from apoptosis.

Two other works occurring in parallel at that time also supported the reversal of hypothesis described above. First, Chung et. al. demonstrated that insulin and Bcl-2 act as survival factors for the hybridoma cell line that they intuited, based on literature reports, exhibited faulty regulation of cell cycle entry presumably due to *c-myc* over-expression (Chung, Sinskey et al. 1998). Second, Sanfeliu et. al. demonstrated that *c-myc* induction and expression by insulin in an anchorage-dependent CHO cell line leads to cell death in the absence of survival factors, and furthermore that cell death is minimized for cultures growing slower as the result of nutrient limitation (Sanfeliu and Stephanopoulos 1999; Sanfeliu, Chung et al. 1999 in press). These fellow works comforted me greatly as I re-formulated my hypothesis for the hybridoma cell cycle and designed experiments with rapamycin. Both of the works point to the emergence of cell death being the result of entry to the cell cycle in the absence of some other factors that

are necessary for cell survival. Achieving faster growth with a deregulated *c-myc* (the hybridoma cell) or by stimulating *c-myc* with insulin (Sanfeliu's CHO cell) is not as simple as just forcing entry to the cell cycle. Depending on environmental and cellular constraints, cells finding themselves "prematurely" in the S-phase may be limited in other necessary components and thus die by apoptosis. As I fortuitously identified, rapamycin acts as another survival factor, presumably through its inhibition of progression into the S-phase.

The essence of this chapter is to demonstrate the positive effects that rapamycin (the best of the four arresting agents tested) has on hybridoma cell culture. At the optimum concentration, the mild arrest in G1-phase leads to only a mild reduction in growth rate, but a dramatic reduction in death rate, allowing an additional day of viable proliferation before viability begins to decline. The extension of viability results in higher viable cell densities, greater integrated viable cell densities, and higher monoclonal antibody titers. Thus, the use of rapamycin is shown to be a valuable tool to enhance culture productivity. Furthermore, it can also help discern the relationship between the cell cycle and metabolism and the underlying causes for apoptotic cell death.

## 4.3 MATERIALS AND METHODS

### 4.3.1 Cell Line and Culture Medium

The cell line used was a murine hybridoma (ATCC CRL-1606) which produces and secretes an immunoglubulin IgG against human fibronectin. Each inoculum culture was prepared from a frozen stock of CRL 1606 hybridomas (previously adapted to serum-free medium) grown in T-flasks (Corning, Cambridge, MA.) and spinner flasks (Bellco, Vineland, NJ) within a 37°C humidified incubator (95% relative humidity) with 10% $CO_2$ atmosphere. The inocula were passaged daily for one to two weeks by subculturing the culture down to 2.5e5 cell/ml (from the approximately 7.5e5 cell/ml reached after 24 hours).

The hybridomas were grown in glutamine-free IMDM basal medium (Specialty Media, Inc., Lavallette, NJ), supplemented with 10 mg/L insulin (USB), 5 mg/L transferrin

(USB), 2.44 μL/L 2-aminoethanol, 3.5 μL/L 2-mercaptoethanol, 7.5 mg/L protease free bovine serum albumin (Sigma Chemical Co.), and 1 U/ml penicillin – 1 μg/ml streptomycin (Sigma). Glutamine was added at a concentration of 4.0 mM prior to an experiment. No serum or hydrolysates were added.

During the reactor experiments, the medium was further supplemented with pluronic F68 (Sigma #P-5556) and antifoam A (Sigma #A-5633). 10 ml of the 10% Pluronic F68 were added per liter of medium to yield a concentration of 0.1% w/v pluronic. Antifoam A stock solution was prepared at a concentration of 0.1 w/v in water and sterilized by autoclaving. The antifoam A stock was stored at room temperature. The stock bottle was shaken vigorously prior to addition to emulsify the solution. 1.0 mL of stock was added per liter of culture, yielding a concentration of 100 PPM antifoam. These additives ensured that the sparging had negligible effect on culture density and viability, as determined by separate trials at much higher sparging rates.

Experiments in which rapamycin (Sigma Chemical Co., St. Louis, Mo.) was added were given the appropriate amount of a rapamycin stock solution. Rapamycin stock solutions were made at either 325 μM or 1 mM by the addition of either 3.37 mL or 1.09 mL industrial grade ethanol to 1.0 mg rapamycin. Stock solution was stored as aliquots of 100 and 250 μL at -70°C for up to six months. Medium for rapamycin batch experiments (other than the optimization batches) were given sufficient rapamycin stock solution to yield a concentration of 100 nM for rapamycin. Fed-batch experiments, in which rapamycin was re-fed daily starting on the second day of culture, were given sufficient rapamycin stock solution to yield an equivalent concentration of 50 nM (not including any residual rapamycin). Based on the culture behavior, the added rapamycin plus the residual concentration yielded an estimated combined concentration between 50 and 100 nM throughout the experiment.

Initial medium for fed-batch experiments consisted of the IMDM medium described above, with the regular amount of glucose and glutamine (25 mM and 4 mM). Supplemental medium was a concentrated stock of nutrients and vitamins prepared according to the stoichiometric feeding strategy developed by Xie and Wang (Xie and Wang 1994; Xie and Wang 1994; Xie and Wang 1994; Xie and Wang 1996). Total

concentration of nutrients in the supplemental medium was set to 600 mM. Supplemental medium was stored frozen at -20°C in 10-mL aliquots. During some fed-batch experiments, cultures were fed additional insulin stock solution as well. In these cases, volumes of the 2-g/L insulin stock sufficient to add an equivalent of 10-mg/L insulin to a culture were added starting on the second day. The depletion or re-feeding of insulin was shown not to impact the emergence of cell death.

### 4.3.2 T-Flask Batch and Fed-Batch Experiments

Batch and fed-batch experiments conducted in T-flasks were performed in either T-25 $cm^2$ flasks (volume = 10 mL), T-75 $cm^2$ flasks (22.5 mL), or T-175 cm2 flasks (50-60 mL) (Corning, Cambridge, MA.). Flasks were placed within a heated and humidified $CO_2$ incubator set at 37°C, 95% relative humidity, and 10% $CO_2$. Cultures were sampled daily for cell counts and cell cycle analysis by transferring momentarily to a sterile hood, rocking back and forth to mix, and retrieving the desired volume with a disposable plastic pipette.

Daily feeding of supplemental medium, insulin stock, and rapamycin stock during fed-batches was accomplished within a sterile hood by removing approximately 10 mL of culture to a sterile 15 mL centrifuge tube, adding milliliter amounts of supplemental medium and microliter amounts of insulin and rapamycin stock, gently vortexing to mix, and transferring the fortified partial culture back to the T-flask. This procedure was adopted to facilitate the transfer of such small volumes for rapamycin and insulin in a reproducible and sterile fashion. The amount of supplemental medium to provide was determined using the material balance program created by Mollborn, et. al. (Mollborn 1996). Apparent growth and death rates and viable and total cell densities from each fed-batch experiment were used as inputs.

### 4.3.3 Bioreactor Batch Experiments

Batch bioreactor cultures were cultured within a 3-liter water-jacketed Applikon reactor (Foster City, CA) at a working volume of 1.5 liters. A New Brunswick Scientific (Edison, NJ) ML 4100 controller was used to monitor the bioreactor temperature, monitor and control the pH and dissolved oxygen, and control mixing of nitrogen, air, and oxygen for

the feed gas via a solenoid control assembly. A heated water jacket maintained the temperature at 37 °C. The pH was controlled at 7.20 +/- .05 by addition of 320 mM HCl and up to 10% $CO_2$ in the gas feed or a solution of 6.2 g/l NaOH and 0.4 g/l KOH as needed. The dissolved oxygen concentration was maintained at 50% of air saturation by a constant 75-150 mL/min (0.05-0.1 VVM) of sparging maintained with a Mass Flow Controller. $CO_2$ concentration in the feed was adjusted manually with a rotameter set at flows of 3.5 to 7.5 ml/min to minimize HCl addition. Agitation was set at 175 rpm. Process data (T, pH, DO, control outputs) were logged through the ML-4100 controller to a computer using the AFS software from New Brunswick Scientific (Edison, NJ).

The reactor experiments were inoculated with approximately 400 ml of inoculum culture of a density of approximately 8 x $10^5$ with high viability (> 95%) to give a starting density of 2.0 x $10^5$ cell/ml. The reactor was sampled daily for analysis and to recalibrate the pH and DO probes using the sampling technique described in Chapter 3.

## 4.3.4 Analytical Methods

### 4.3.4.1 Cell Number and Viability

Cell number and viability were determined by cell counts using a hemacytometer with trypan blue dye exclusion. One slide was counted daily for each T-flask culture, two slides for the reactor cultures. Due to an overwhelming body of evidence showing that the hybridomas die by apoptotic cell death during batches and fed-batches, it was taken for granted that the dominant mode of death was apoptosis. An occasional check of some of the experiments for the extent of apoptosis, performed as described in Chapter 3, confirmed that this was true.

### 4.3.4.2 Metabolite Concentrations

Supernatant samples taken from a flask or reactor were prepared, stored, and analyzed as described in Chapter 3. Supernatants were analyzed for glucose, ammonia, and lactate concentrations during some of the experiments.

### 4.3.4.3 Insulin Concentration

Supernatant from cultures for insulin analysis was stored in the same manner as that for the metabolite concentrations. The concentration of insulin in each sample was determined using an insulin ELISA kit (ALPCO #10-1113-01, New Hampshire), per manufacturer's instructions.

#### 4.3.4.4 MAb Antibody Concentration

MAb antibody concentrations were determined by ELISA as described in Chapter 3.

#### 4.3.4.5 Calculation of Growth and Death Rate and Specific Uptake and Production Rates

The apparent culture growth and death rates ($\mu$ and $k_d$) were determined from cell balances using total and viable cell densities ($n_T$ and $n_V$) obtained by Trypan blue counts:

$$\frac{dn_T}{dt} = \mu n_V = \mu f_V n_T \qquad (4\text{-}1)$$

$$\frac{dn_V}{dt} = \mu n_V - k_d n_V \qquad (4\text{-}2)$$

where $f_V$ is the fraction of viable cells. Between two time points, and taking the growth rate, death rate, and viability as constants at their average values over the time interval, these balances yield the following expressions for the average culture growth and death rates over a specific time interval:

$$\bar{\mu}_{1\to 2} = \frac{\ln(n_{T2}/n_{T1})}{\bar{f}_V(t_2 - t_1)} \qquad (4\text{-}3)$$

$$\bar{k}_{d\,1\to 2} = \bar{\mu}_{1\to 2} - \frac{\ln(n_{V2}/n_{V1})}{(t_2 - t_1)} \qquad (4\text{-}4)$$

Integrated viable cell densities (IVCDs) were calculated by numerical integration using the trapezoid rule:

$$IVCD_{i\to j} = \int_{t_i}^{t_j} n_v dt \approx \frac{1}{2}(n_{Vi} + n_{V(i+1)})(t_{(i+1)} - t_i) + .... + \frac{1}{2}(n_{V(j-1)} + n_{Vj})(t_j - t_{(j-1)}) \qquad (4\text{-}5)$$

Specific metabolite production or uptake rates (or MAb production rate) were determined by a species balance:

$$\frac{dC}{dt} = q_C n_V \qquad (4\text{-}6)$$

Using the IVCD, this balance yields the following expression for an average specific rate over two time points:

$$\bar{q}_{1\to 2} = \frac{(C_2 - C_1)}{IVCD_{1\to 2}} \qquad (4\text{-}7)$$

#### 4.3.4.6 Estimation of ATP Production

With only glucose, lactate, and ammonia as measurements, total ATP production was estimated with the help of the physiology observed in the chemostat experiments discussed in Chapter 3. First, ATP from glycolysis was determined from the measured glucose uptake rate and the observed glucose-to-lactate ratios. Second, ATP from TCA cycle was taken to be the same as that for a chemostat culture that exhibited a similar GUR and L/G ratio. Control cultures had similar GUR and L/G as chemostat steady state A (D=0.04 $hr^{-1}$), whereas rapamycin cultures had similar GUR and L/G as chemostat steady state C (D=0.02 $hr^{-1}$).

#### 4.3.4.7 Cell Cycle Distribution

Cell cycle distributions and progression times were measured by the same method described in Chapter 3. The determination of cycle distribution was performed once per day per T-flask culture, in duplicate for reactor cultures. Cell cycle phase progression times are calculated using the average growth rate for an interval and the average of the cell cycle distribution fractions from the beginning and the end of the interval.

# 4.4 RESULTS

## 4.4.1 Effects of Rapamycin on Culture Cell Density and Viability

#### 4.4.1.1 T-flask Batch Cultures

The first evidence of rapamycin's positive effect on hybridoma cell cultures was obtained during four to five day batch experiments performed in T-flasks. The optimum rapamycin concentration was determined from a series of four T-flask cultures given 0 nM, 50 nM, 100 nM, or 200 nM (data not shown). 50-100 nM provided the best effects in

terms of viable cell density and IVCD, whereas 200 nM showed a detrimental inhibition of growth and lesser reduction in death rate.

Cell density and viability plots are provided for the averages of batch cultures grown with or without rapamycin. The plots are composed of three control cultures and four rapamycin cultures, taken from the initial experiment, the optimization experiments, and an additional confirmatory experiment. The cultures included in the rapamycin group are those that were given the optimum concentration of 100 nM. The 95%-confidence error bars shown are more representative of variations of inoculum state and experimental outcome rather than experimental error. The error bars indicate the outcome one would expect based on the three to four independent trials. Furthermore, the average trend holds true for each individual experiment: a rapamycin culture always outperformed the control regardless of other factors.

Plots of total and viable cell density and viability for the control and rapamycin experiments show the reduction in growth rate and the extension of viability for the rapamycin cultures (Figure 4-1 a,b). Control cultures grow viably for two days in batch. The presence of rapamycin slows the growth of a culture, yet allows the culture to outperform the control by proliferating viably up through the third day in a batch. Thus, viable cell density and the IVCD are enhanced by the presence of rapamycin through its effects on culture physiology.

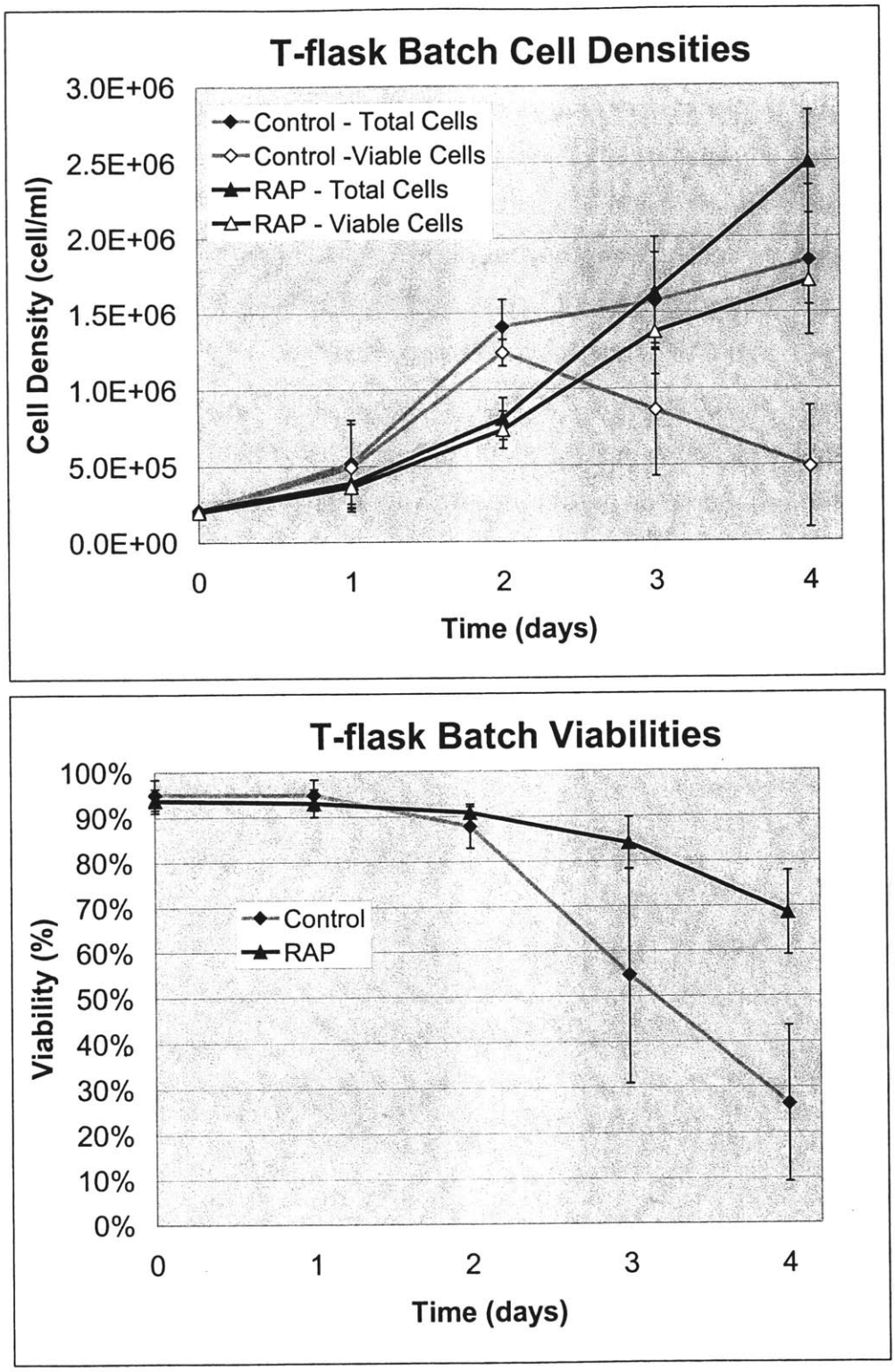
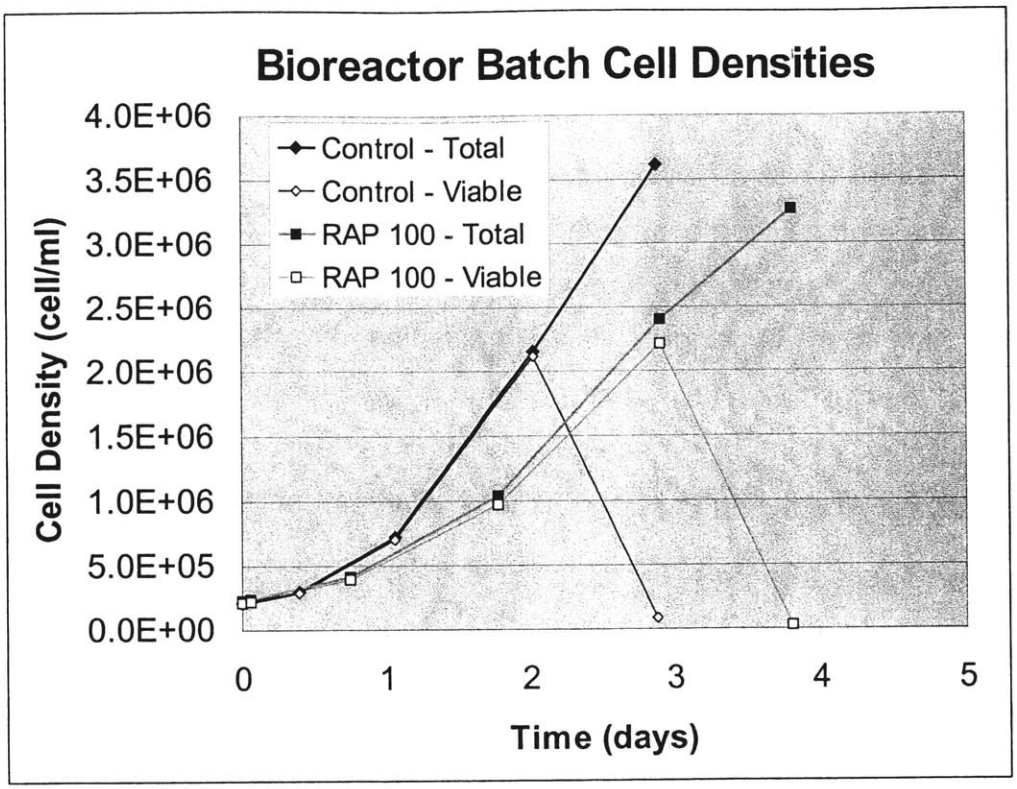



**Figure 4-1 a,b. Comparison of T-flask hybridoma batch cultures with and without rapamycin. a. total and viable cell density b. viability.**

The control data is the average from three separate experiments. The rapamycin data is the average of four separate experiments.

#### 4.4.1.2 Bioreactor Batch Cultures

To further test the enhancement of cultures using rapamycin, hybridoma cells were cultivated in a 3-liter bioreactor. The aim of this test was to confirm the effect at a larger scale, as well as to see if the better-controlled environment of a bioreactor would yield further increases in cell density beyond those already seen in T-flasks. The figure for the control and rapamycin reactor runs depicts a similar effect of rapamycin on the outcome of the reactor experiments that was seen for the T-flask experiments (Figure 4-2). Though the reactor cultures had higher viability and higher peak densities than the T-flask cultures (possibly the result of the better environment), the control culture nevertheless died after 2 days and the rapamycin culture died after 3 days. Thus, the effect of rapamycin was maintained at the bioreactor scale. The emergence of cell death which occurs with or without rapamycin must be attributed to other factors beyond nutrient depletion, dissolved oxygen depletion, pH changes, or ammonia and lactate accumulation (data measured but not shown).



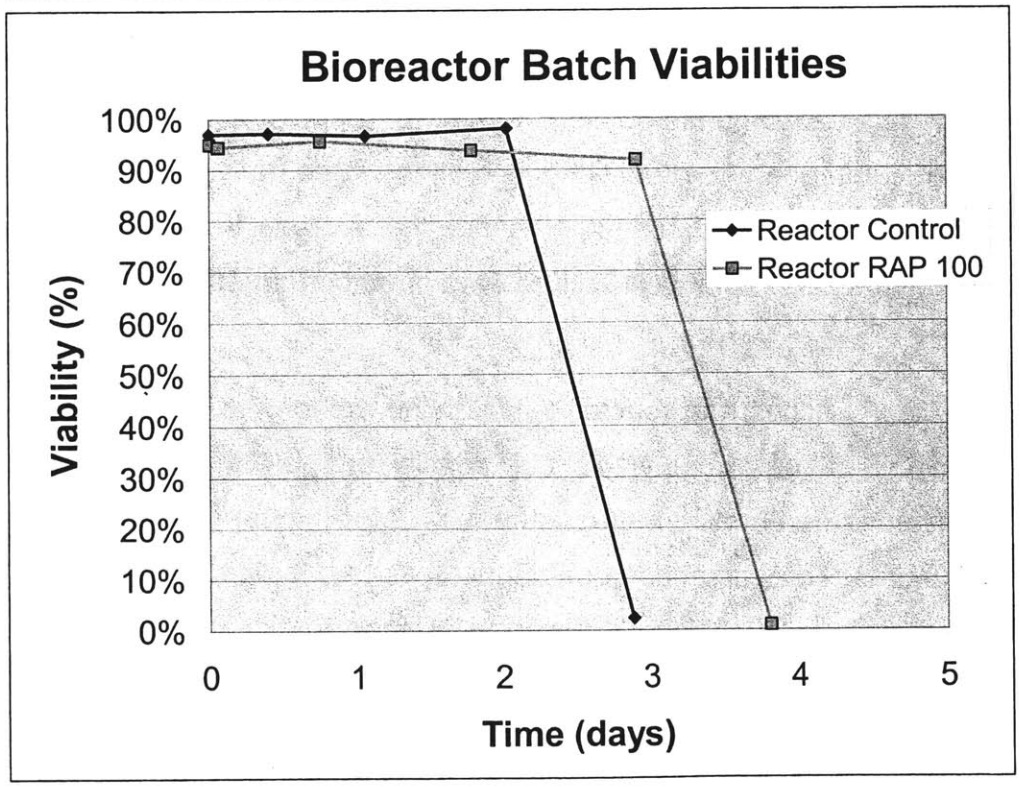



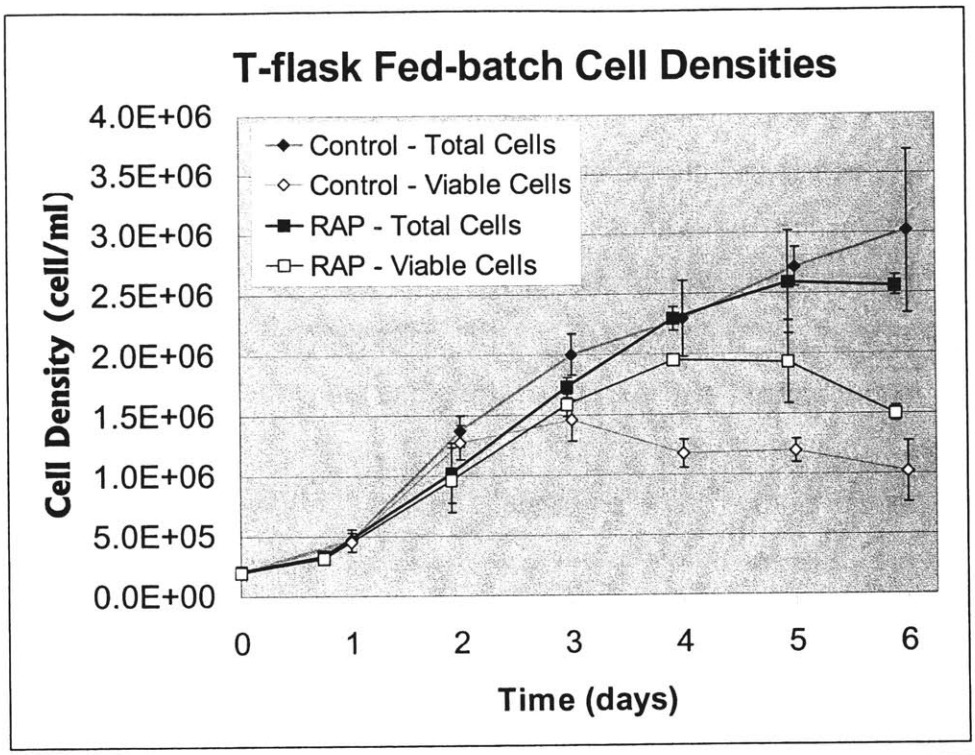

**Figure 4-2 a,b. Comparison of bioreactor hybridoma batch cultures with and without rapamycin. a. total and viable cell density b. viablity.**

#### 4.4.1.3 T-flask Fed-Batch Cultures

The use of rapamycin was combined with the stoichiometric feeding strategy developed by Xie and Wang that had already been successful in extending culture growth by maintaining the optimal nutritional environment and minimizing the formation of the undesired by-products lactate and ammonia (Xie 1997). Previously, the feeding strategy was successful in maintaining a viable population of cells, although viability was declining as the result of cell death. The aim of coupling nutritional control with rapamycin addition was to see if even further improvements could be achieved for fed-batch cultivation of the hybridomas and for increasing product titer.

The following figure (Figure 4-3 a,b) depicts the average outcomes for control and rapamycin-fed fed-batches. The control data is the average of 5 fed-batch control cultures, while the rapamycin data is that for 3 fed-batch rapamycin-fed cultures. Other fed-batch experiments in which rapamycin was fed only initially (data not shown), showed intermediate results, which prompted us to feed additional rapamycin on a daily basis. The 95%-confidence error bars shown are more representative of variations of inoculum state and experimental outcome rather than experimental error. The error bars indicate the outcome one would expect based on the three or five independent trials. As for the averages for T-flask batch experiments, the average trend holds true for each individual experiment: a rapamycin culture always outperformed the control regardless of other factors.

As for the T-flask batches and reactor batches, the rapamycin cultures outperform the controls in terms of maximum viable cell density and IVCD. Yet despite supplying additional nutrients, death still emerges after day 2 for the controls and after day 3 for rapamycin cultures. The presence of nutrients slows the decline in viability but does not prevent the emergence of cell death. It was determined that lactate and ammonia levels were not inhibitory for these cultures. Dissolved oxygen was estimated and measured via blood gas analyzer to be sufficient. Though pH could be a cause for death in T-flasks (it was measured to be 6.8 to 7.0 for some of the fed-batches), the reactor trials, in which pH was controlled, point to something other than pH as acting as a possible trigger of apoptosis.



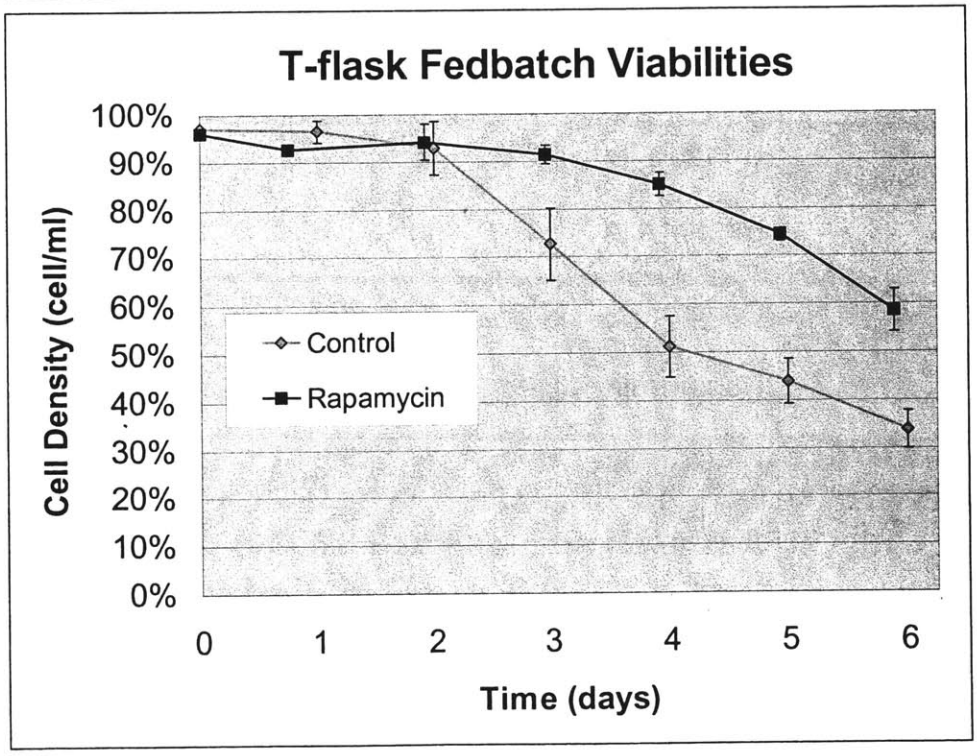




**Figure 4-3 a,b. Comparison of T-flask hybridoma fed-batch cultures with or without rapamycin.**

The control data is the average from five separate experiments. The rapamycin data is the average of three separate experiments.

#### 4.4.1.4 Summary of the Effect of Rapamycin on Cell Density

Overall, in terms of maximizing viable cell densities and IVCD's, the use of rapamycin is very effective despite slightly inhibiting culture growth. The table below compares the achieved IVCD's for the various types of experiments, where the error ranges correspond to 95% confidence intervals for the observed cultures (Table 4-1). Two IVCD's are of interest. The first is the IVCD corresponding to period during which the culture has high viability (> 90%). For controls this is a two-day period, for rapamycin, a 3-day period. From this measure, it appears that rapamycin allows cells to grow viably to a higher density before death emerges. The second IVCD is calculated over the total process time, 4 days for batches and 6 days for fed-batches. It is clear that rapamycin enhances viable cell density and yields IVCD's that are 20-30% greater overall.

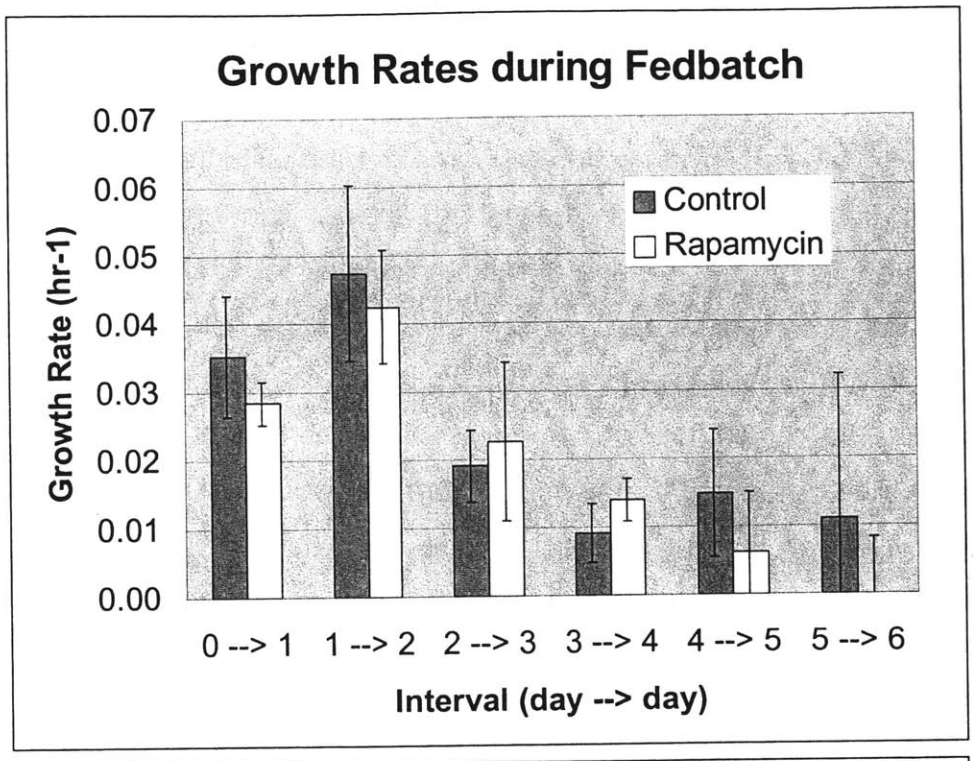
**Table 4-1. Comparison of culture cell density enhancement by rapamycin.**

| | | IVCD \| high viability ($10^7$ cell-hr/ml) | IVCD \| total ($10^7$ cell-hr/ml) |
|---|---|---|---|
| **T-flask BATCHES** | Control | 2.9 ± 0.8 | 7.0 ± 1.9 |
| | Rapamycin | 4.5 ± 0.6 | 8.2 ± 0.7 |
| **Reactor BATCHES** | Control | 4.4 ± 20% | 7.0 ± 20% |
| | Rapamycin | 7.2 ± 20% | 10.0 ± 20% |
| **FED-BATCHES** | Control | 3.0 ± 0.3 | 15.0 ± 0.8 |
| | Rapamycin | 5.4 ± 0.8 | 18.0 ± 0.5 |

### 4.4.2 Effect of Rapamycin on Growth Rate and Death Rate

Data from the fed-batch cultures is used to calculate the apparent growth and death rates for control and rapamycin cultures (Figure 4-4 a,b). The rates are calculated over daily periods using the equation described above. The error bars are the 95% confidence intervals for the average of rates calculated for each experiment in a given set. Though the observed ranges are large, their average holds true for the observed changes in growth and death witnessed within any of the runs. Rapamycin inhibits the growth rate 10-25% during the first two days, when both control and rapamycin cultures have high viability. Afterward, rapamycin cultures grow slightly faster than the controls, though at lower rates than at the beginning. Finally, at the end when death has fully emerged, control cultures show faster growth rates again. Overall, in both cases, the initially fast growth of the first two days is observed to slow down on the third and following days.

More dramatic than the differences in growth rate is the suppression of death rate for rapamycin cultures. Both cultures exhibit little death initially (< 0.004 hr-1) during the first two days, but then death within the control culture emerges sharply between the $2^{nd}$ and $3^{rd}$ days, while the rapamycin culture shows no increase in death yet. Even as the death rate for rapamycin begins to increase between days 3 and 4, it does so much more gradually. This delay and reduction is death rate is the dominant positive effect of rapamycin on the hybridoma cells. It appears that the growth rates for the control and rapamycin cultures follow the similar paths (at first fast, then slow), but it is the lack of death in the rapamycin culture that allows it to outperform the control.



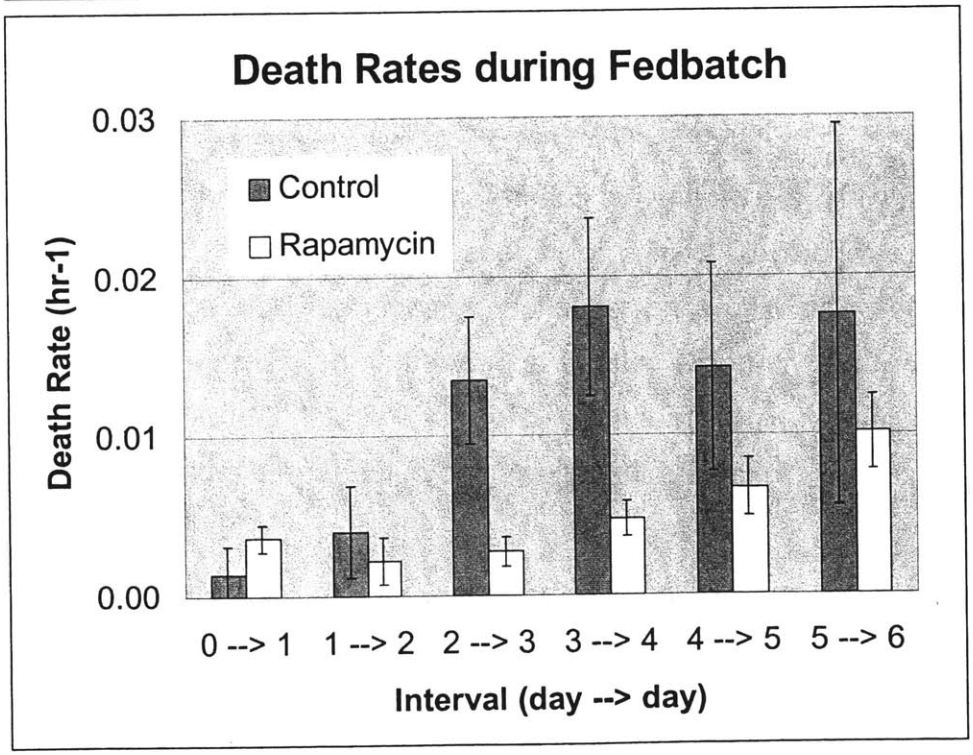




**Figure 4-4 a,b. Growth and death rates during hybridoma fed-batches with and without rapamycin.**

The control data is the average from five separate experiments. The rapamycin data is the average of three separate experiments.

### 4.4.3 Effect of Rapamycin on Cell Cycle Distribution and Cell Cycle Progression

As described in the literature, and the reason I adopted it as a good candidate for an arresting agent for our studies, rapamycin is shown to increase the percentage of cells in the G1-phase of the cell cycle. Below is data for the cell cycle distribution for fed-batch cultures with or with out rapamycin (Figure 4-5 a,b,c) (rapamycin cultures were given 100-nM rapamycin initially and 50 nM daily beginning on day 2). Cell cycle data was not acquired during the first day of the rap-fed cultures, although other data confirms arrestment is already visible. Over the four day period on which we can compare, it is evident that the percent of cells in G1 for the control remains approximately at 30% (the same fraction seen during the chemostats), but that the percent of cells in G1 for rapamycin ranges from 37-48%. At day two, when both cultures are proliferating viably, G1 for rapamycin cultures is roughly 30% greater (28% vs. 37%), which agrees adequately with the observed reduction in growth rate. In terms of the other phase of the cell cycle, rapamycin appears to minimize the increase of cells in the G2/M phase of the cell cycle and also maintains a lesser fraction of cells in the S-phase. Overall, in terms of cell cycle distribution, rapamycin cultures have a lesser percentage of cells within the cycle (S plus G2/M) as compared to the control.

Interestingly, for both control and rapamycin cultures, the fraction of cells within the G1-phase remains steady despite the observed slowing of culture growth and the emergence of cell death. The analysis for the progression time for each of the phases of the cell cycle between the first and second days and the second and third days during the fed-batches reveals that progression times for *all phases* are increasing as death emerges (Figure 4-6 a,b), and regardless of the presence of rapamycin. The total cycling time (the doubling time for growth) is calculated from the growth rate during an interval. As I discussed for the chemostat data, the application of an accepted cell cycle model (Suzuki and Ollis 1989) must be done with the assumption that cells do not arrest in late G1 phase, since otherwise would require that a higher percentage of the culture be in G1, which is in direct contradiction to the measured distributions. Thus, I allow the

total cycle time to vary with growth rate as I set the fraction of arrested cells ($f_A$) equal to zero in the following equation:

$$t_C = (1 - f_A) \cdot \ln(2) / \mu \qquad (4\text{-}8)$$

If I were to calculate $f_A$ based on a constant $t_C$ for fast growing cells, a decrease of 50% in growth rate would yield a fraction of arrested cells of 50%. But in reality I never measure G1 (which would be the sum of arrested cells plus those cycling) to be greater than 45%, regardless of growth rate. Thus, with the fraction arrested set to zero, the total cycling time increases for decreases in growth rate. After calculating the total cycling time, I calculate the progression times for each phase based on the measured distribution. The result is that fed-batch cultures appear to progress more slowly throughout all the phases of the cell cycle. The relative progression times are two to three times longer for G1 and G2/M, where as progression through S-phase is 1.5 to 2 times longer.

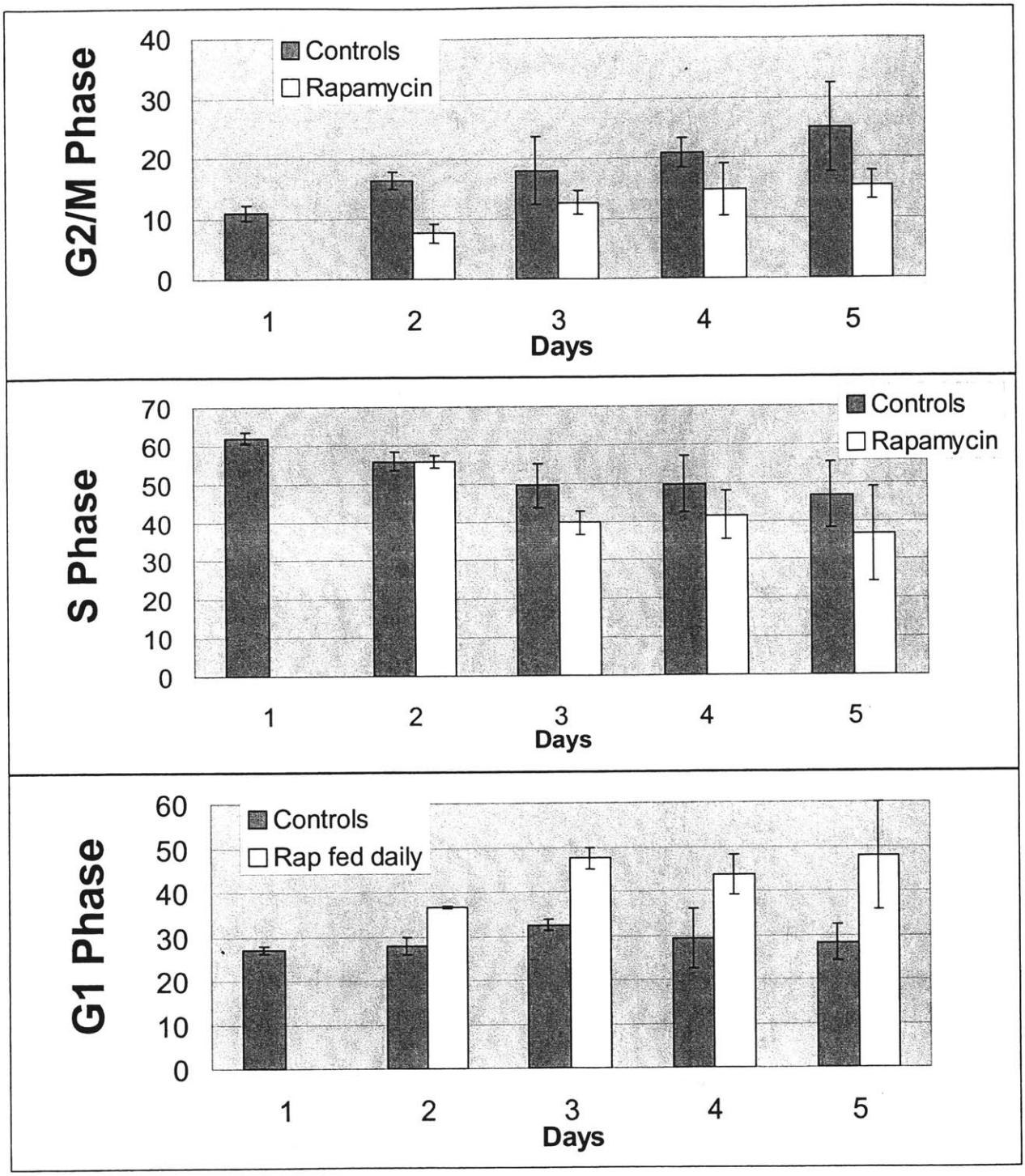



**Figure 4-5 a,b,c. Cell cycle distribution during hybridoma fed-batches with and without rapamycin.**

The control data is the average from five separate experiments. The rapamycin data is the average of three separate experiments. No data was obtained for the first day of rapamycin fed-batches.

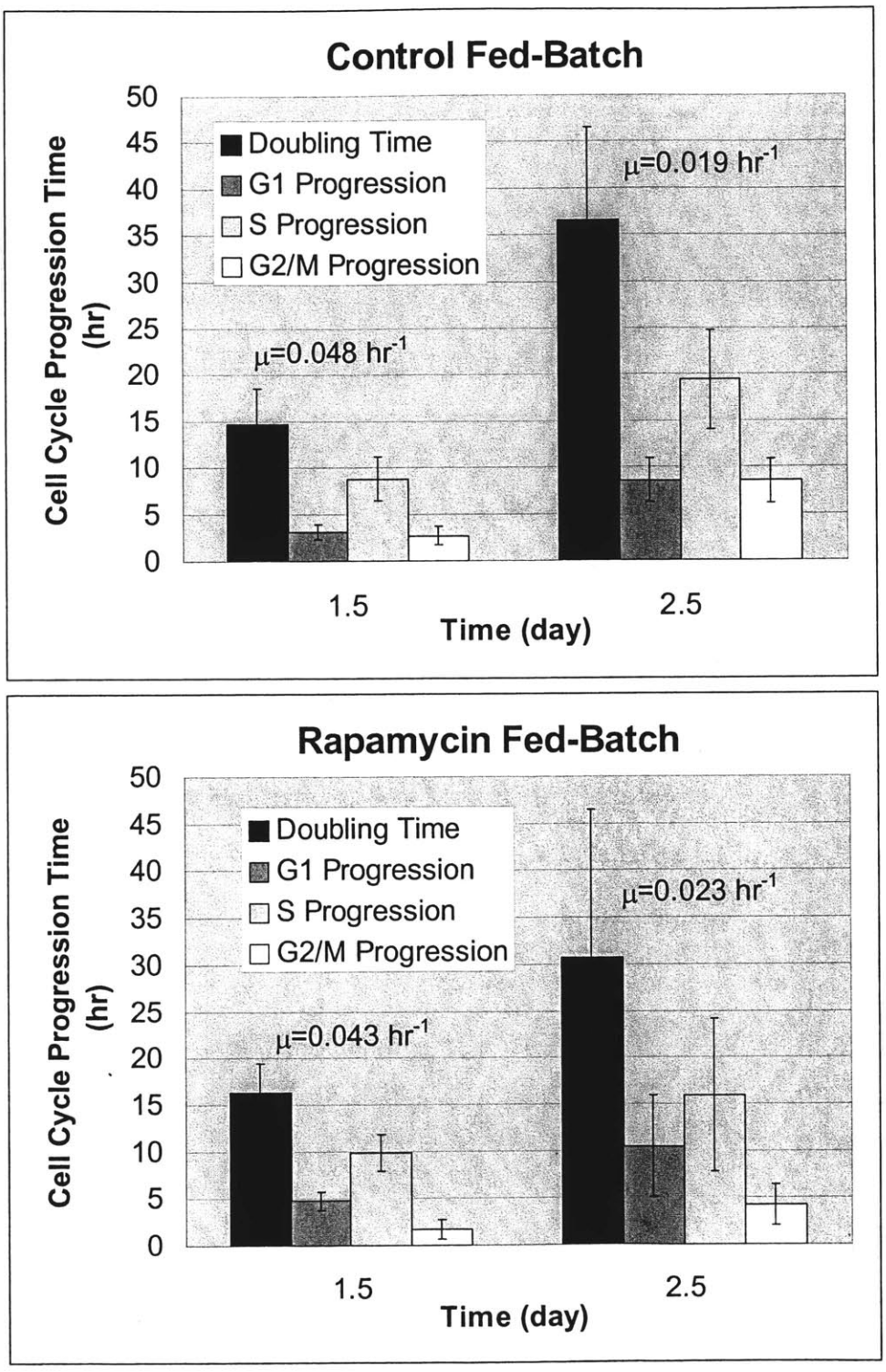



**Figure 4-6 a,b,c. Cell cycle progression times during hybridoma fed-batches with and without rapamycin.**

The control data is calculated from the growth rate and cell cycle distribution for the average of five separate experiments. The rapamycin data is similarly calculated from three separate experiments.

### 4.4.4 Enhancement of Monoclonal Antibody Production

In support of the primary goal for research in the field of therapeutic protein production, a principle effect of rapamycin on the hybridomas is to increase monoclonal antibody titers. The effect is the result of two complementary phenomena. The first is the increase in viable cell density that results from rapamycin's reduction of culture death rate. With increases of total IVCD's about 20-30%, we can expect proportional increases in product titers as well. The second, fortuitous phenomenon is that higher hybridoma production of MAb's is associated with the G1 phase of the cell cycle. Thus, an increase in the apparent specific productivity is also observed. Combining the two effects, it is shown that monoclonal antibody titers can be doubled by the presence of rapamycin (Figure 4-7 a). For my serum-free, 6-day fed-batch process, the use of rapamycin increases the titer from 0.25 g/L to 0.55 g/L (120% greater). Data shown is for two controls and three rapamycin experiments conducted simultaneously from a single inoculum. Data for other fed-batches shows typical variation in specific productivity and titer, yet the effect of rapamycin to yield greater titers and increased specific productivity over the control cultures is conserved for all experiments.

The specific productivity (Figure 4-7 b) observed during control fed-batches is within the same range as those measured for the chemostats, specifically being similar to that of steady state A ($D=0.04\ hr^{-1}$). The specific productivity during rapamycin fed-batches is slightly greater than the maximum observed for the chemostats at steady state B ($D=0.03\ hr^{-1}$). Such an increase in specific productivity is plausible for the slower growing rapamycin culture with a greater fraction of cells in G1, with a possibly shifted metabolism, and in the absence of nutrient limitation. The increase in specific productivity is believed not to be the result of increased passive release of antibodies from stationary phase cells. Measurements of intracellular MAb concentration for both control and rapamycin cells indicates that the amount of MAb's inside the cells is insignificant and can not account for increases in titer (data not shown).

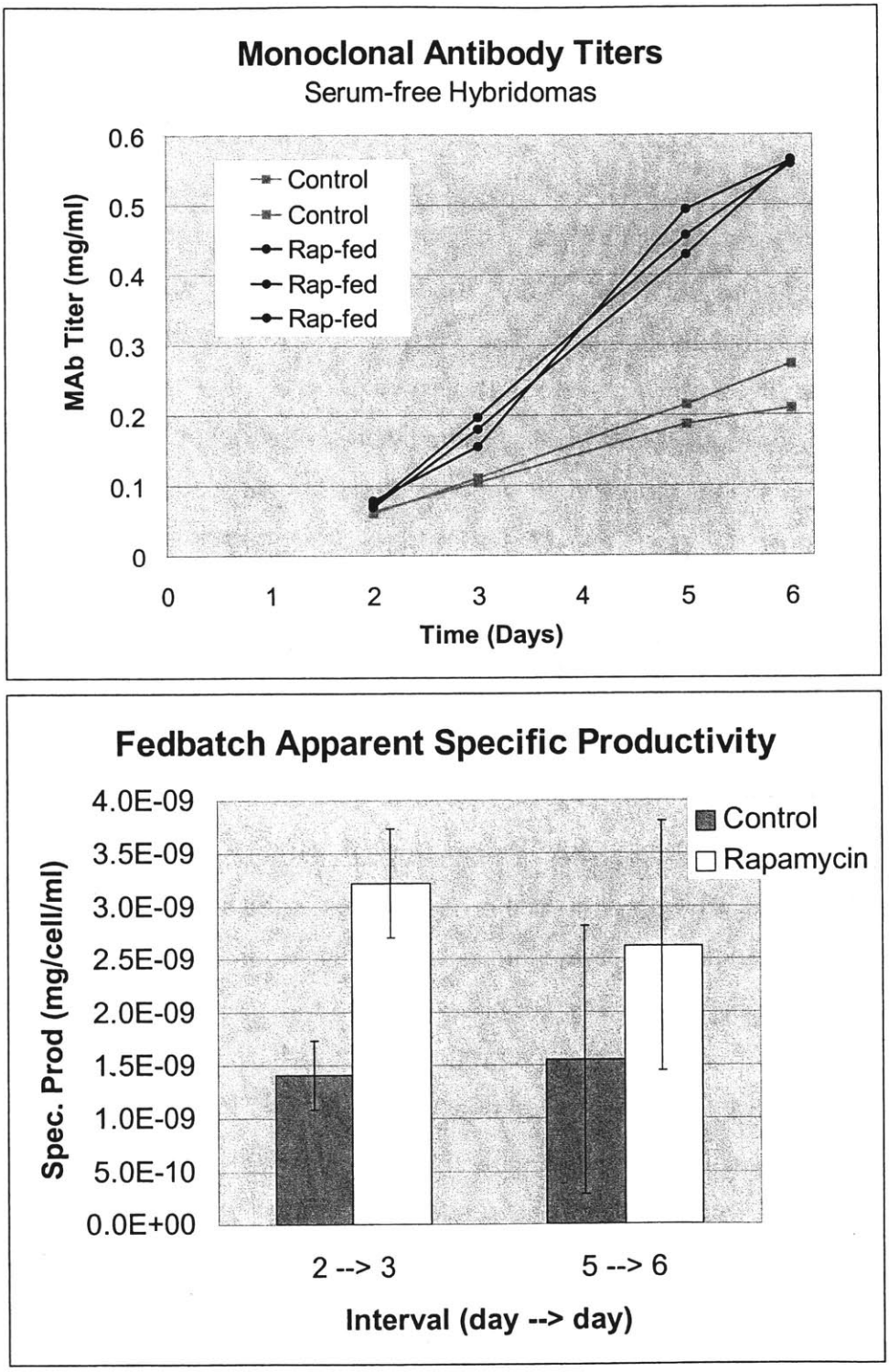



**Figure 4-7 a,b. Monoclonal antibody titers and specific productivity during fed-batches with and without rapamycin.**

Errors for titer data are within 10%. Specific productivity for the control is the average calculated from two experiments. Specific productivity for rapamycin is that from three experiments.

# 4.5 DISCUSSION

## 4.5.1 Mechanism of Rapamycin

The effect of rapamycin on culture physiology is an active area of research. To date there is substantial literature that supports the arrestment of cells in G1 by the presence of rapamycin (Terada, Franklin et al. 1993; Aagaard-Tellery and Jelinek 1994; Hung, Jamison et al. 1996; Wadsworth and Siekierka 1996; Metcalfe, Canman et al. 1997; Hashemolhosseini, Nagamine et al. 1998; Peterson, Desai et al. 1999; Sabatini 1999). The arrestment phenomena, however, is a downstream event which follows somewhat after the interaction of rapamycin with the protein signaling FRAP pathway. As shown in the diagram below, which is a consolidation of knowledge taken from several sources, the FRAP pathway is one which controls the translation of proteins and the activity of ribosomal proteins (Figure 4-8).

Rapamycin diffuses into cells and binds to the protein FKBP12, a complex that then interacts with the protein FRAP. This interaction draws FRAP away from its role as an active kinase for $p70^{S6k}$ and 4E-BP1. A shift in p70S6k phosphorylation in turn causes shifts in several other downstream protein phosphorlyations, such as for S6 (a ribosomal protein). A shift in 4E-BP1 phosphorylation impacts the availability of translation initiation factors. There are a number of papers describing how rapamycin affects proteins associated with cell cycle control. However, the connection between the FRAP pathway and its ultimate downstream targets remains unclear. In yeast cells, rapamycin causes the upregulation of survival genes and starvation genes, and the down regulation of glucose and amino acid transport genes (Schreiber 1999). The effect of rapamycin on mRNA translation appears to have a global impact on cellular physiology. Some of the impacts, such as on cell cycle progression, are well characterized; others, such as on metabolism and apoptosis, are still being elucidated.

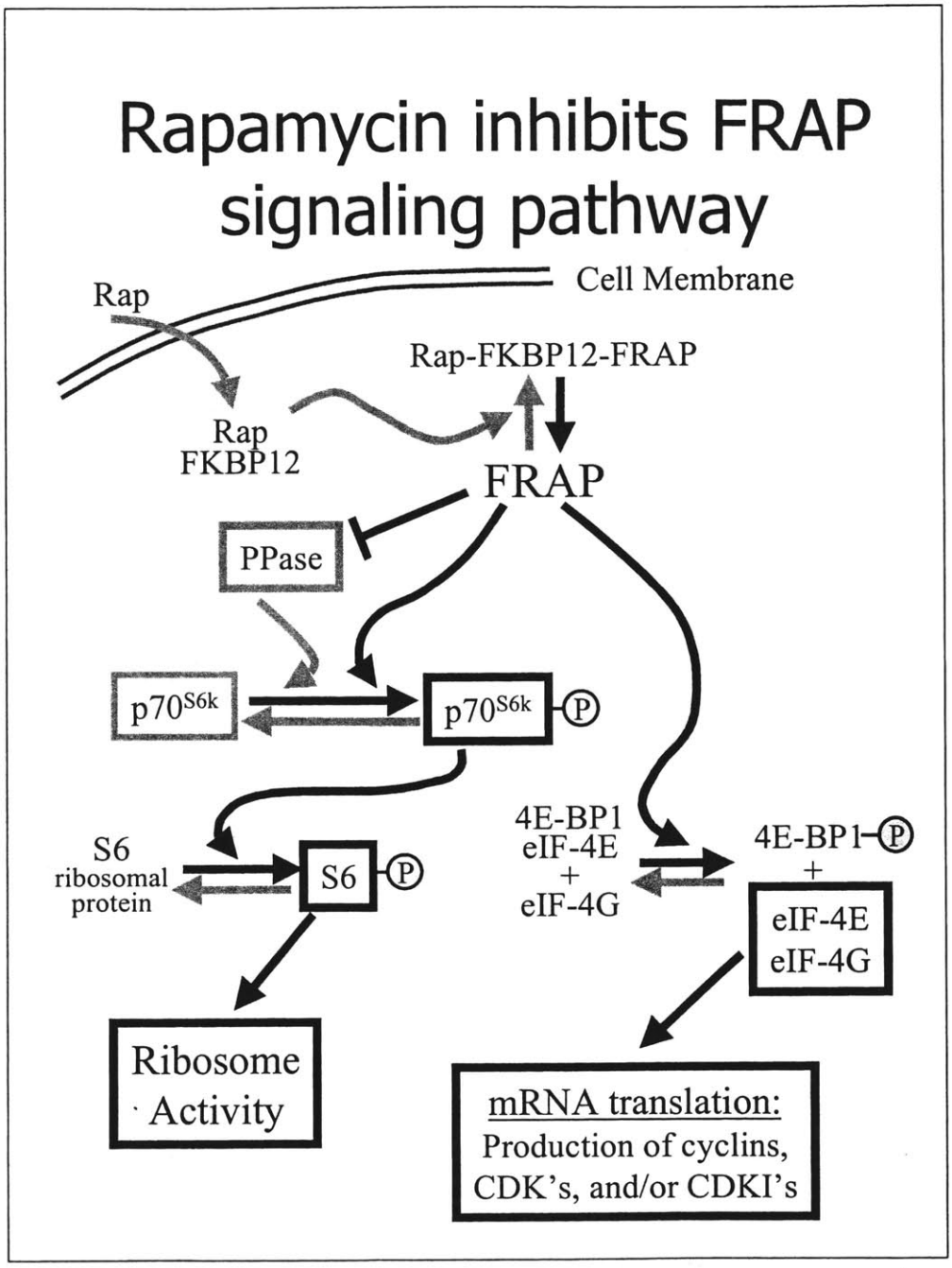



**Figure 4-8. Interaction of rapamycin with the FRAP signaling pathway**

### 4.5.2 Cell Cycle Arrest

One well-established phenomenon downstream of the FRAP pathway and rapamycin's primary effect is the arrestment of cells in the G1 phase of the cell cycle. Measurement of cell cycle distributions for my hybridoma confirms that the arresting agent inhibits entry to the cell cycle, even with defective *c-myc* regulation. A recent literature report confirms rapamycin's direct action on cell cycle control (Hashemolhosseini, Nagamine et al. 1998). It claims that rapamycin destabilizes cyclin D1 mRNA (cyclin D1 is associated with the latter part of the G1 phase of the cell cycle) and also accelerates the degradation of cyclin D1. Both these effects lead to an extension of residence in the G1-phase by inhibiting the accumulation of cyclin D1, and hence the cyclin D1-cdk complex needed to activate additional cellular processes. Rapamycin's ability to inhibit cell cycle entry in my hybridoma cell line agrees with the findings regarding cyclin D1. Rapamycin can successfully inhibit cell cycle entry despite the *c-myc* deregulation because *c-myc* expression precedes that of cyclin D1.

The effect of rapamycin on delaying passage through the G1-phase of the cell cycle agrees with the extent of reduction of the apparent growth rate for the hybridoma cells. For example, looking at the data for fed-batches on their second day (shown above), a control culture exhibits a growth rate equal to 0.0475 $hr^{-1}$ and spends approximately 3.1 hr (the G1-phase progression time) of its 14.6 hr doubling time in G1. The rapamycin culture, on the other hand, exhibits a growth rate of 0.0425 $hr^{-1}$ and spends 4.8 hr in G1. Holding progression times for S-phase and G2/M as being equal (by calculation, S is an hour longer and G2/M an hour shorter for rapamycin, differences which cancel here), the increase in the G1-phase residence time from 3.1 hr to 4.8 hr should increase the overall doubling time from 14.6 to 16.3 hr. Indeed, 16.3 hr corresponds exactly to the growth rate of 0.0425 $hr^{-1}$. Thus, it is reasonable that rapamycin's effect on cell cycle progression is the cause for the observed reduction in growth rate for rapamycin cultures, at least when comparing fast growing, high viability cultures.

It is interesting that both the control and rapamycin fed-batch cultures exhibit the same symptoms of growth arrest and increases in progression times for all phases of the cell cycle. The increase in G1 progression for both cultures is in fact greater than the 1.7-

hour increase for the rapamycin culture during viable proliferation. The cause for this phenomenon in fed-batches is unknown. Yet, in the following chapter, the same phenomenon will be observed upon the removal of insulin from steady state chemostats, depending on the environment and metabolic state of the cells. Thus, in these cultures, it appears that the trend for fed-batches is one of decreasing metabolic capacity as time passes. The metabolic challenge may be reflected in the uniform extension of progression through the cell cycle and the emergence of cell death.

### 4.5.3 Rapamycin Delays Apoptotic Cell Death

The major finding in this work is that another one of rapamycin's effects is the reduction of apoptotic cell death for hybridoma cultures. I believe this to be a novel finding because literature regarding rapamycin does not mention such an anti-apoptotic effect for rapamycin. Yet attributing the delay of apoptosis solely on cell cycle phenomenon (rapamycin's well-known effect) may not be entirely justified. There is preliminary evidence (see next section) to show that metabolism is also affected, being shifted towards more efficient consumption of glucose. From the chemostat work and subsequent fed-batches performed by Brian Follstad, an increase in metabolic efficiency can be associated with increases in viable cell density and sustained viability during fed-batches. Thus, a complete explanation for the effect of rapamycin must address both cell cycle and metabolism as playing a role in reducing apoptosis. As for other survival factors such as insulin and Bcl-2, metabolism, cell cycle, and apoptosis are intimately related and affected by changes in one another. The certainty here is that rapamycin's effect on cell physiology has a protective effect against apoptotic cell death during batch cultivation.

The evidence presented here is insufficient to determine how the altering of a cell's protein profile by inhibiting FRAP signaling leads to reducing cell death. With FRAP inhibition leading to changes in mRNA translation and ribosome function, it could be changes in one or many proteins that could be responsible for suppressing apoptosis. Changes to cell cycle control proteins have been reported, and help explain the delay in G1. Even still, a mere delay cannot fully explain the reduction in cell death. If it is the delay that really helps the cells, I believe other changes beyond those for cell cycle

control proteins must occur during the delay that serve to protect the cells later in the cycle. This is the essence for my hypothesis for the relationship between cell cycle entry and apoptosis: by aiming to slow down entry to the cell cycle, I implicitly agreed that extended time in G1 confers better survivability for cycling cells. The delay in progression may allow for (more complete) production of additional protein factors that enhance survival. If so, the identification and control of such protein factors would be an ideal goal for genetic manipulation.

In trying to understand rapamycin's anti-death activity, it may be beneficial to consider the similar, though less dramatic, effects that sodium butyrate and olomoucine also had on G1-arrest and reduction of apoptosis for this hybridoma cell line during preliminary experiments. Sodium butyrate inhibits histone deacetylation, leading to cycle arrest in G1 and G2 (Hung, Jamison et al. 1996). Olomoucine also leads to G1 and G2 arrests through its inhibition of CDK activity (Hung, Jamison et al. 1996). That these substances exclusively effect cell cycle related phenomena and yet also influence the emergence of cell death, could further support the effect of delayed cell cycle progression on reducing apoptosis. At the same time, such evidence (given that sodium butyrate and olomoucine are not as effective for preventing cell death) could also be used to support a hypothesis that rapamycin may act on other cellular functions (such as metabolism) to minimize cell death.

### 4.5.4 Metabolism of Rapamycin Cultures

While there is very good evidence for the relationship between cell cycle progression and apoptosis, it is nonetheless important to consider other observed differences in culture physiology. Culture metabolism is also known to change in conjunction with the emergence of cell death (Chapter 3). During rapamycin experiments, differences in metabolism were visibly observable in the differences in the color of conditioned culture medium of control and rapamycin flasks. The rapamycin culture medium looked noticeable redder in color versus the control culture medium that was more orange-yellow (culture medium contains phenol red as a pH indicator). The significance of this observation is that the metabolism of rapamycin cultures (at least in terms of producing lactic acid and ammonia, which affect the pH) is apparently different from the controls

since they both were reaching similar total cell densities though producing different quantities of acids and bases. This observation is supported by lactate and ammonia measurements for some of the rapamycin experiments. Furthermore, the measurement of glucose consumption indicates that central carbon metabolism may be shifted as well. Cultures with rapamycin present have lesser specific uptake rates for glucose than that measured for control T-flasks and reactor cultures. Furthermore, the measured Lactate/Glucose ratios for rapamycin cultures are lower than their control counterparts, the exception being the rapamycin culture with the smallest sGUR. (Figure 4-9a)

Lesser glucose uptake rates and lesser production of lactate point to a metabolism that is more efficiently shuttling glucose carbon to the TCA cycle. This shift in metabolism of glucose and production of lactate is similar to those shifts in metabolism that have already been observed as being associated with enhanced viability of cell cultures. Based on early chemostat data with CHO cells, we saw how glucose limited cultures metabolize more carbon through the TCA cycle, hence operating more "efficiently" (Nyberg, Balcarcel et al. 1999). Brian Follstad and I also observed a "more efficient" metabolism in glutamine-limited hybridoma chemostats (Chapter 3, (Follstad, Balcarcel et al. 1999)) Cultures with a metabolism relying more on TCA cycle than glycolysis help explain the multiple steady states with higher viable cell densities that were observed at same dilution rates. Dr. Anna Sanfeliu observed that her CHO cells grew slower under glutamine-limited conditions (they produce glutamine from glutamate using Glutamine Synthetase), yet they exhibited more robust behavior under sub-optimal conditions (Sanfeliu and Stephanopoulos 1999). Finally, I have now shown that the hybridoma cells grow more robustly when their growth rate is somewhat inhibited by rapamycin. A personal communication with Dr. Sabatini of the Whitehead Institute provides additional support for rapamycin's effect on metabolism (Sabatini 1999). During our discussion Sabatini mentioned that rapamycin effects are drastically reduced when a cell line is transfected with an ecotrophic receptor, which is used to make a cell line infectable to retroviral vectors. He said he believed that rapamycin was causing the cells to mimic amino acid limitation behavior, and that the ecotrophic receptor, which is actually a leucine transporter, might be alleviating the "amino acid limitation" imposed by rapamycin. His observations are in line with my own observations regarding the kind of

shift in metabolism that occurs in my hybridoma cultures in the presence of rapamycin, as well as those of Schreiber (Peterson, Desai et al. 1999; Schreiber 1999).

Thus, already knowing that metabolic phenomena are also associated with cell death (from Chapter 3 and Chapter 5), I cannot discount the possible connection between rapamycin's effects on metabolism and cell death. The possible enhancement of metabolic capacity may explain why rapamycin cultures outlive the control cultures during fed-batches where both exhibit growth inhibition and uniform cycle arrest.

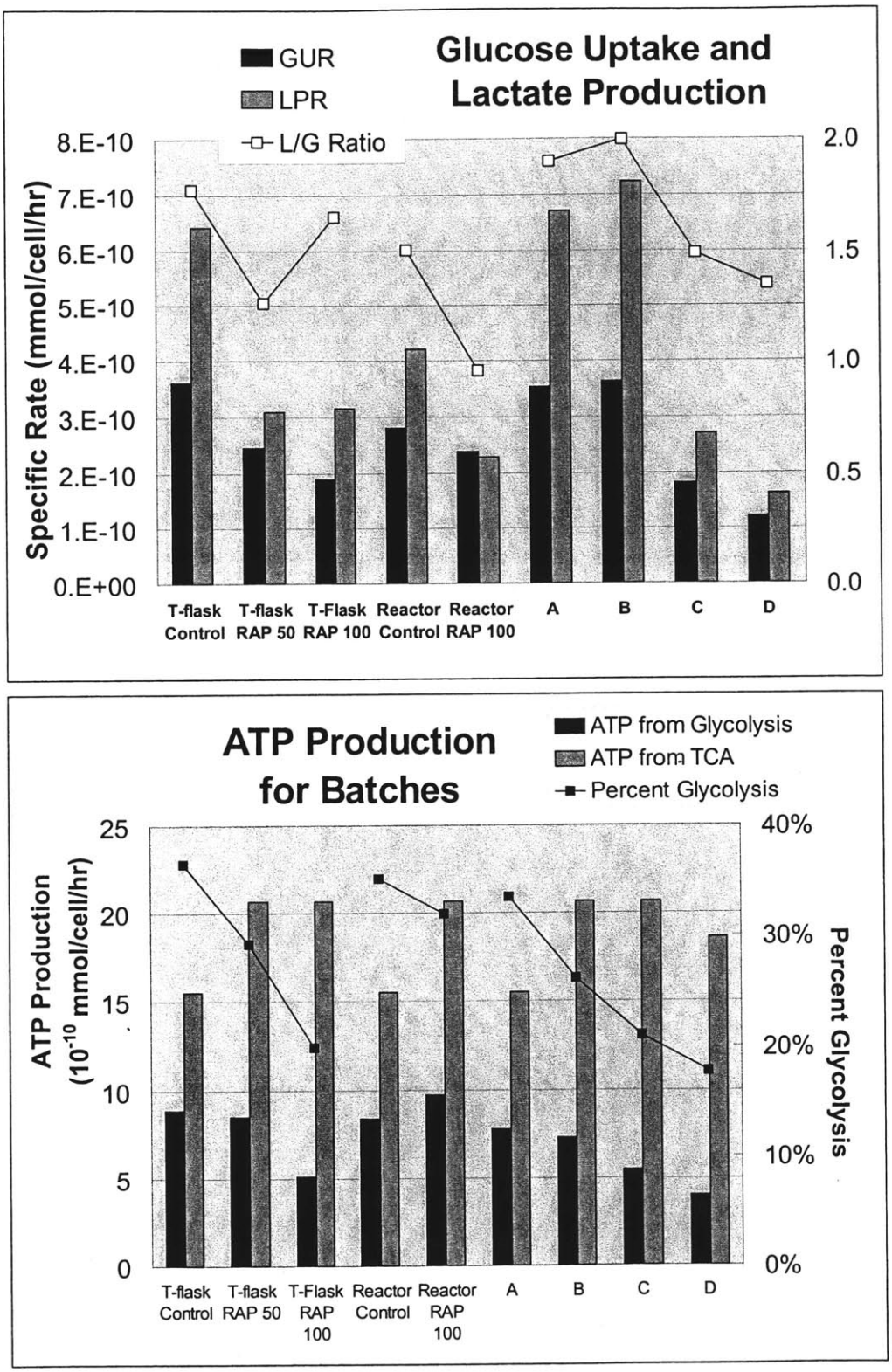



**Figure 4-9 a,b. Metabolism and ATP production for control and rapamycin batch cultures compared with chemostat data.**

A, B, C, and D refer to chemostat cultures at dilution rates of 0.04, 0.03, 0.02, and 0.01 $hr^{-1}$.

### 4.5.5 Beyond Hybridomas

The beneficial effects of rapamycin are strongly associated with controlling cell cycle entry. Understandably, this is an especially important effect for the hybridoma cell line that exhibits defective cell cycle entry. Possible applications of rapamycin for other cell lines, such as Chinese Hamster Ovary cell lines, which retain proper cell cycle control, are not futile, however. The growth stimulation of these cell lines by growth factors within serum or by insulin essentially stimulate cells to enter the cell cycle, even under conditions that could be sub-optimal. It already being demonstrated that such entry in and of itself is not sufficient for viable proliferation, it may be possible for rapamycin to provide a beneficial inhibition of cell cycle entry for cell lines which are being externally signaled to enter the cell cycle. Preliminary evidence with CHO cells within the Stephanopoulos group indicates rapamycin's possible anti-apoptosis effect, though it is harder to observe due to the greater degree of growth arrest that occurs.

## 4.6 CONCLUSIONS

The cell cycle arresting agent rapamycin is observed to have beneficial effects on hybridoma culture. At the optimum concentration of 50-100 nM, rapamycin mildly inhibits cell cycle entry resulting in a mild reduction in growth rate. In addition to changes in growth rate, cell cycle distribution, and metabolism, rapamycin also postpones the emergence of apoptotic cell death for one day, and reduces its extent thereafter. As a result, hybridoma cells grow with higher viability over the course of batch and fed-batch cultures, reaching higher peak viable cell densities and achieving greater integrated viable cell densities. Such improvements in cell density result in the doubling of monoclonal antibody product titer. The increase in productivity results from the higher IVCD's, as well as the greater apparent specific productivity for hybridoma cultures with a higher percent of cells in the G1-phase of the cell cycle.

Rapamycin's effect on culture physiology is partially understood in the context of its inhibition of the FRAP protein signaling pathway and the regulation of mRNA translation and ribosome function. Such effects are known to be associated with altering cell cycle control proteins, such as cyclins and cdk's. The link between rapamycin and the anti-

apoptosis effect observed in this work remains unclear. The complexity of the connection is due to the changes in growth rate, cell cycle distribution, and metabolism that occur simultaneously with apoptotic cell death.

## 4.7 ACKNOWLEDGEMENTS

I thank Steve Meier for lending his expertise on the best way to protect the cells in reactors when oxygen is delivered by sparging aeration, two undergraduates researchers, Ryan Gilbert (University of Michigan – Ann Arbor) and Jose Alemán (Cornell University), for their help with the rapamycin cultures, and Glen Paradis of the MIT Cancer Research Center for his help with the flow cytometric analysis of cell cycle. I acknowledge support from the National Science Foundation through Cooperative Agreement 9843342 to the Biotechnology Process Engineering Center.

# 5 THE PROTECTIVE EFFECT OF INSULIN ON THE VIABILITY OF HYBRIDOMA CELL CULTURES

## 5.1 ABSTRACT

Accumulated experience with the growth of the hybridoma cell line in serum free media with and without insulin points to the presence of other, as of yet undetermined, environmental factors which play a role in the proliferation and apoptosis of the hybridoma cells. Insulin, the only growth factor added to our serum-free medium, plays a dual role as a growth factor and also as protective factor against cell death. Nevertheless, it is found that cells can sometimes grow viably without insulin, albeit at a reduced growth rate, as long as the cells are passaged daily and the cell density remains low. At higher cell densities and with less frequent passaging, insulin is necessary to extend viable proliferation.

In the course of a long term study of the role of insulin for steady state hybridoma growth, we observed that cells were able to survive for many residence times despite complete insulin withdrawal in three of five insulin-free chemostat cultures. Two steady state cultures, one at a high (D=0.04 $hr^{-1}$) and another at a low (D=0.02 $hr^{-1}$) dilution rate, each exhibited metabolic changes, cell cycle arrest, and apoptotic cell death within 10 residence times (10-20 days) after removing insulin from the feed medium. However, sustained cell growth was observed in three chemostat cultures run at an intermediate dilution rate (D=0.03 $hr^{-1}$) for an additional 10 to 30 residence times beyond insulin withdrawal. The physiology of these cultures, in terms of cell density, growth and death rates, and metabolism, remained the same despite the absence of insulin.

These differing responses to the withdrawal of insulin were further investigated in batch experiments using (1) cells obtained from an insulin-free chemostat culture and (2) chemostat-conditioned medium from an insulin-free chemostat; each collected at the end of an insulin-free chemostat culture that survived without insulin. We observed that the chemostat cells grew significantly slower and began dying after 24 hours when cultivated in batches without insulin, hence reproducing the previously seen behavior of

stock cells. Yet, at the same time, we observed how batch cultures (of both chemostat cells and fresh stock cells) proliferated relatively faster and with high viability for two full days despite the lack of insulin when they were inoculated using 30% of chemostat-conditioned medium. Thus, it appears that the seemingly anomalous behavior of hybridoma cells in insulin-free chemostats is the result of differences in their environment rather than changes in the nature of the cells. The environment that allows for the proliferation of cells in the absence of insulin is observed to possess unknown factors that stimulate growth and lack unknown factors that stimulate death.

## 5.2 INTRODUCTION

In order to improve cell culture processes, be it by designing better a medium, choosing targets for genetic manipulation, or creating new cell lines, we need to understand the factors which affect the ability of cells to proliferate viably. One highly investigated and employed factor is insulin. Insulin has a positive effect on viable proliferation, through its known stimulation of the map kinase cascade and its various endpoints.

The adaptation of the hybridoma cell line to serum-free medium demonstrated the positive effects that insulin has on hybridoma growth rate and death rate. Chung, et. al. showed that insulin allowed the viable proliferation of the hybridoma following serum withdrawal (Chung, Sinskey et al. 1998). Cells given insulin grew faster and lived longer, with high viability up through two days. It is well known that insulin acts in a variety ways through signal transduction pathways to support metabolism and cell cycle function. From the chemostat experiments of Chapter 3, however, I know that insulin does not play a role in entry to the cell cycle: the c-Myc overexpression essentially short-circuits the effect of insulin in this regard. Nonetheless, insulin remains a necessary media component. It has a positive effect on cell physiology that allows cultures to proliferate faster and for longer times with high viability. Thus, in addition to being a "growth factor", insulin serves as a survival factor that protect cells from apoptotic cell death. The primary goal of this work is to better understand how insulin protects cells from apoptotic cell death.

While insulin has been shown to be very beneficial for growth of cultures, it has also at the same time, been implicated in stimulating cell death under suboptimal culture conditions. Sanfeliu, et. al., showed how serum-arrested CHO cells could be stimulated by insulin to grow viably for a few days, but that such stimulation by insulin eventually led to apoptotic cell death (Sanfeliu, Chung et al. 1999 in press). This behavior contrasted the behavior of cells grown with serum, which grew viably and then arrested viably once again when a limitation arose. Thus, though insulin is a very effective and necessary factor for promoting proliferation, by itself it cannot completely protect cells against cell death, and may even exacerbate cell death by "over-stimulating" entry to the cell cycle. Taking both Chung's and Sanfeliu's findings together, it appears that c-Myc stimulation and cell cycle entry is the real culprit for cell death, and that insulin confers some protection for cycling cells.

Understanding the key role insulin has in cell culture engineering technology, it was quite shocking to learn that insulin degrades very rapidly in batches, fed-batches, and chemostats. Work by Goswami verified that insulin was dramatically degraded during fed-batches of CHO cells (Goswami 1998). This was alarming in light of the reduction in glucose uptake and the cell cycle arrest in G1 (physiological parameters associated with insulin stimulation) that were observed to occur along with the emergence of cell death and insulin depletion. The degradation of insulin was attributed to proteases that are present in conditioned medium from CHO cell cultures. Goswami found that glycocholic acid, an amino peptidase inhibitor that prevents degradation from the N-terminus, was successful in minimizing the degradation of insulin in cell-free conditioned medium. The high degradation rate of insulin in cultures is also observed for hybridoma cells grown in batches, fed-batches, and steady state chemostats. From insulin measurements taken in chemostat cultures, the insulin degradation rate is observed to be a complex function of cell density, dilution rate, and insulin feed concentration (it is not simple first order decay). Despite the observed 50-fold reduction in insulin concentration from the feed medium to the reactor medium (100,000 mU/L down to approx. 2000 mU/L), the residual insulin concentration is apparently sufficient to maintain the steady states.

I investigated the use of glycocholic acid in conjunction with insulin feeding to maintain higher residual insulin concentrations during fed-batches of hybridoma cells. Though the

insulin feeding strategies were successful in keeping insulin levels higher than that of the control, the presence of additional insulin is shown not to influence the emergence of cell death. Thus, insulin is necessary to reach higher viable cell densities, but it is not sufficient to prevent the emergence of cell death.

To better understand insulin's role in protecting cells against apoptotic cell death, I investigated the effect of insulin withdrawal from hybridoma steady states containing insulin (100,000mU/L = 10 mg/L) by switching to an insulin-free feed. The physiology of the cultures was measured daily for at least 10 to 15 residence times (15 to 30 days), as insulin washed out exponentially. Initially, the goal of such experiments was to document the changes in metabolism, intracellular fluxes, and cell cycle distribution that occurred along with the emergence of cell death that was expected. While two experiments provided cultures which exhibited changes in metabolic and cell cycle distribution that were associated with apoptotic cell death, three of the experiments did not exhibit any observable physiological changes at all, even for many residence times beyond complete depletion of insulin. Thus, the goal shifted from studying the physiological changes associated with cell death due to insulin depletion, to probing the apparent lack of need for insulin altogether that was exhibited by the majority of the experiments.

Toward this new objective, I re-visited the role of insulin during batch cultivation. A series of batch experiments were performed to compare the insulin-dependent behavior of fresh stock cells and cells taken from the insulin-free reactors. Both types of cells behaved similarly, being able to grow viably *without* insulin when passaged daily and kept at low cell density, and needing insulin to proliferate viably to higher cell density during 2 days in batches. The major finding came from growing either type of cells in medium containing either 30% regular conditioned medium (from the inoculum) or 30% conditioned medium from the insulin-free reactors. Cells placed in the former and without insulin could only grow viably for 24 hours (normal behavior), but the cells grown with the chemostat-conditioned medium and without insulin grew viably for 48 hours, slower in growth than the insulin(+) case, but with high viability. Altogether, the evidence presented herein will show that the emergence of cell death for the hybridomas is dependent on the as of yet undetermined factors present in conditioned medium. Insulin

protects cells against cell death in more highly conditioned environments, but insulin is not necessary for survival when the environment is not as conditioned.

## 5.3 MATERIALS AND METHODS

### 5.3.1 Cell Line and Culture Medium

The cell line used was a murine hybridoma (ATCC CRL-1606), described in Chapter 3, and maintained in a similar manner. Inocula were passaged daily by subculturing the approximately 7.5e5 cell/ml cultures down to 2.5e5 cell/ml. The hybridomas were grown in glutamine-free IMDM basal medium, as described in Chapter 3. For some fed-batches, glycocholic acid was added to cultures at a concentration of 2.0 mM to minimize insulin degradation. Glycocholic acid (Sigma, #G7132) stock was prepared in MilliQ water at a concentration of 150 mg/mL and stored at 4°C.

### 5.3.2 T-Flask Batch and Fed-Batch Experiments

Batch and fed-batch experiments were conducted in the same manner as described in Chapter 4. Daily feeding of supplemental medium and insulin stock during fed-batches was accomplished as described in Chapter 4.

### 5.3.3 Bioreactor Operation

Five separate insulin-withdrawal experiments from steady state chemostat cultures were obtained using 2-liter and 3-liter Applikon reactors (Foster City, CA), at working volumes of 1.2 and 1.5 liters. The chemostats were run in the same manner described in Chapter 3, scaling liquid and gas flows accordingly for the different working volumes. Agitation for the 3-liter reactor was set at 175 rpm. Each experiment was inoculated at a density of 2e5 to 3e5 cell/ml from an inoculum culture at a density of approximately 8e5 cell/ml with high viability.

Insulin withdrawal was performed holding the dilution rate fixed. Experiments were performed at the following dilution rates (and in the given order): 0.04 $hr^{-1}$, 0.02 $hr^{-1}$, 0.03 $hr^{-1}$, and two simultaneously at 0.03 $hr^{-1}$. The first and the last 0.03 hr-1

experiments were eventually shifted to dilution rates of 0.02 hr-1 and 0.04 hr-1, but only after insulin was fully depleted. The last three runs were inoculated with 2.0-mM glutamine in the batch reactor, without any detrimental effects, to facilitate startup and the reaching of a first steady state. The last two runs were also immediately started with lower insulin concentration in the feed medium (5100 and 510 mU/L respectively) to expedite the insulin washout, and, furthermore, insulin was reduced stepwise, rather than all at once.

## 5.3.4 Analytical Methods

### 5.3.4.1 Cell Number, Viability, and Apoptosis

Cell number and viability were determined by cell counts using a hemacytometer with trypan blue dye exclusion. One to two slides were counted for batch and fed-batch cultures. Three slides were counted for bioreactor cultures.

For bioreactor cultures, the extent of apoptosis was determined by the acridine orange / ethidium bromide (AO/EB) assay using a hemacytometer (Mercille and Massie 1994). Apoptosis cell counts consisted of counting 200 cells for 6 separate hemacytometer chambers, for a total of 1200 cells. Such cell counts were used to calculate the observed percentage of cells that were viable, apoptotic (early and late), or necrotic. The cell death in batches and fed-batches was presumed to be due to apoptosis given the overwhelming evidence and experience that apoptosis is the dominant mode of death under the conditions studied. This assumption was periodically verified by an AO/EB count.

### 5.3.4.2 Metabolite Concentrations

Samples for metabolite concentration analysis were taken and stored as discussed in Chapter 3. Full metabolite analysis was performed for the first three insulin-withdrawal experiments. Analysis of samples from batch, fed-batch, and the last two insulin-withdrawal experiments consisted of glucose (Sigma protocol 16-UV), lactate (Sigma protocol 826-UV), and ammonia (Sigma protocol 171-UV) assays, as needed.

### 5.3.4.3 Calculation of Specific Uptake and Production Rates

The steady state metabolic production or uptake rates were determined for bioreactor experiments as described in Chapter 3:

Metabolic production or uptake rates for batches, fed-batches, and dynamic chemostats were determined as described in Chapter 4.

#### 5.3.4.4 Oxygen Uptake Rate

During bioreactor experiments, the oxygen uptake rate (OUR) was obtained as described in Chapter 3.

#### 5.3.4.5 Cell Cycle Distribution

Cell cycle distribution was determined by the method described in Chapter 3. Single measurements were performed for batch and fed-batch experiments, whereas bioreactor measurements were performed in duplicate.

## 5.4 RESULTS

### 5.4.1 Insulin Degradation

Accepting the presence of insulin as a necessary prerequisite for extending viable proliferation in the hybridoma cell line, I investigated daily feeding of insulin as well as the addition of glycocholic acid, a protease inhibitor, to help combat the near complete degradation of insulin observed during batch and fed-batch experiments. Goswami determined glycocholic acid (10 mM) to be the protease inhibitor that best minimized insulin degradation in highly conditioned, cell-free medium samples at 37°C for 10 hours (Goswami 1998). To make use of this finding for my batches and fed-batches, I needed to determine if glycocholic acid minimized insulin degradation for hybridoma-conditioned medium and at what concentration glycocholic acid could be used without being toxic to a growing hybridoma culture.

Both these questions were answered in a series of five hybridoma batches given 0 mM (control), 0.3 mM, 1.0 mM, 3.0 mM, or 10.0 mM glycocholic acid [hereafter abbreviated as GA]. Starting with 100,000-mU/L insulin, it is evident that glycocholic acid minimized degradation over the 40 hours observed (Figure 5-1). The protection against insulin

degradation appears to linear with respect to the log concentration of glycocholic acid. The culture without any GA showed about 75% degradation, whereas the culture with 10.0-mM GA showed only 23%. In terms of toxicity however, it appears that the culture with 10.0 mM was severely inhibited by the presence of GA at that concentration: cells did not grow and were even found to be fewer in number than had been inoculated. Cells at lower concentrations of GA faired better, exhibiting normal growth with high viability. Thus, the optimum level of GA for hybridoma cultures was determined to be 2.0 mM, where cell growth was not apparently impacted and insulin degradation was reduced to 40% vs. 75% for the control over a forty-hour period.

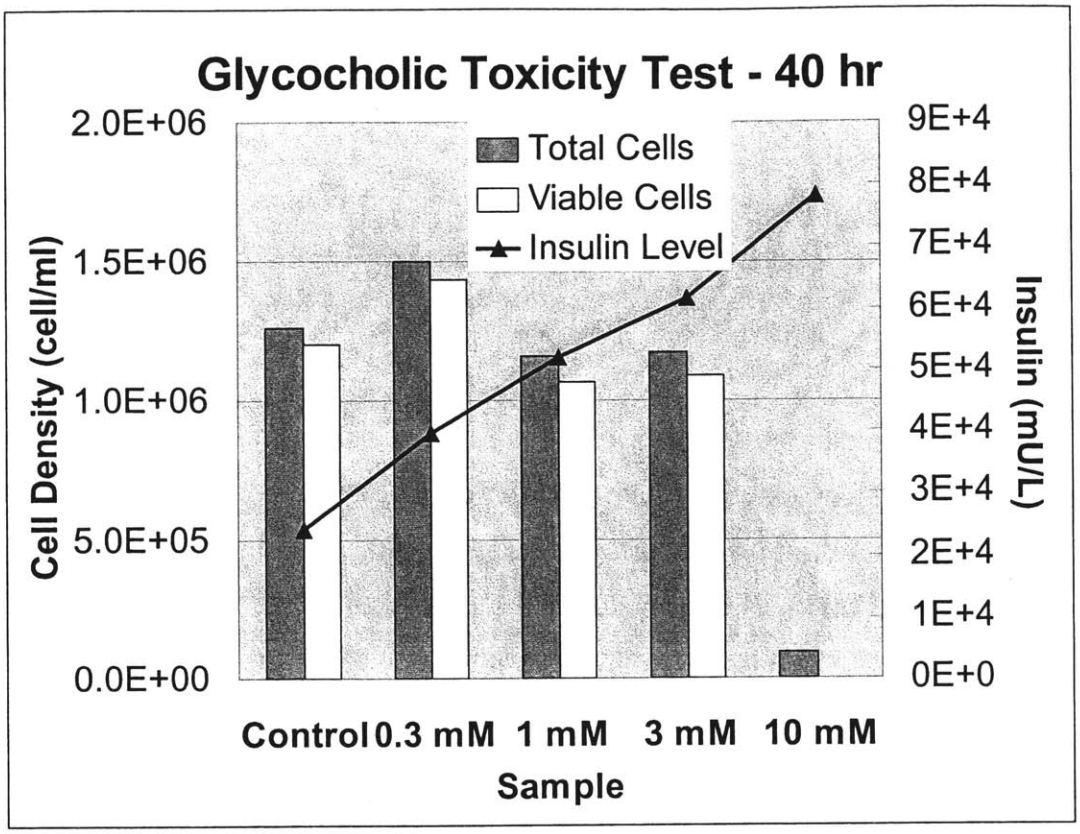



**Figure 5-1. Effect of glycocholic acid on hybridoma proliferation and insulin degradation.**

The impact of insulin feeding and glycocholic addition on the emergence of cell death was investigated in a series of three fed-batch hybridoma cultivations. All three experiments were begun with the full amount of insulin (100,000 mU/L). The control did not receive any more insulin or any GA. The second culture was re-fed another full amount of insulin daily beginning on the second day. The third culture was also re-fed insulin in the same manner as the second culture, but was also given a one time shot of 2.0 mM GA at the beginning.

In terms of cell densities, the three cultures behave nearly identically for the first two days, each growing quickly and with high viability (Figure 5-2 a). The plot of insulin concentration shows that the amount of insulin left after 48 hours (before any insulin is re-fed) is roughly 18,000-19,000 mU/L for the control and the "insulin-fed" culture, and about 28,000 mU/L for the culture with GA (Figure 5-2 b). Yet after the second day, and despite feeding insulin to the "insulin" and "GA" cultures, death emerges within all the cultures, although at varying degrees. On the third day, insulin concentration is 3000 mU/L for the control, and greater than 20,000 for both the "Insulin" and the "GA" cultures. Thus, it appears that the higher insulin levels between day two and three do not prevent the emergence of cell death for the hybridoma cells. The control and the "insulin" culture behave very similarly, and ironically, due to normal variation or for unknown reasons, the "GA" culture expresses the most death. Furthermore, as is evident in the chemostat experiments, insulin levels of the order of 2000 are sufficient for steady state growth. Thus, the emergence of cell death in these experiments must be attributed to other factors besides insulin concentration. Insulin is necessary for viable proliferation up through approx. 50 hours (see following sections), yet its presence does not protect the culture from death beyond 50 hours.

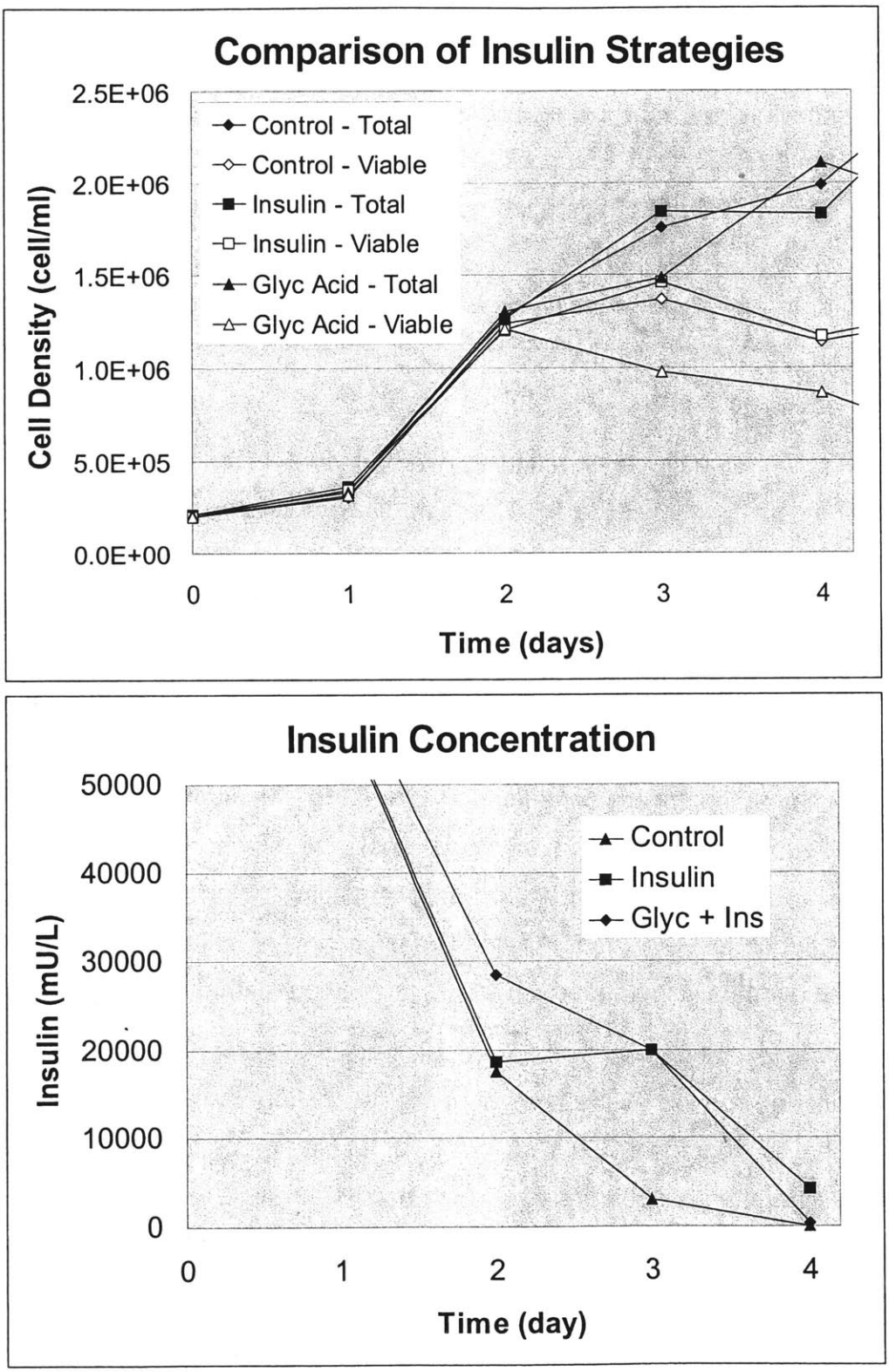



**Figure 5-2 a,b. Effect of insulin concentration on the emergence of cell death.**

Note: values for insulin concentration for "Insulin" and "Glyc + Ins" on day three were above detection limit – they are greater than 20,000 mU/L on that day.

### 5.4.2 Withdrawal of Insulin from Steady State Chemostat Cultures

To investigate the role that insulin plays as a growth factor and a protective factor, I measured the physiology of continuous hybridoma cultures after switching to an insulin-free feed medium. A total of five such experiments were conducted, at dilution rates of 0.04 $hr^{-1}$, 0.02 $hr^{-1}$, and 0.03 $hr^{-1}$ in triplicate. The physiology of each culture was measured in terms of cell density, degree of apoptosis, cell cycle distribution, and in some cases, metabolism for glucose and glutamine, production of lactate, and uptake of oxygen.

Two of the five steady states cultures were unable to continue proliferating upon removal of insulin (Figure 5-3 a b). The high dilution culture (performed first) exhibited cell cycle arrest, apoptotic cell death, and a shift in metabolism within 10 residence times (10 days) of removing insulin from the feed. The low dilution rate culture (performed second) likewise exhibited its dependence on insulin within 10-15 residence times (20-30 days) after removing insulin from the feed. The next culture, however, run at D=0.03 $hr^{-1}$ (#1), survived unchanged for 20 residence times. An additional two insulin withdrawal experiments were conducted at D=0.03 $hr^{-1}$, this time starting out with lower insulin feed concentrations and lowering insulin in steps. Both such experiments reproduced the previous behavior seen for D=0.03 $hr^{-1}$, this time surviving for greater than 20 residence times without insulin. The dilution rate for the experiment at D=0.03 $hr^{-1}$ (#3) was eventually increased to D=0.04 $hr^{-1}$ without detriment to the culture.

Plots of viable, apoptotic, and necrotic cells determined from the acridine orange/ethidium bromide assay show that the two cultures that exhibited physiological changes died by apoptosis (Figure 5-4 a b c). The emergence of apoptosis coincides with the decline of viable cell density, which begins at day 9 for D=0.04 and day 21 for D=0.02. The three experiments that survived insulin withdrawal do not show any changes with respect to apoptosis. Experiment D=0.03 #3 showed lesser apoptosis when its dilution rate was increased to 0.04, a normal response.

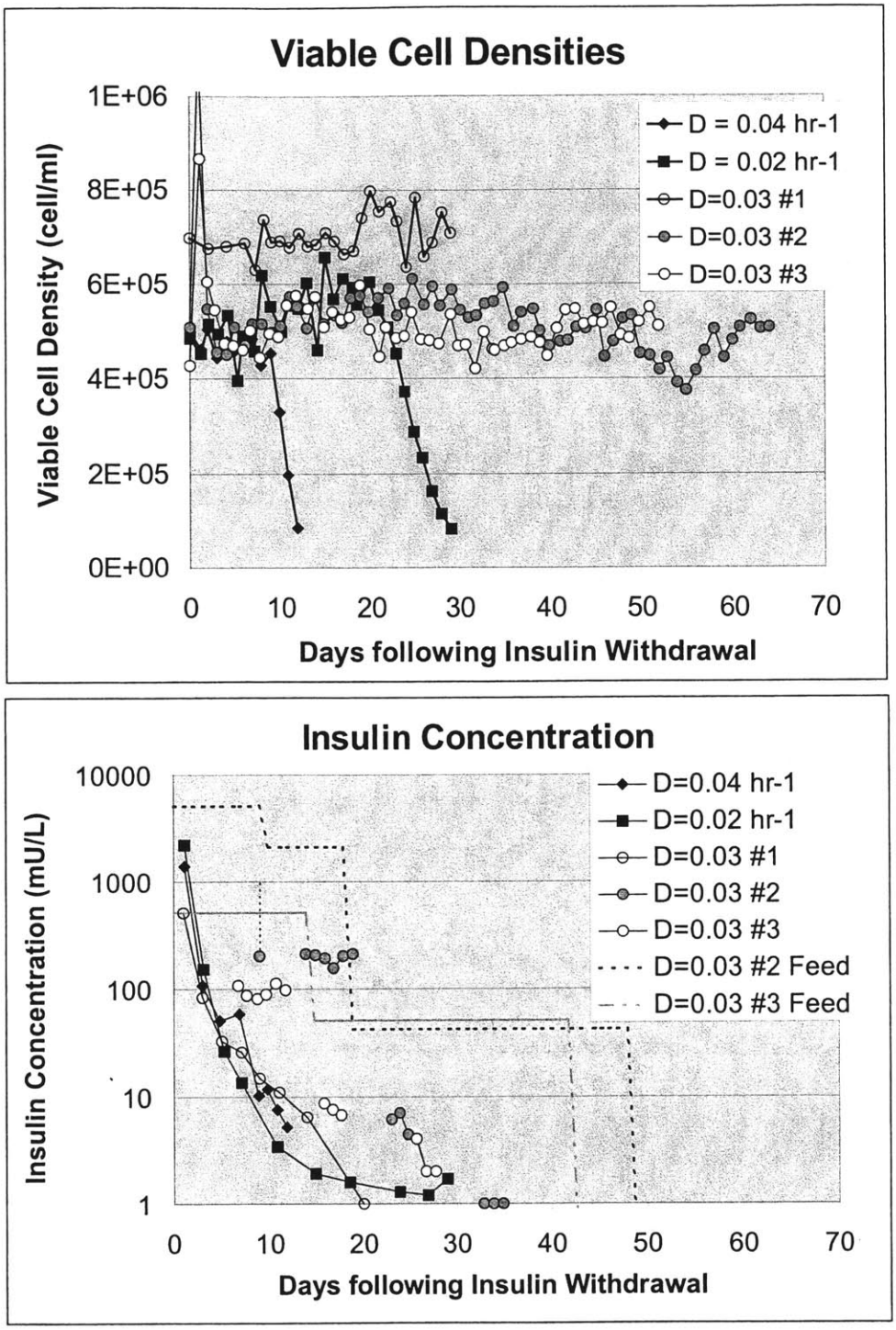



**Figure 5-3 a,b. Viable cell density and residual insulin concentration during the withdrawal of insulin from steady state hybridoma chemostats.**

Insulin feed concentration was switched from 100,000 mU/L to zero in one step for D=0.04, D=0.02, and D=0.03 #1 at day 0. Insulin feed for D=0.03 #2 was started at the lower level of 5100 mU/L, and was reduced 2100, then 42, and finally zero. Insulin feed concentration for D=0.03 #3 was initially 510 mU/L and was reduced to 51 and then zero.

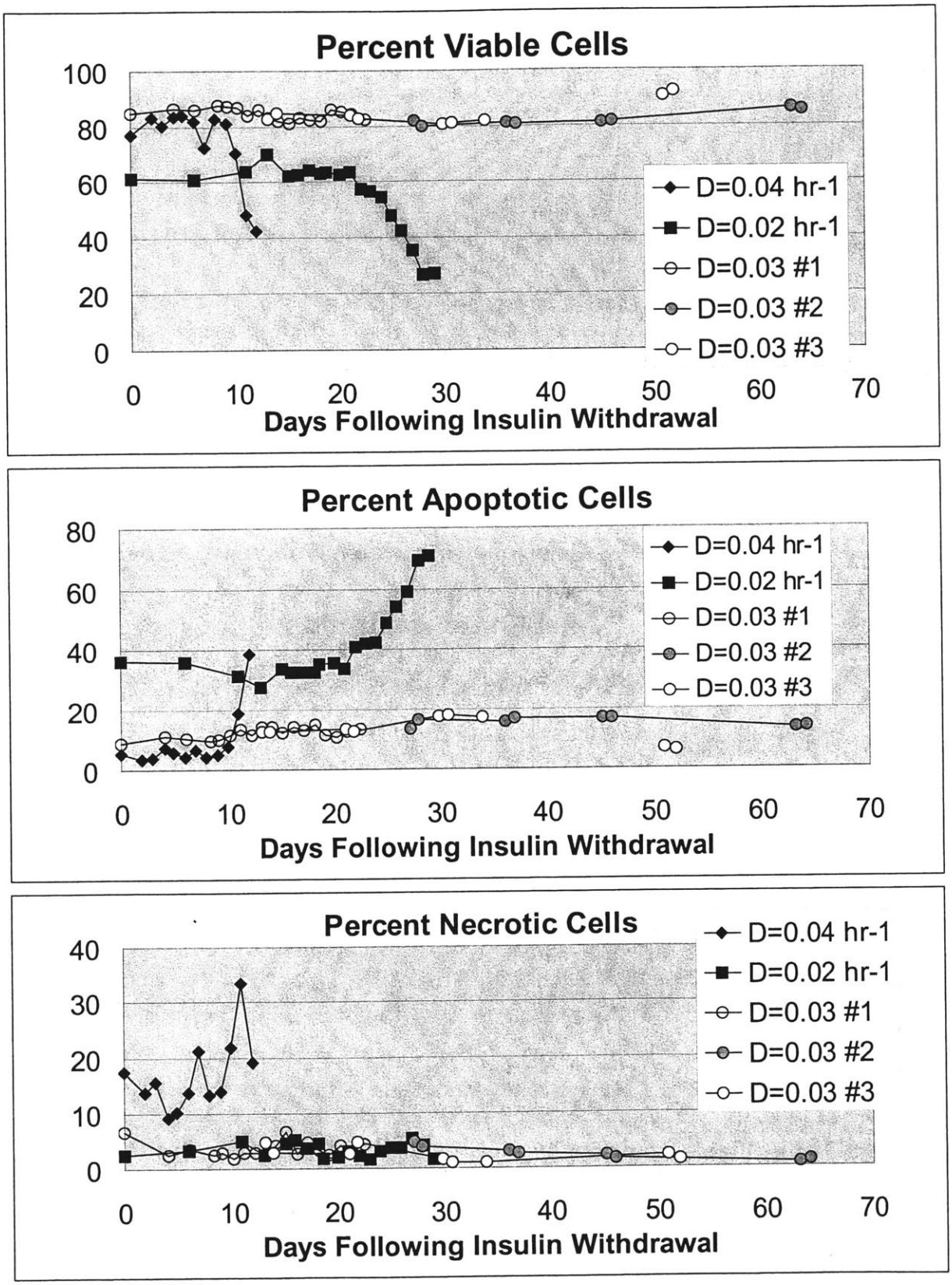
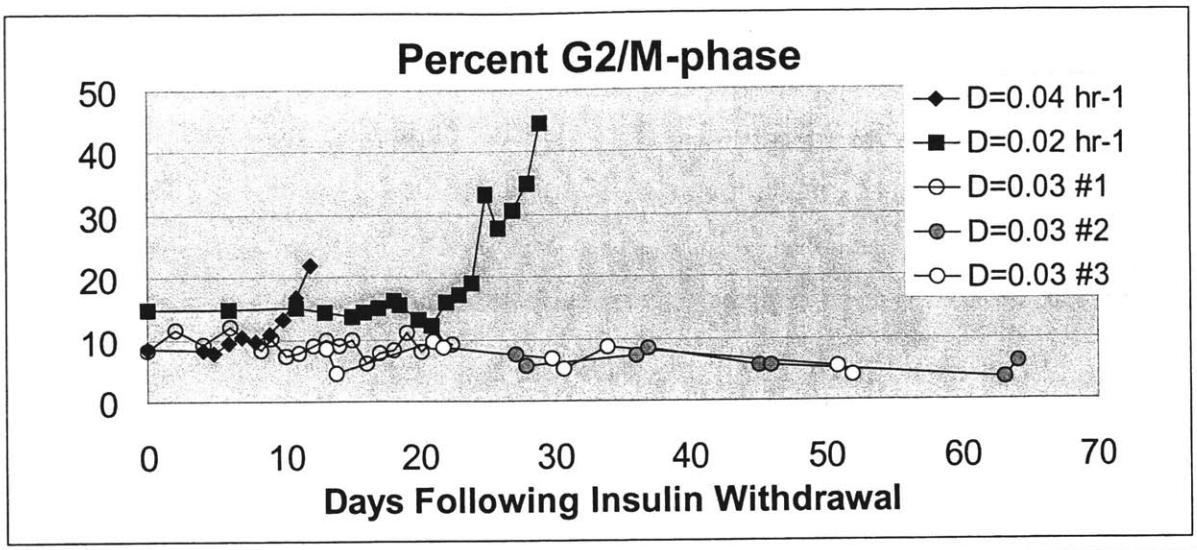
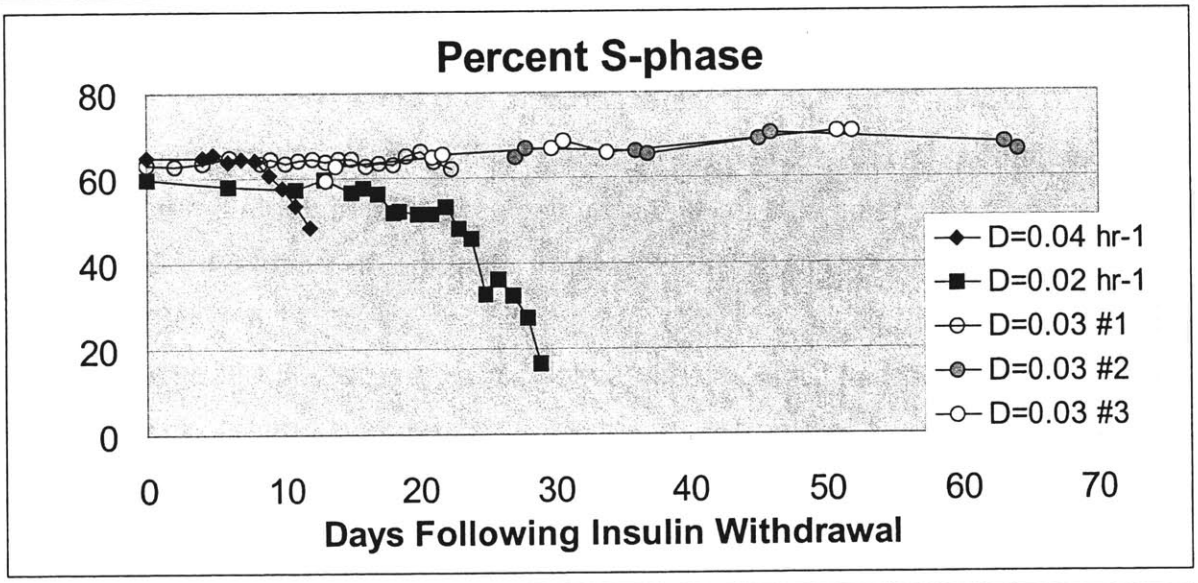



**Figure 5-4 a,b,c. Viable, apoptotic, and necrotic cell populations during the withdrawal of insulin from steady state hybridoma chemostats.**

The dominant change in cell cycle distribution that is associated with the growth arrest and apoptosis seen in experiments at D=0.04 and D=0.02 is an arrest in the G2/M-phase of the cell cycle (Figure 5-5 a b c). The beginning of arrest coincides with apoptosis and occurs at insulin levels of 10 mU/L for D=0.04 and 2-3 mU/L for D=0.02. Both of these cultures also exhibit mild arrestment in the G1-phase. The other experiments show no such changes in cell cycle distribution.

In terms of cell cycle progression times (Figure 5-6 a b), both of the experiments that failed to maintain their steady state show increases in the progression times for G1 and G2/M. These increases in cell cycle progression upon insulin withdrawal are reminiscent to those seen during fed-batch cultures (Chapter 4). Compared to the relative changes in the other progression times, the S-phase does not appear to be as affected, only increasing 50% for the experiment at D=0.04 and actually decreasing for the experiment at D=0.02. Changes in progression times for the experiment at D=0.04 start close to the same time that cell density begins to fall and death emerges. However, for the experiment of D=0.02, it is evident that the increases in G1 and G2/M progression times begin much earlier. Progression times for G1 and G2/M are initially 4.3 and 4.8 at for the insulin(+) steady state (day zero) (data not shown), but steadily increase to 6.6 and 5.7 by day 18 (shown below), three days prior to culture arrest and cell death. Then they increase further thereafter.



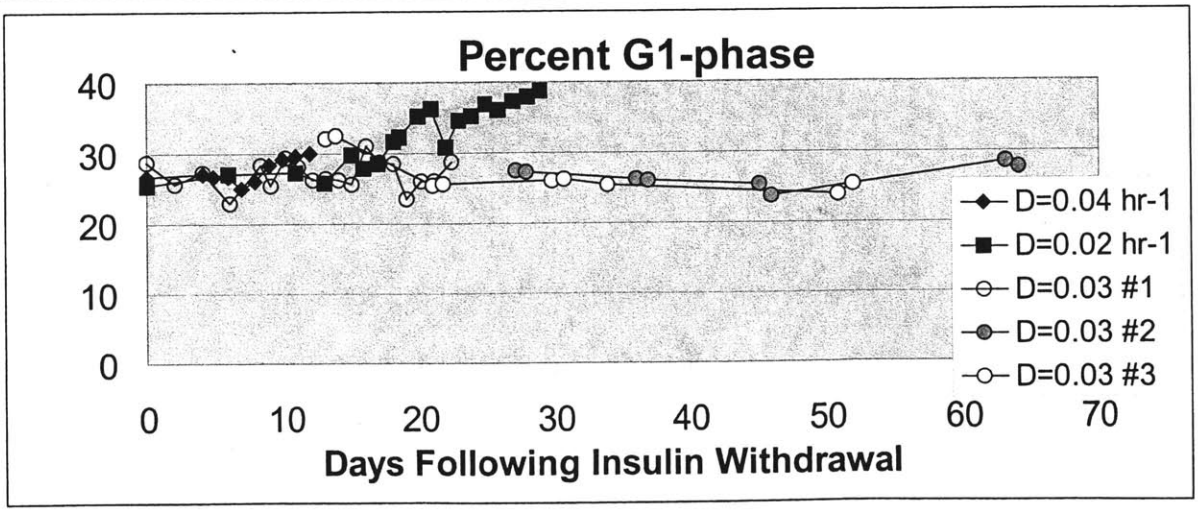




**Figure 5-5 a,b,c. Cell cycle distributions among G1, S, and G2/M during the withdrawal of insulin from steady state hybridoma chemostats.**

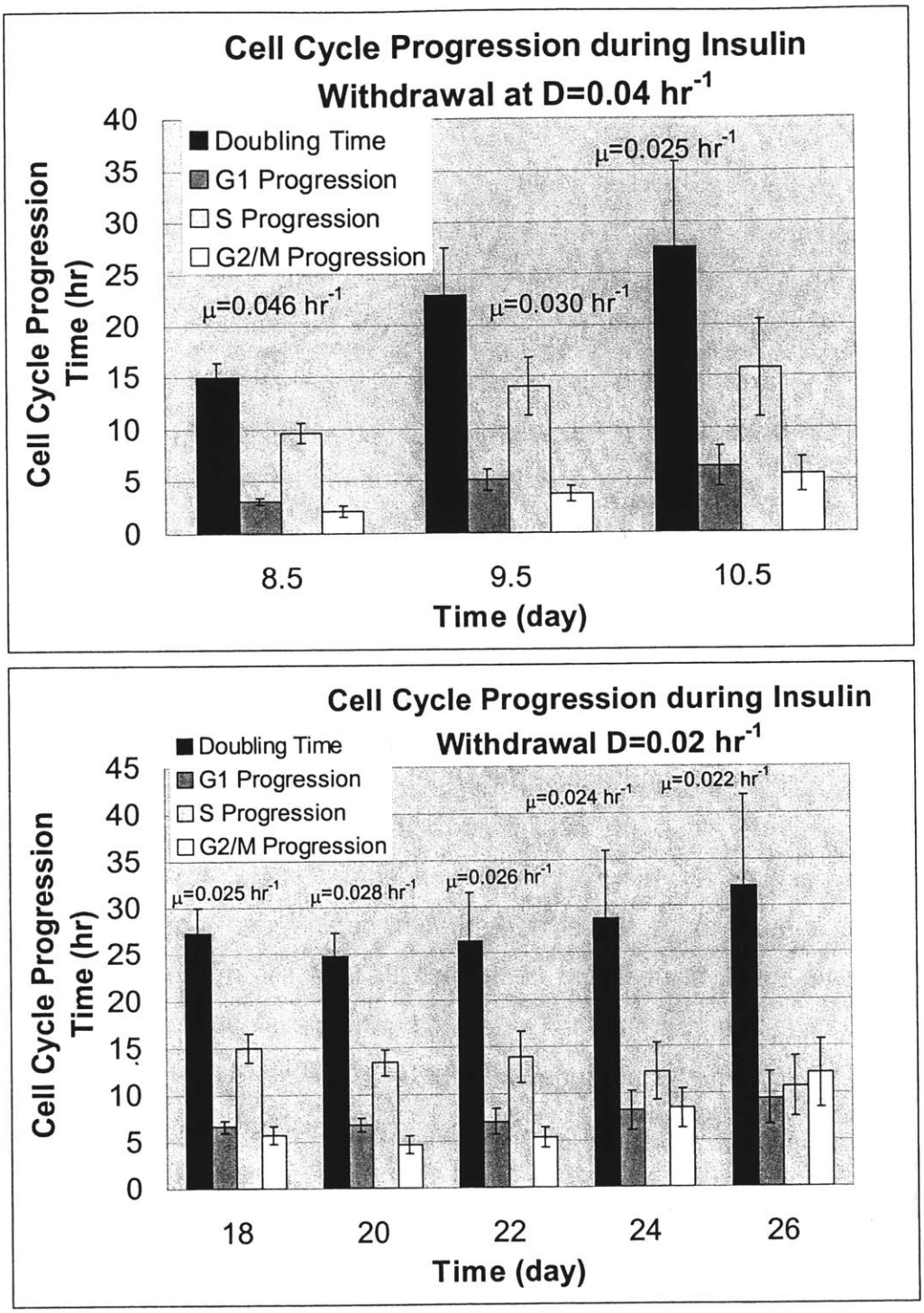



**Figure 5-6 a,b. Cell cycle progression times during the withdrawal of insulin from steady state hybridoma chemostats.**

Changes in metabolism only occur for the two cultures that eventually died without insulin. Both cultures show increases in glucose uptake and lactate production as insulin washes away (Figure 5-7 a b c). These shifts appear to coincide with the other physiological changes. Changes in glutamine uptake and oxygen uptake also occur, yet apparently beginning up to 5-10 days prior to the changes in cell density, apoptosis, and cell cycle distribution (which begin to occur at day 9 for D=0.04 and day 21 for D=0.02) (Figure 5-8 a b). Inferences about the role of insulin in metabolism are to be made with caution. Not only are the cells dying (which has been seen to be associated with an increase in TCA cycle activity) but the viable cell number is also declining, and hence the residual nutritional supply is increasing and therefore relaxing the nutrient limitation. Still, the trends observed for metabolism are reasonable. For D=0.04, we see a slight increase in glutamine and oxygen consumption prior to cell death, which may perhaps reflect cells' response to the loss of metabolic support from insulin (i.e. less efficiency without insulin). For D=0.02 I observe the reverse trend – glutamine and oxygen consumption drop slightly prior to cell death. Here, for cells already in a much more constrained environment (and hence presumably already depending on more oxidative phosphorylation), the loss of insulin perhaps limits the cells' ability to consume glutamine and oxygen for energy. For both experiments, glutamine uptake and oxygen consumption appear to increase once death begins and nutrients become more available as the result of declining cell density. Distinct from the two chemostats that could not survive, metabolism for D=0.03 #1 remains steady, as did the rest of its physiology. Metabolism was not measured for D=0.03 #2 and D=0.03 #3.

Thus, continuous cultures at dilution rates of 0.04 hr-1 and 0.02 hr-1 were unable to maintain their steady states due to metabolic changes and cell cycle arrest. The enigma surrounding the insulin-free chemostat cultures is that those grown at an intermediate dilution rate (D=0.03 hr-1) were able to survive for many residence times beyond the point at which insulin was completely washed away.

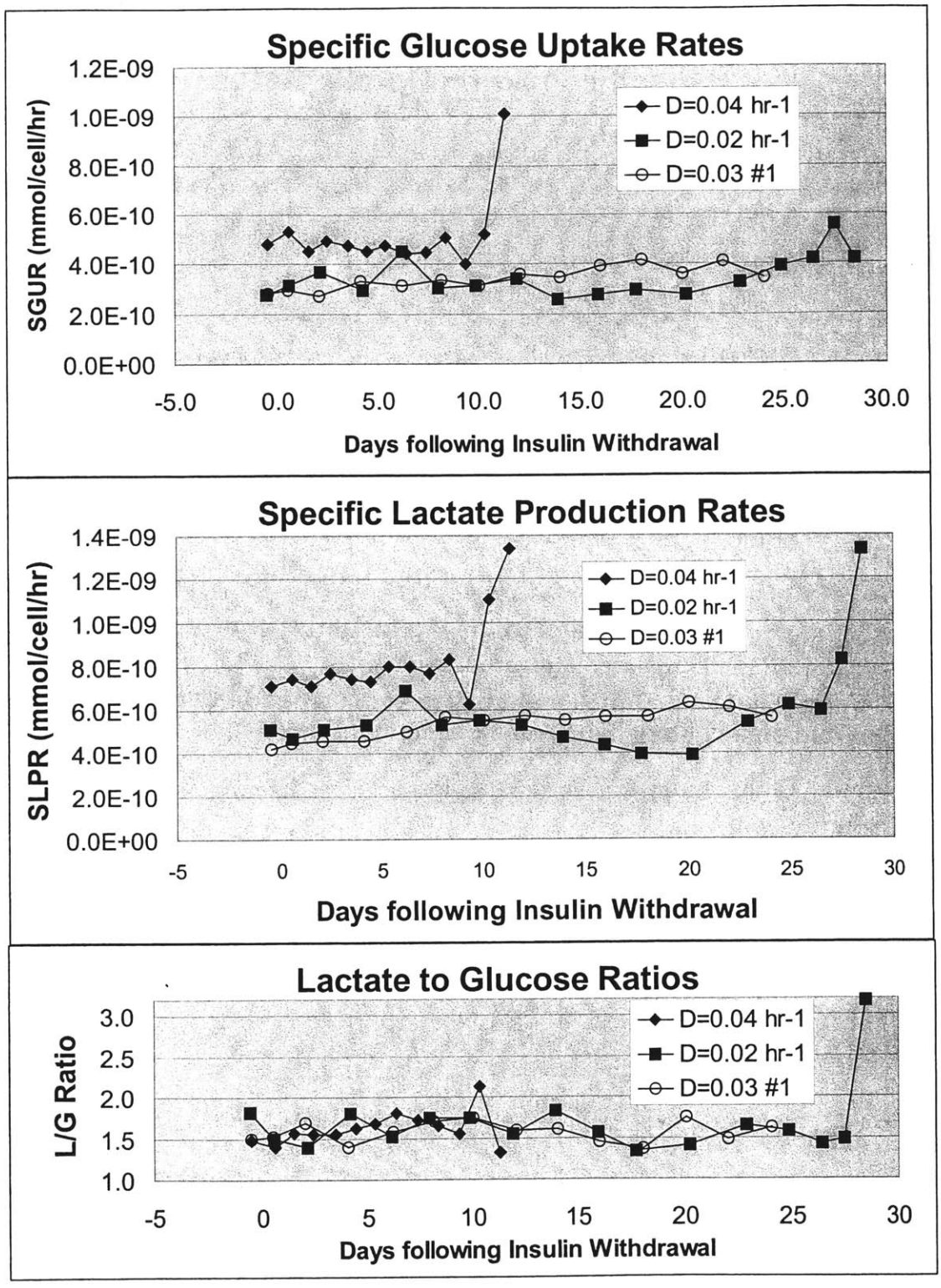



**Figure 5-7 a,b,c. Glucose uptake, lactate production, and L/G ratio during the withdrawal of insulin from steady state hybridoma chemostats.**

Note: The final data points of each series are less reliable due to the transient nature of the culture and the decreasing viable cell number.

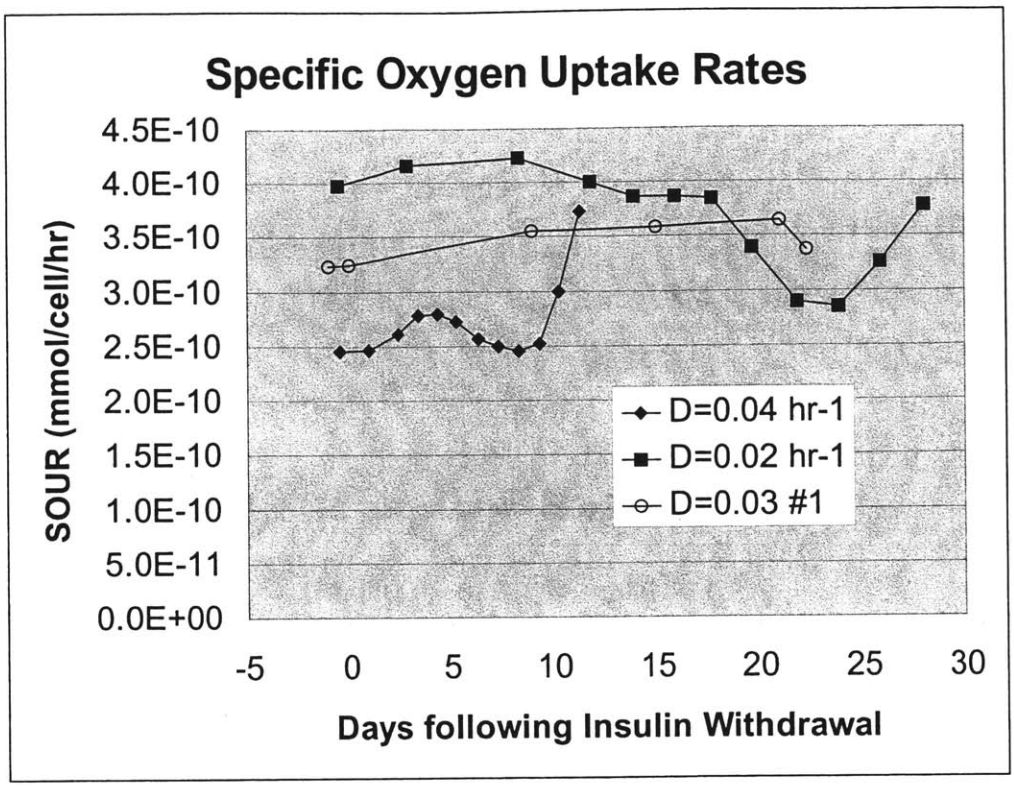



**Figure 5-8 a,b. Glutamine uptake and oxygen consumption during the withdrawal of insulin from steady state hybridoma chemostats.**

### 5.4.3 Insulin(-) Chemostat Cells

To further investigate the ability of cells to grow viably in the absence of insulin, cells frozen from the end of chemostat experiments D=0.03 #2 and D=0.03 #3 were defrosted and passaged daily in 100% fresh insulin(-) medium and 100% fresh insulin(+) medium. The goal of such experiments was to see if the cells were permanently adapted to an insulin-free medium. For simplicity, results are only presented for chemostat cells from D=0.03 #2 - chemostat cells from D=0.03 #3 behaved similarly during all the experiments. As controls, a fresh stock of cells were also defrosted and passaged daily in the same insulin(-) and insulin(+) media.

As shown below, both the chemostat cells and the fresh stock cells could be defrosted and passaged to high viability regardless of the presence of insulin (Figure 5-9 a b). Insulin(+) cultures grew faster than the insulin(-) ones, but both types of culture reached greater than 90% viability after several days.

Having essentially obtained healthy insulin-free inocula, the chemostat cells and the fresh stock cells were seeded into insulin-free batches and allowed to grow for 48 hours (Figure 5-10). Each culture was seeded at 2.0e5 viable cells/ml using roughly 30% of its own conditioned medium (*lightly* conditioned for 24 hours at an average viable cell density of 4-5e5 cell/ml). Again both cultures performed well despite the absence of insulin. The chemostat cells and the stock cells without insulin grew more slowly than the insulin(+) control, yet they maintained high viability for 48 hours. Such viable growth of the stock cells was thus far an apparent contradiction to the earlier work that showed cells dying within 48 hours (Chung, Sinskey et al. 1998).

The contradiction was resolved when I repeated the batch experiments, this time using *more highly* conditioned medium for the inoculation of the flasks. The more highly conditioned medium used was that from the insulin-positive control of the previous experiment, which had supported cell densities up to $1.4 \times 10^6$ cell/ml (it was conditioned for 48 hours by an average of 8e5 cell/ml). Each new experiment was seeded into 30% of more highly conditioned medium and either 70% insulin(-) fresh medium or insulin(+) fresh medium. It was now that I observed that the chemostat cells still required insulin for viable proliferation up through 50 hours in batches (Figure 5-11 a

b). Moreover, the same behavior was observed for the stock cells seeded under similar conditions. Stock and chemostat cells grew at quite different rates, but their dependence on insulin was shared.

Overall, the dependence on insulin for viable proliferation was apparent only in an environment that contained more highly conditioned medium. These batch experiments demonstrate that it was the chemostat environment that was dictating the behavior of the cells, and not any permanent changes in gene expression or protein control networks of the cells themselves.

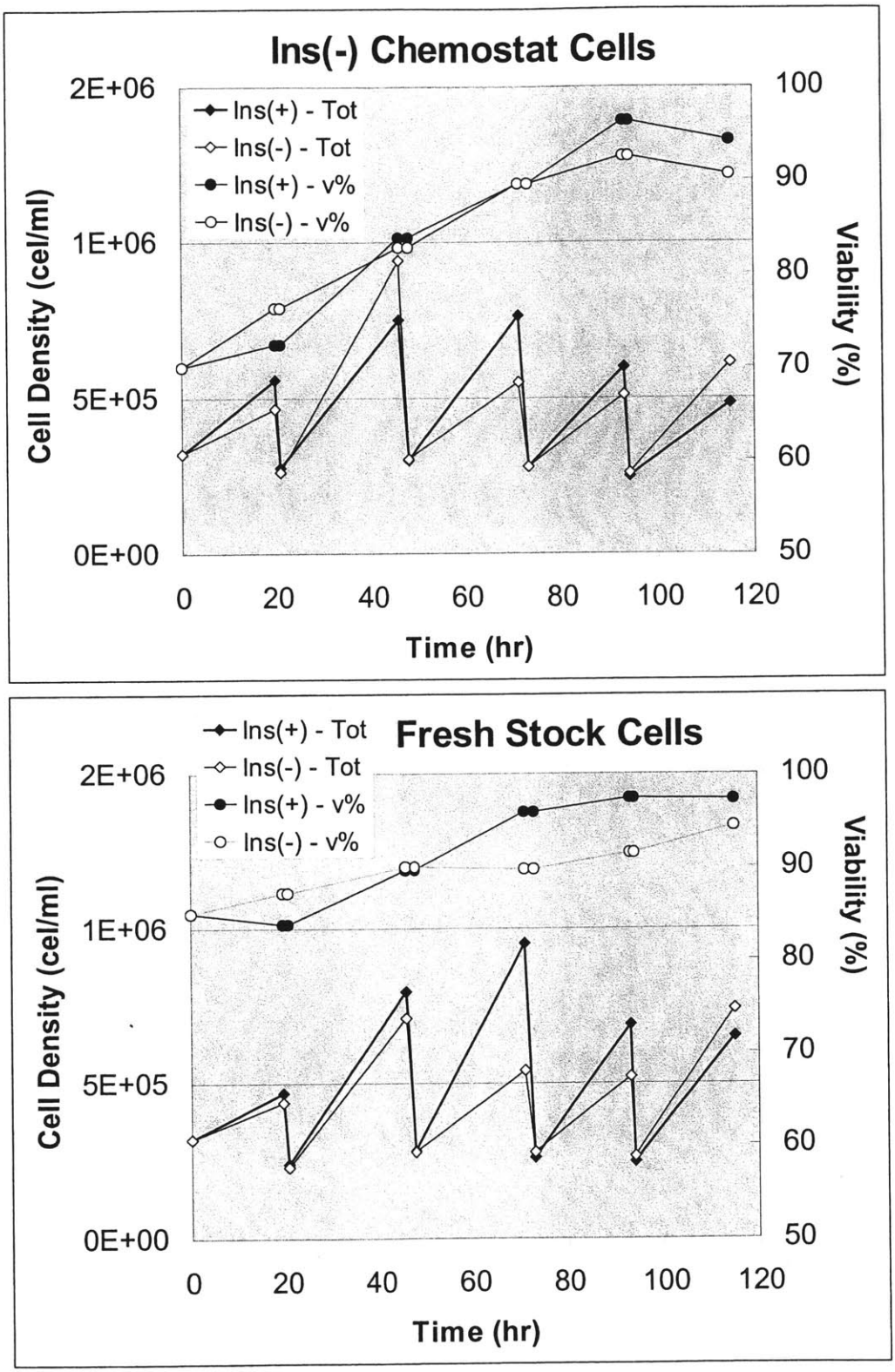



**Figure 5-9 a,b. Passaging of Insulin(-) Chemostat cells and fresh stock cells in insulin(-) and insulin(+) medium.**

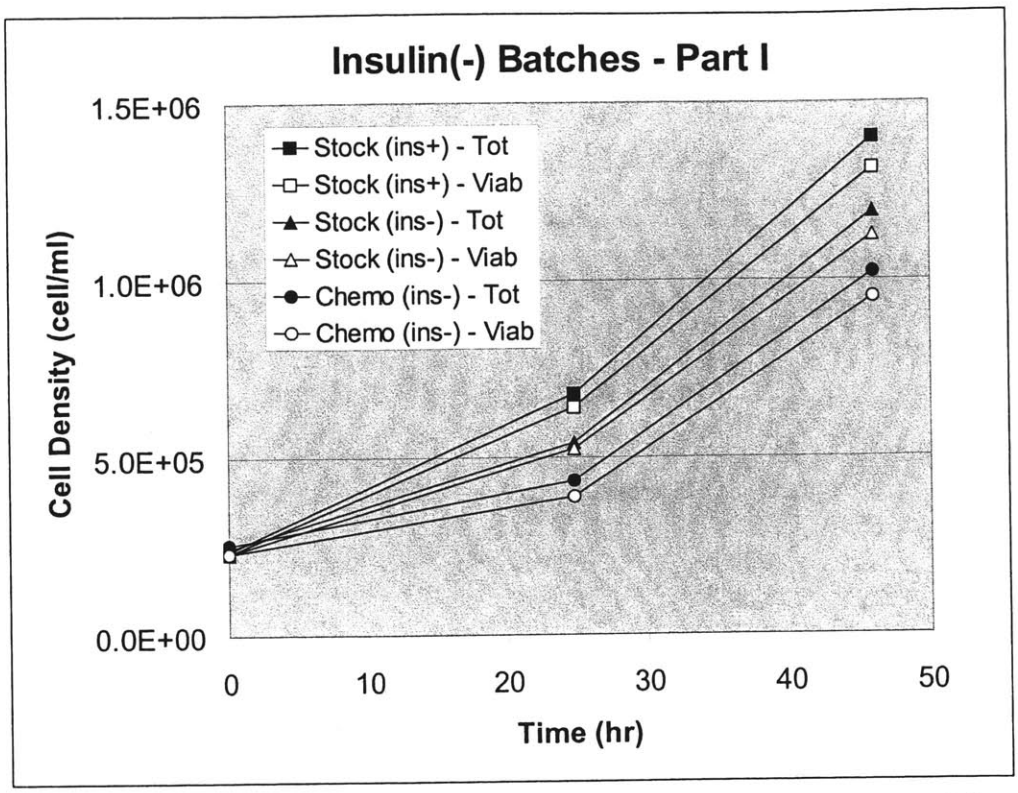



**Figure 5-10. Insulin(-) batch cultures of insulin(-)-chemostat cells and fresh stock cells**

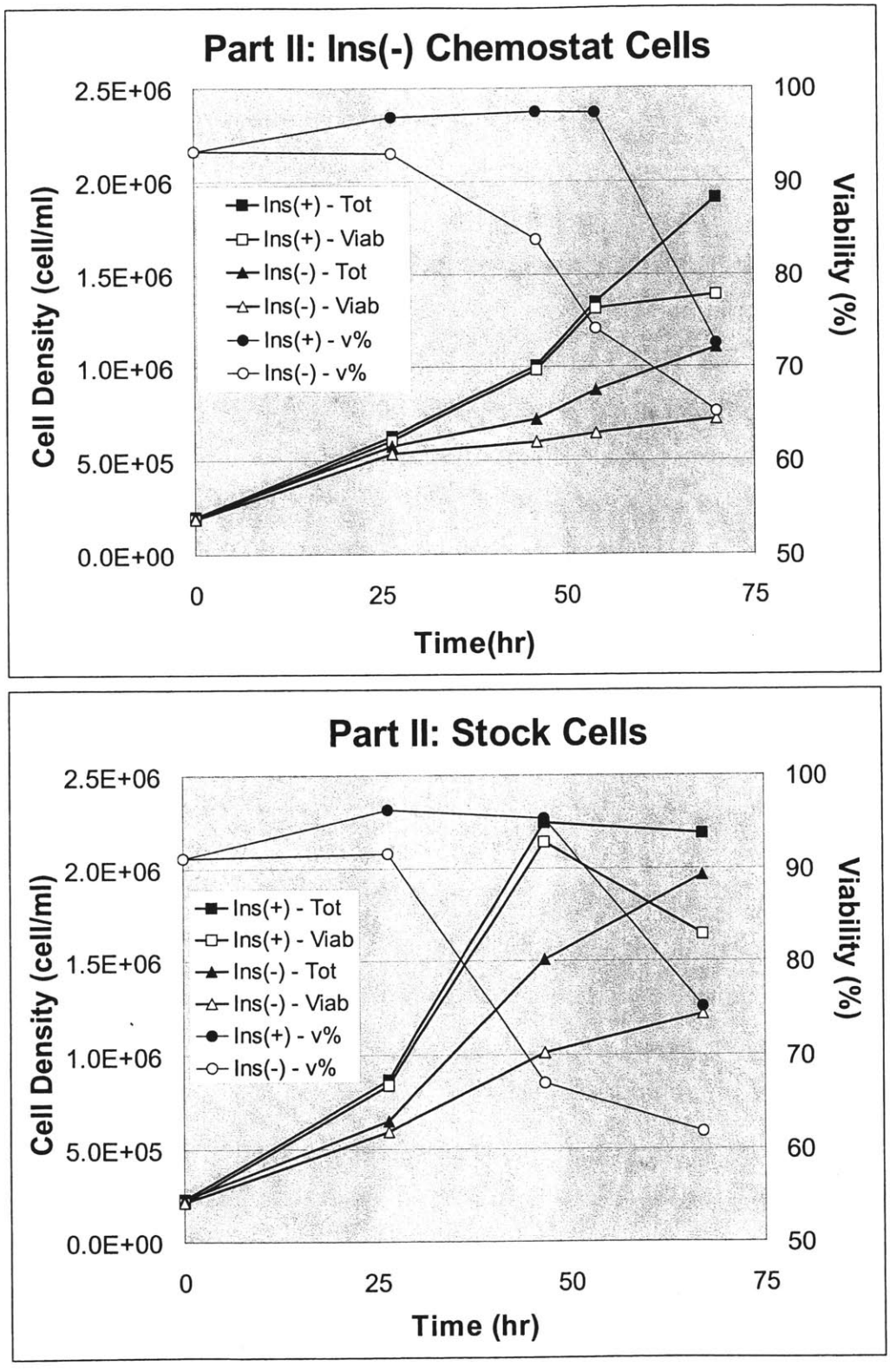



**Figure 5-11 a,b. Insulin(+/-) batch cultures of insulin(-)-chemostat cells and fresh stock cells**

### 5.4.4 Insulin(-) Chemostat-Conditioned Medium

I next investigated the effect of chemostat-conditioned medium that was stored as cell-free supernatant from the end of the D=0.03 #2 experiment. This special conditioned medium was used to seed a batch of fresh stock cells. Fresh stock cells, which were cultivated in insulin(+) medium, were seeded into insulin(-) medium, using either 30% of regular conditioned medium (from the inoculum) or 30% of chemostat-conditioned medium. Cells were also seeded into insulin(+) medium with regular conditioned medium for the control. The cells with regular conditioned medium grew slower and died earlier than the insulin positive control (Figure 5-12 a b). Interestingly, the stock cells with chemostat-conditioned medium, though growing a little slower than the control, proliferated with high viability for two days, and then even outperformed the control on the last day in terms of viable cell density.

To see if the robustness of the cells grown in the special chemostat-conditioned medium was due to the absence or presence of unidentified conditioned medium factors, stock cells were also grown with or without regular conditioned medium. Cultures containing insulin grew quickly and viably for 48 hours regardless of the type or the absence of conditioned medium (data not shown). Growth and death for cultures without insulin did depend on the conditioned medium used (Figure 5-13 a b). Cells seeded into insulin-free medium consisting of 30% regular conditioned medium faired the worst, while cells seeded into insulin-free medium consisting of 30% chemostat conditioned medium faired the best. Cells seed into 100% fresh medium grew the slowest yet with an intermediate viability.

These experiments indicate that the chemostat-conditioned medium (which is insulin-free) contains, at the very least, one unknown factor that supports growth, and possibly lacks another unknown factor that stimulates death. The unknown growth factor may be an autocrine factor. Indeed interleukin-6 can enhance viability of Sp2/0 hybridoma cells (Chung, Zabel et al. 1997), as well as the hybridoma of this study (data not shown). The factor that seemingly leads to more cell death in the experiment with regular conditioned medium is hypothetical, yet the proposed accumulation of such a death factor, as will be

discussed in Chapter 6, may be used to describe the extent of conditioning of the culture medium that apparently affects the emergence of apoptosis.

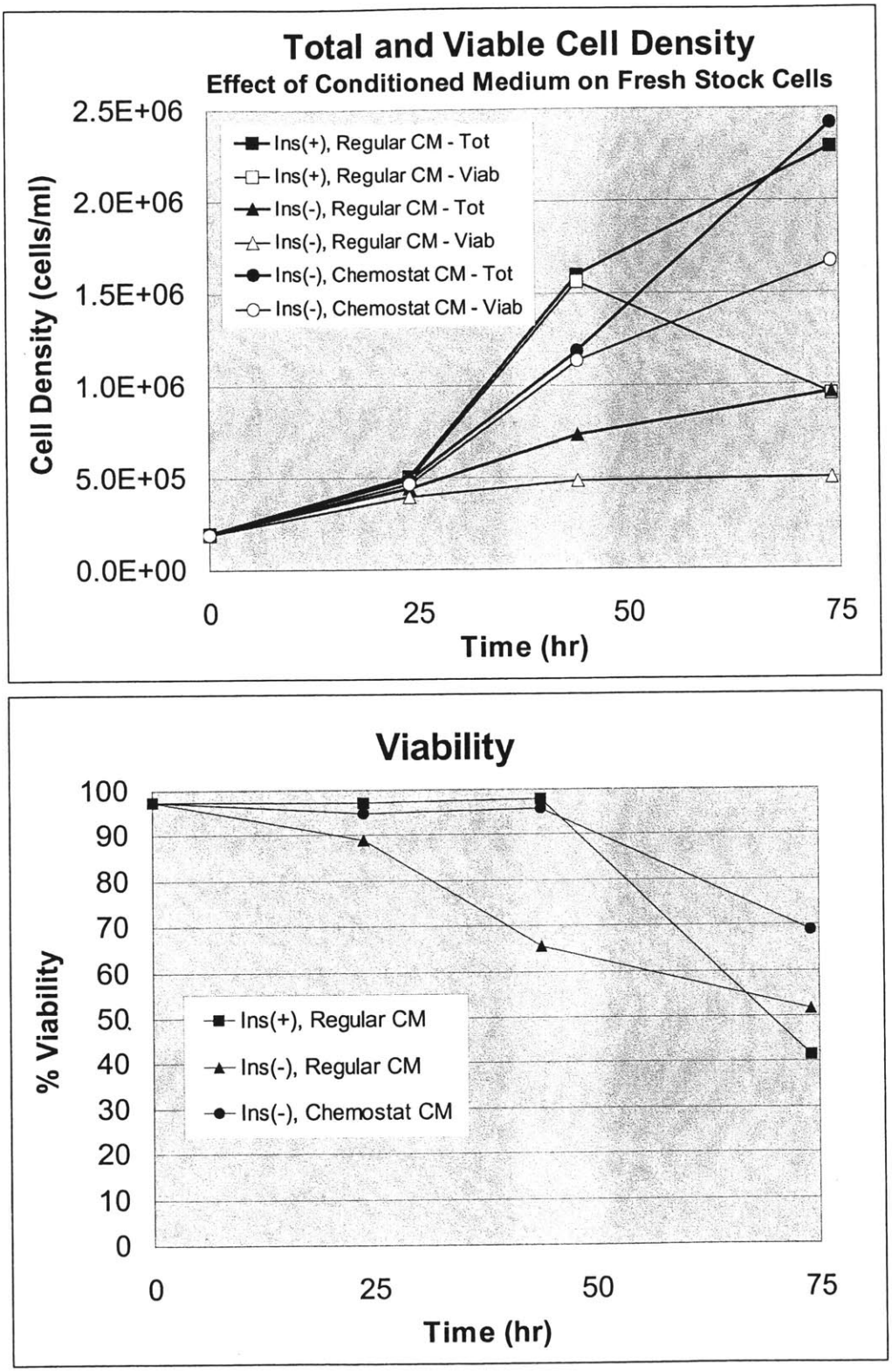
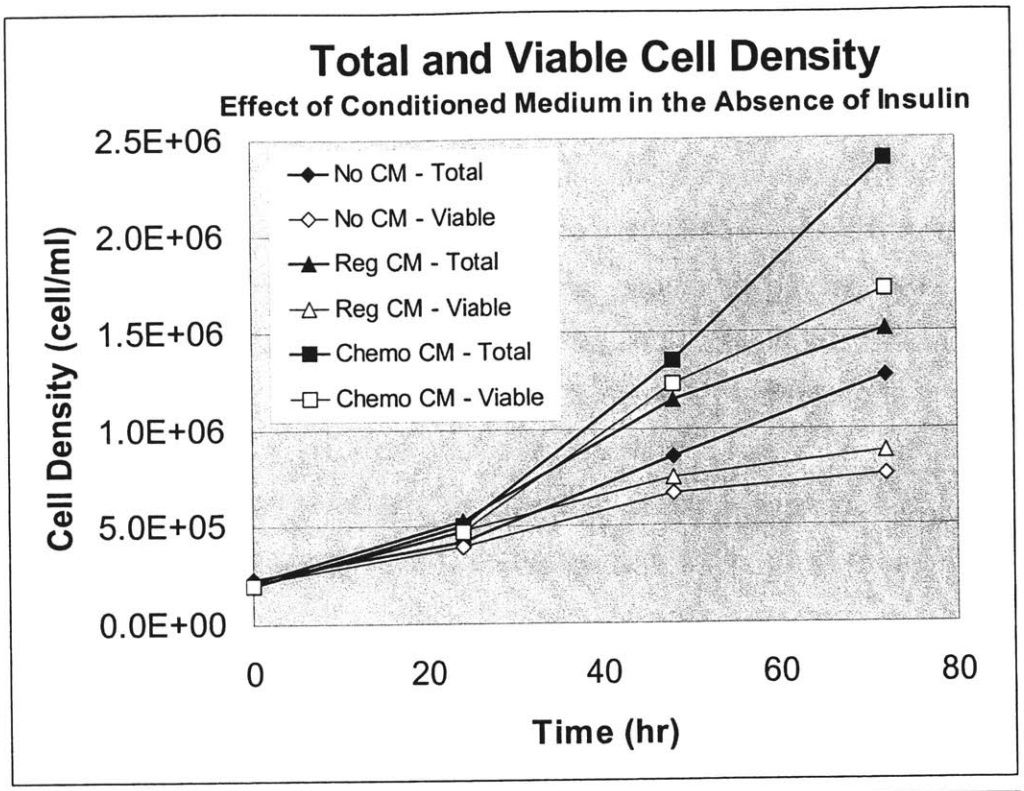



**Figure 5-12 a,b. Cell density and viability for fresh stock cells in insulin(+ or -) medium, with either regular or chemostat-conditioned medium.**



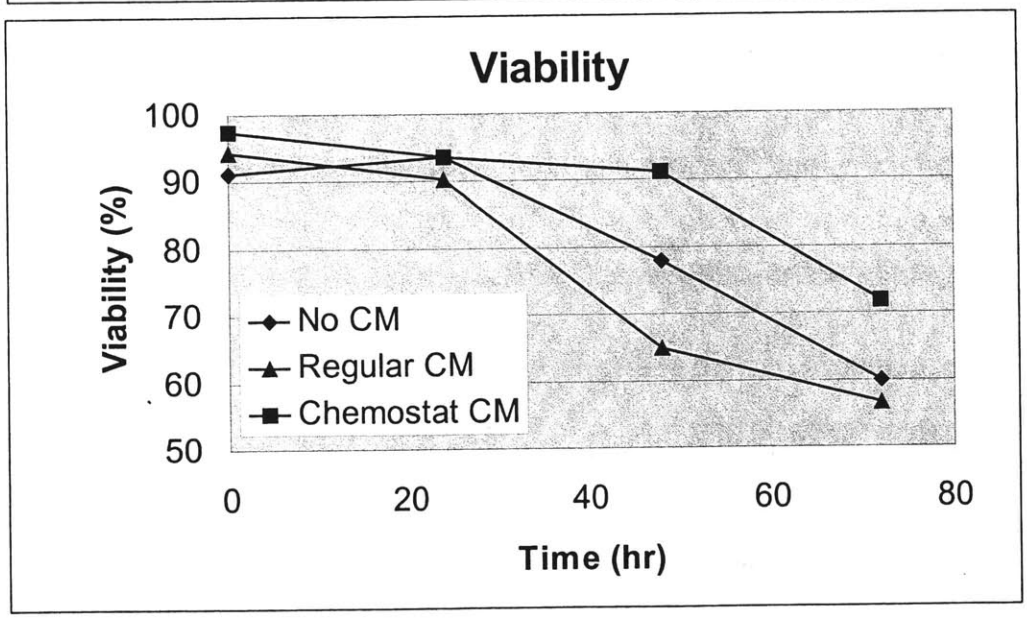




**Figure 5-13 a,b. Cell density and viability for fresh stock cells in insulin(-) batch cultures cultivated with regular conditioned medium, chemostat conditioned medium, or none at all.**

## 5.5 DISCUSSION

The growth of hybridoma cells in the absence of insulin reveals the presence or absence of positive and negative factors that affect proliferation and the emergence of cell death. The last figure above (Figure 5-13 a b) reveals that the chemostat-conditioned medium contains a positive factor that is not present in regular conditioned medium nor is supplied by 100% fresh medium. Even at 30% of its concentration in a chemostat (30% chemostat-conditioned medium is mixed with 70% fresh medium for the batches), the unknown positive factor allows for faster growth with high viability for 48 hours compared to the other insulin-free batches. However, the factor does not allow for growth as fast as that seen in fresh insulin(+) medium, with or without regular conditioned medium (Figure 5-12 a b, 100% fresh insulin(+) not shown). Perhaps this is because the factor has been diluted three-fold. Yet another explanation is that the factor is only replacing some of insulin's various effects on cellular physiology. This would be feasible since it has been hypothesized that insulin acts both dependently and independently of insulin-receptor tyrosine kinase activity. The independent signaling involves G-proteins and intracellular receptors, which are proposed to interact with metabolic enzymes in mitochondria (Term Project for Molecular / Cell Bioengineering submitted by Brian Follstad) (Follstad 1997).

### 5.5.1 Dependence on insulin for cell cycle progression is either not unique or not necessary

One of insulin's important effects is that of its activation of cell cycle related genes through signal transduction via the MAP kinase cascade. Though in this hybridoma it has been shown that *c-myc* expression (one of the endpoints of the MAP kinase cascade) is independent of nutrients and insulin, MAP kinase activity should still be required for other genetic factors. Phosphorylation of the MAP protein (the acronym refers to *m*itotic *a*ctivating *p*rotein and *m*icrotubule *a*ssociated *p*rotein) is necessary to allow the reorganization of microtubules during mitosis. In the unphosphorylated state, MAP binds microtubles to provide structural support, and upon phosphorylation, MAP's affinity for microtubles is reduced, and microtubules are destabilized and able to

reorganize (Lodish, Baltimore et al. 1995). Thus, in the absence of insulin stimulation, it is possible that the mitotic spindle (which is comprised of microtubles) would not be able to form (as readily) and the cells may arrest in G2-M. Given that cells can proliferate without insulin stimulation, implies that the MAP kinase cascade is either constitutively activated for the hybridoma (which would agree with the *c-myc* deregulation as well) or that the unknown factor in conditioned medium can replace insulin's stimulation of the map kinase cascade. Thus, by one mechanism or another, it appears that the removal of insulin does not absolutely lead to cell cycle inhibition. Insulin's effect through signal transduction pathways is not unique or may even be moot for this cell line.

### 5.5.2 Insulin provides support for metabolism

Ruling out insulin's role as a growth factor for cell cycle progression, I thus attribute the protective role of insulin against growth arrest (exhibited as increases in both G1 and G2/M or all phases of the cell cycle) and against apoptosis to its other well-known effects on cellular metabolism. Though the exact mechanism of regulation is debatable, the presence of insulin can lead to significant increases in the activity of metabolic enzymes, such as that for pyruvate dehydrogenase (Gottschalk 1990). Furthermore, insulin is reported to enhance mitochondrial Kreb's cycle activity (which "is the energy source for the anabolic activity of the cell") by 30% (Bessman and Mohan 1997). As opposed to the stimulation of the genetic factors that are absolutely necessary for progression through the cell cycle, I view a metabolic enhancement of 30% by insulin as an additional resource that a cell can harness when cellular energetics so require.

The results presented in this chapter can be cast in a framework that assumes that it is primarily the metabolic capacity of a culture that is dictating its dependence on insulin as a survival factor. Essentially, insulin may provide protection against apoptosis by enhancing metabolic capacity. As for the fed-batch cultures in Chapter 4, it appears that cultures shift their metabolism and exhibit uniform increases in cell cycle progression under conditions of increasing environmental stress. The metabolic shift indicates an increase in maintenance and slower progression through the cell cycle implies some sort of limitation. The presence of insulin allows cultures to grow viably for an additional 25 hours despite the progressively harsher environment. In this framework, insulin may

forestall apoptosis by giving the cells more metabolic potential with which to respond to the limitations. In this sense, the requirement of insulin then depends on both the metabolic state of the cells and the nature of the environment.

### 5.5.3 Insulin protects cells against detrimental environmental factors

The relationship between environmental factors and insulin protection is demonstrated by the ability to passage cells without insulin as long as the medium is changed frequently (daily) and the cell density is maintained low. Cultivation past 25 hours requires the presence of insulin. The influence of environmental factors on insulin requirement is also highlighted by the differences between chemostat-conditioned medium and regular conditioned medium. For unknown reasons, not all conditioned media are of the same nature. It is unclear why regular conditioned medium from cells passaged with insulin contains different types or amounts of factors than chemostat-conditioned medium obtained from an insulin-free chemostat. Presumably, just as for the production of the product MAb, the variation in production of such factors may depend on culture physiology as well.

The survival or death of cells in insulin-free chemostats is then an indication of both the metabolic state of the culture and the degree of conditioning of the chemostat medium. At the lowest dilution rate of 0.02 $hr^{-1}$, the culture is the most limited on glutamine and has the greatest dependence on TCA cycle and oxidative phosphorylation, as indicated by its glucose, glutamine, lactate, and oxygen metabolic rates. Furthermore, in terms of conditioned medium, the lowest dilution rate culture should have the highest accumulation of factors (assuming constant production). Thus, this culture appears to be doubly taxed in its metabolic needs to survive in the presence of more severe nutrient limitation and possibly harsher environment. Thus, it is reasonable that the removal of insulin could be detrimental. Indeed, well before the culture density begins to fall and death emerges, the cycle progression in G1 and G2/M have become extended. Eventually, the removal of metabolic potential causes severe enough of a limitation that cell growth is comprised and death emerges.

If the culture at the highest dilution rate (D=0.04 $hr^{-1}$) had survived without insulin, I could simply state that the increase in nutrient supply (and hence less of a metabolic

shift) and the decrease in factor accumulation (less environmental stress) associated with faster dilution rates of 0.03 and 0.04 would be sufficient to allow the survival of insulin-free cultures. But that was not the case. Three different experiments survived at 0.03, but the initial experiment at 0.04 did not. Essentially, the lack of survival at D=0.04 is evidence that either the metabolic state of the cells was suboptimal or that the environment was conditioned to a greater degree than that of the 0.03 experiments. Yet the metabolic state is observed to be "normal" for the insulin(+) steady state (meaning that it was not shifted towards TCA cycle) and only changes once death begins. Thus, I am left with trying to rationalize why the environment would be less optimal at D=0.04 $hr^{-1}$ than that at D=0.03 $hr^{-1}$ in terms of negative conditioned medium factors. My hypothesis for such a difference in environment for the 0.04 and 0.03 experiments is presented in Chapter 6.

The evidence presented in this chapter reveals something of the nature of the as of yet undetermined cause for apoptosis during batches and fed-batches. Insulin's effect on cell physiology is one that allows cells to grow faster and more viably despite increased levels of negative conditioned medium factors. Insulin's role as a protective factor is to support the normal metabolic and cell cycle function of cells. Yet with the ability of cells to grow viably even in the absence of insulin, insulin's role for metabolism seems to be the dominant effect in protecting cells against apoptosis which results from increasing environmental stresses.

## 5.6 CONCLUSIONS

The role of insulin in hybridoma cell culture was investigated in terms of its ability to protect cells against apoptotic cell death in serum-free medium. Because of its known positive effects, the severe insulin degradation observed in batch, fed-batch, and continuous cultures is minimized through the use of insulin feeding and the protease inhibitor glycocholic acid. Yet despite successfully maintaining higher insulin concentrations through the first three days of batch culture, the higher levels of insulin do not extend viable proliferation beyond two days in batches.

Insulin-free chemostats were achievable with insulin-dependent hybridoma cells at an intermediate dilution rate of 0.03 $hr^{-1}$, yet not at dilution rates of 0.04 or 0.02 $hr^{-1}$. Such survival at the intermediate dilution rate is shown to be related to the nature of the conditioned medium environment rather than the nature of the cells. Cells taken from the insulin-free chemostat still exhibited insulin-dependence in batch experiments. Moreover, conditioned medium from the insulin-free chemostat was able to support viable proliferation of fresh stock cells for 50 hours (just as long as the insulin(+) case).

The ability of insulin to extend viable proliferation is attributed to its role in supporting metabolism. Apoptotic cell death in fed-batches with insulin (Chapter 4) and apoptotic cell death upon removal of insulin (this chapter) exhibit the same symptoms of growth arrest and metabolic changes. With the growth arrest not occurring as the result of cell cycle impediment in any particular phase (all phases are delayed), it is an impediment in cellular metabolism that is taken to be the cause for growth inhibition and the emergence of cell death. In this framework, insulin serves as a protective factor through its stimulation of additional metabolic capacity. As cultures exhibit a shift in metabolism to one of higher maintenance, insulin may confer a culture the ability to more robustly meet the increase in energy demand. The protection of insulin is observed to be linked to the extent of conditioning of the culture environment. The additional metabolic capacity provided by the presence of insulin is necessary for longer survival in more highly conditioned environments, which are presumably the principle cause for the increase in maintenance energy.

## 5.7 ACKNOWLEDGEMENTS

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# 6 DISCUSSION AND IMPLICATIONS OF EXPERIMENTAL FINDINGS

## 6.1 REDUCTION OF APOPTOSIS IN FED-BATCH CULTURE

The investigation of hybridoma physiology in continuous culture provided useful evidence for the relationship between metabolism, cell cycle progression, and apoptosis. Such evidence can and has been applied to the improvement of fed-batch cultures with the aim of maximizing the production of therapeutic proteins. Yet, the chemostat evidence is not directly applicable to fed-batch cultures due to the differences in nutritional environments. In the chemostats, the cells are limited on glutamine and cell death emerges at lower dilution rates primarily due to the nutrient limitation (though other factors may play a role as well, as is discussed later). In fed-batches, no nutrient limitation (or any other known limitation for that matter) is present by design. In terms of growth factors, residual insulin levels in chemostats of greater than ~ 1000 mU/L (1% of that in fresh medium) are apparently sufficiently high despite degradation. Though insulin levels in fed-batches can become fully depleted, as demonstrated in Chapter 5, the depletion of insulin to levels lower than those found in the chemostats *follows* the emergence of cell death. Thus, in terms of known medium factors which we can control and measure, the major difference between chemostat experiments and the fed-batch experiments is nutrient levels. All the other process variables, such as temperature, pH, and osmolarity, can be monitored and controlled at optimal setpoints. Hence, the question remains as to why cell death emerges in fed-batch cultures. The emergence of death at lower dilution rates in the chemostats is expected for a continuous cell line, but the explanation for the emergence of cell death in a well designed fed-batch still eludes us. The investigation of the protective roles of rapamycin and insulin in my work show that survival in fed-batch environments depends on how the cells respond (in terms of shifts in metabolism and changes in cell cycle progression) to environmental limitations, whatever they may be. Interestingly, as I hypothesize in this chapter, the very same environment to which the cells must adapt

may also be conditioned in different ways or at different rates depending on the cellular physiology.

### 6.1.1 Metabolism and Apoptosis

Despite differences in nutrient levels, the physiology of cells in chemostats is still ultimately very useful because of the similarity between the physiology of cells at different dilution rates and cells at different time points in a fed-batch. Culture physiology in a fed-batch during the first two days before death emerges is comparable to the physiology of cells in a chemostat culture at a high dilution rate. Both are fast growing and exhibit high viability. They also both produce more lactate and consume less oxygen. Culture physiology for the fed-batch on later days after death emerges is comparable to the physiology of cells in a chemostat culture at lower dilution rates. Both are slower growing and exhibit lower viabilities. Also, both tend to produce less lactate and consume more oxygen. Thus, even though the cause for cell death is apparently different for each system, the physiology associated with cell death is remarkably similar. In both cases, cell death is associated with a growth arrest and a shift in metabolism towards TCA cycle and oxidative phosphorylation.

The observed shift in metabolism, towards better efficiency for glucose metabolism and higher maintenance, as the result of nutrient limitation or other causes, implicates the compromising of cellular energy production as cells die by apoptosis. In response to known or unknown causes for cell death and in the presence of sufficient nutrients and growth factors, the cells appear to respond robustly by shifting their metabolism in a direction to supply additional energy. Even though cell death emerges, the extent of cell death is less than I imagine would occur if the cells did not shift their metabolism at all. Hence, a shift towards efficiency should be taken as a positive, anti-apoptotic response. Indeed, the viable cells from the chemostat at the lowest dilution rate exhibited the ability to reach twice the viable cell density when returned to the better environment at a higher dilution rate. This multiplicity of steady states dramatically emphasizes the influence of metabolism and cellular energetics on viable cell density and apoptotic cell death. Brian Follstad is pursuing this finding concerning the metabolic effects on

apoptosis. He has linked higher metabolic efficiency and mitochondrial potential with a higher culture viable cell density.

### 6.1.2 Cell Cycle and Apoptosis

The cell cycle phenomenon observed in the chemostats points to the inhibition of S-phase progression as the cause for the increase in doubling time. Both G1 and G2/M progression times appear to remain fairly unchanged, while progression time through the S-phase increases from 10 to 20 hours as dilution rate is decreased. It is reasonable that glutamine limitation (or any nutrient limitation in general) may delay S-phase progression as the result of a shortage of synthetic precursors for DNA. The apoptosis that results from within the S-phase of a proliferating culture could be due to p53 activity or another DNA checkpoint. The emergence of apoptosis in my particular hybridoma cell line is particularly severe since entry to the cell cycle is deregulated. Similarly, for the anchorage CHO cell line, the stimulation of cell cycle entry by growth factors can also lead to cell death under sub-optimal conditions. The dramatic improvement in culture performance by employing a rationally designed medium rests for the most part on the minimization of cell death due to delays in S-phase progression by nutrient limitation.

In the absence of nutrient limitation, cells in a fed-batch culture still exhibit growth arrest and cell death. Cell cycle analysis reveals that arrestment occurs uniformly for each phase of the cycle as growth declines and apoptosis emerges. The elongation of the G1-phase is particularly interesting because it sharply contrasts the behavior seen in the chemostats, as well as the finding that cell cycle entry is defective in the hybridoma. As opposed to the chemostat phenomenon, there is something different about the fed-batch cultivation that leads to a delay in G1. The delay of G1-progession, as well as the delay of the other phases, implicates another phenomenon as the cause for growth arrest and cell death.

From the insulin-withdrawal chemostat at D=0.04 $hr^{-1}$ (that could not maintain an insulin-free steady state and did exhibit apoptosis), it appears that growth arrest and apoptosis also are associated with the uniform elongation of the cell cycle. Thus, to explain the death in fed-batches, one could propose that cell death emerges as the result of insulin

depletion. However, as was shown in Chapter 5, the insulin levels when death emerges in fed-batches were sufficiently high and did not effect the emergence of cell death. Thus, the similarity in cell cycle phenomenon for the insulin-free chemostat at D=0.04 $hr^{-1}$ and fed-batches that apparently contain sufficient insulin also implies that there may be another underlying phenomenon causing growth arrest and cell death which the two culture systems share in common. Furthermore, insulin cannot be uniquely required for proliferation since the hybridomas have been observed to grow without insulin altogether.

The phenomenon at the intersection of cell death due to insulin depletion in chemostats and cell death occurring in fed-batches may very well be metabolism. The role of insulin for metabolic function, rather than cell cycle function, could explain the duality of culture responses when it is removed from steady state chemostats. Depending on the culture physiology and the environment for a particular steady state, insulin may or may not be required for additional metabolic capacity. In this context, the removal of insulin is thought to limit metabolic capacity, which in turn can lead to slower cell cycle progression, growth arrest, and cell death. That fed-batch cultures exhibit similar cell cycle phenomenon is thus additional evidence, though indirect, that it is possibly the obstruction of metabolic function that is leading to growth arrest, uniform cell cycle arrest, and apoptosis.

The ability of this cell line to proliferate viably in the absence of insulin points to the presence of other factors (autocrine, interleukins) in insulin-free conditioned culture medium. Though insulin stimulation of *c-myc* via the MAP kinase cascade is not required for this hybridoma cell line, insulin should presumably still be required for MAP (microtubule associated protein) phosphorylation and microtubule reorganization. Viable proliferation in the absence of insulin indicates that insulin' effect as a growth factor is not unique - the hybridomas can somehow still perform cell cycle division. Thus, once again, the arrest of cells in fed-batches is probably the result of other factors beyond the direct expected effect of depletion of growth factors.

The evidence for and discussion of the role of cell cycle in apoptotic cell death for this hybridoma cell thus further implicate the role that metabolism has on the emergence of

apoptosis. Obviously, proper cellular function is key for viable proliferation. Here it is plausible that unhindered progression through all phases of the cell cycle depends on a robust metabolism.

The mounting evidence for metabolic impediment as the underlying phenomenon for cell death raises questions as to the role of rapamycin in delaying apoptosis. As shown in Chapter 5, rapamycin's primary effect on the cell cycle is to extend G1-phase progression. However, it also leads to a lesser degree of uniform cell cycle arrestment as compared to the controls. It is not exactly clear why longer G1-phase residence results in lesser inhibition for transition through S-phase and G2/M-phase. As I believed in my initial hypothesis regarding the role for arresting agents, it may be that a delay in G1 allows for the more complete production of intracellular factors that support cell cycle function and viability. Yet, the possible link between a uniform cell cycle arrestment and metabolism support a newer hypothesis that rapamycin's positive effects on viability could be due to its effects on metabolism.

As shown in the Chapter 5 discussion, metabolism data do show that rapamycin cultures appear to have the more efficient metabolic profile. The mechanism behind the metabolic effect of rapamycin could be direct or indirect. As described for yeast cells by Schreiber (Schreiber 1999), rapamycin causes many up-regulations and down-regulations in related cell cycle and metabolism genes. Perhaps, these genetic changes are homologous in mammalian cells and thus rapamycin can have a direct impact on metabolism. Or, barring the existence of such a direct connection, perhaps cell cycle progression itself can influence metabolism. Though it is clear that metabolism can vary despite a constant cell cycle distribution (chemostat data), it is also well established that metabolism is a function of growth rate. It is possible that a shift in metabolism may simply follow a shift in cell cycle distribution towards slower growth. However, it is intriguing that such a shift be similar to that of a nutrient-limited or fed-batch culture that is undergoing cell death. Evidence for rapamycin addition causing a starvation response, as if there were an amino acid limitation, makes the shift in metabolism appear to be more complex than just a growth rate related downshift.

The differences in cell cycle progression and cell death for chemostats with and without insulin, and fed-batches with or without rapamycin are depicted below. Here the delay in progression of cells through G1 is seen to be related to the absence of insulin (chemostat D=0.04 and D0.02) or the arrestment of cell growth during fed-batches. The rapamycin culture shows the slowest progression throughout, but the same increasing trend as the control nonetheless. The insulin-free chemostat culture at D=0.02 hr-1 appears already delayed within G1 even prior to cell death, as was discussed in Chapter 5. Overall, in terms of the relationship between cell death and cell cycle arrest, it appears that the arrest during fed-batch cultures and insulin-free chemostats is associated with greater cell death than is observed when the arrest occurs in the presence of rapamycin. Cell death for the insulin(+)-series of chemostats is associated with arrest in S-phase rather than G1.

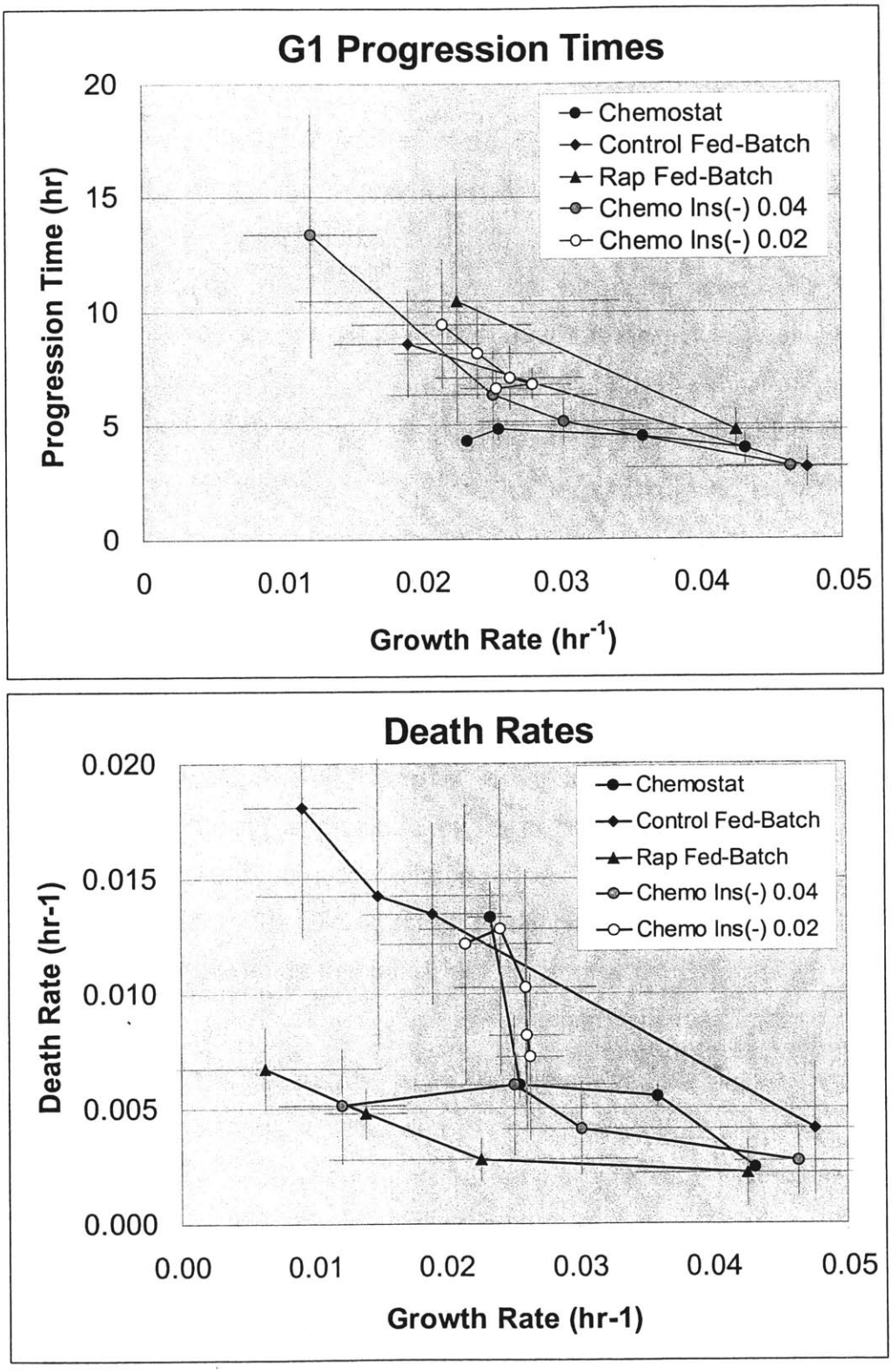


**Figure 6-1 a,b. G1 progression time and death rate versus growth rate for hybridoma cells.**

Error bars represent 95% confidence limits.

## 6.2 MONOCLONAL ANTIBODY PRODUCTION

The monoclonal antibody titer of 0.55 g/L from my six-day rapamycin fed-batch cultures is a significant improvement over the non-rapamycin counterpart titer of 0.25 g/L. Relating back to the state of the art objective fed-batch described in Chapter 2, the use of rapamycin is observed to put the production of MAbs for a serum-free fed-batch process on par with the serum-based ones. On a ten-day basis (the length of time theoretically sufficient for the production of 10.0 g/L in a death-free fed-batch), the rapamycin-fed fed-batch is estimated to yield 0.95 g/L, thus outperforming the previously mentioned records for MAb production in fed-batches.

The apparent specific monoclonal antibody production for this hybridoma is observed to vary over quite a broad range. Data from chemostat steady states span about a 2.5-fold range in apparent specific productivity, from 0.9 to 2.3 x $10^{-9}$ mg/cell/hr. Apparent specific productivity in the control and rapamycin fed-batches spans a similarly large range, from 1.4 to 3.2 x $10^{-9}$ mg/cell/hr. Such variance is attributed to differences in the extent of nutrient limitation, changes in G1 residence, and shifts in metabolism. The observed constant productivity in previous work is still plausible nonetheless. During extended fed-batches with significant apoptosis occurring, the metabolism of the viable cells would be expected to be pinned at the most efficient / highest maintenance state and the cell cycle distribution would be fairly constant as arrestment would be occurring uniformly in each phase.

From the two multiple steady states at 0.04 $hr^{-1}$ discussed in Chapter 3, it is not clear whether a metabolic shift or a change in cell cycle progression is responsible for the 40% lower specific productivity for the second steady state (E). G1 progression time (MAb production is believed to be highest in G1) for the first steady state at D=0.04 (A) was 3.9 hr, whereas it was 3.1 hr for the second steady state at D=0.04 (E), a decrease of about 20%. Though the decrease in G1 residence would be expected to detract from apparent specific productivity, specific productivity is observed to depend on more than just G1 progression time (Figure 6-2). The scatter in data is attributed to differences in culture viability and metabolism. Also shown is the observed progression of decreasing

specific productivity observed during the withdrawal of insulin from a steady state at D=0.02 $hr^{-1}$. Even before cell death emerges for that insulin withdrawal experiment (Day 21), specific productivity has decreased significantly while G1 progression time increased.

The value 0.9 x $10^{-9}$ mg/cell/hr observed for specific productivity for the steady states D and E (D has viability = 40%, E is the multiple steady state at D=0.04 hr-1 that exhibits a shifted metabolism) is comparable to the value of 0.85 x $10^{-9}$ mg/cell/hr quoted for this cell line during extended fed-batches (Xie 1997). This is reasonable since the cells in the fed-batch environment are controlled at lower nutrient levels, levels that can shift metabolism toward the more efficient state, and are exhibiting higher death rates. Furthermore, the higher value quoted by Glacken of 1.2 x $10^{-9}$ (data from Xie, (Xie 1997)) is also reasonable since that number is calculated from the early portion of the culture run where metabolism may not be fully shifted and cell death was not as severe. Thus, in addition to providing consistent data with previous investigators, I believe my observations add additional insight as to the underlying causes that can influence specific productivity.

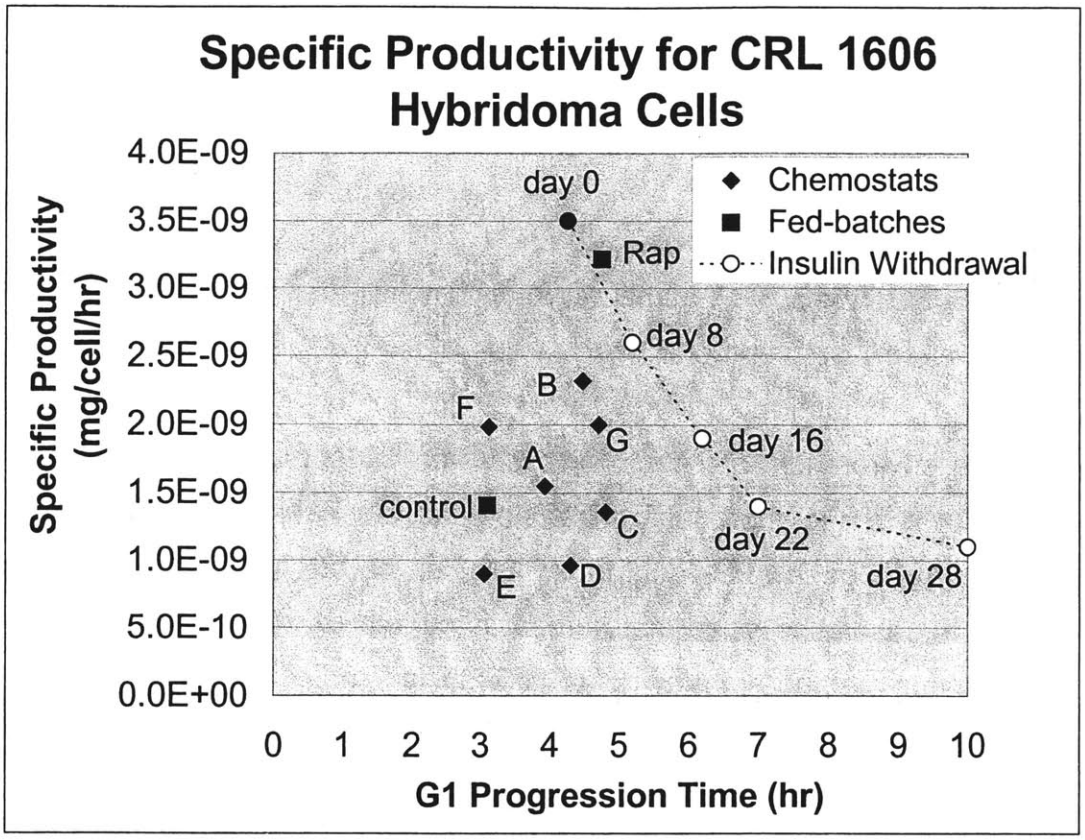


**Figure 6-2. Specific production of monoclonal antibody for chemostat and fed-batch experiments versus progression time through the G1-phase of the cell cycle.**

Chemostat data A, B, C, D, and E are from a single series of steady states at dilution rates of 0.04, 0.03, 0.02, 0.01, and 0.04 $hr^{-1}$, respectively. Chemostat data F and G and from the insulin(+) steady state prior to insulin withdrawal at dilution rates of 0.04 and 0.03 hr-1, respectively. The insulin withdrawal series data is for a chemostat at D = 0.02 $hr^{-1}$. Fed-batch data for rapamycin and control cultures is that measured between days 2 and 3. 95% confidence limits are approximately 10-15% of productivity values and approximately 1 hour for G1 progression times.

Thus, the dependence of apparent specific productivity on growth rate, cell cycle progression, metabolism, and nutrient supply, and cell death (all of which are interrelated themselves) can explain the variable productivity from this hybridoma cell line. In this context, the significant enhancement of apparent specific productivity by rapamycin cannot be attributed solely to the increase in residence in the G1-phase of the cell cycle. The interrelation of the cellular phenomena should be considered in the explanation of variance in specific productivity. For rapamycin-fed fed-batches, the dramatic increase in apparent specific productivity up to 3.2 x 10-9 mg/cell/ml is attributed to the combined effects of the elongated G1 progression, the shift towards a more efficient metabolism, and the minimization of cell death. An increase in G1 residence of 50% cannot alone explain the tripling in specific productivity. The beauty of rapamycin treatment is that higher productivity can be achieved without having to "stress" the cells by reducing nutrient levels or by waiting for the stationary phase and cell death. With rapamycin, the robustness of the cells can be measured in terms of the decrease in death rate as well as the significant improvement of specific productivity.

## 6.3 CONDITIONED MEDIUM FACTOR PRODUCTION AND ACCUMULATION

Despite all the evidence relating metabolism, cell cycle, and cell death phenomenon, the mechanism that drives shifts in metabolism and cell cycle progression in conjunction with the emergence of cell death remains a mystery. As has been raised throughout this thesis, it appears that deteriorating cellular energetics may be the cause for the shift in metabolic efficiency and the uniform delay in cell cycle progression. If this is indeed the case, the question then remains as to what is the cause for the deterioration of cellular energetics. It is within this framework that I hypothesize the existence of a conditioned medium factor that stimulates cell death. The existence of such a factor has been previously proposed and modeled in a similar way that I do below (Lee, Yap et al. 1995; Zeng, Deckwer et al. 1998). For my analysis, I add the physiological impact that the hypothesized factor has on cultures in terms of metabolism and cell cycle, and I also allow for variation in its production. I claim that the factor acts as a "poison" to the

production of energy for proper cell function. The result of such poisoning of metabolic potential would trigger apoptosis by indirectly impeding cellular functions, including cell cycle progression.

This hypothesis for cell death stems from my accumulated experience with the cultivation of hybridomas under a variety of conditions: under nutrient limitation, in the presence or absence of insulin, in the presence or absence of rapamycin, and in batches, fed-batches, and chemostats. Following the description of my model for the conditioning of culture medium, I present applications of the model, which in fact comprise the phenomenon on which the model was based. Though the exact nature of the conditioned medium factor is unknown, I believe this model is useful for explaining some of the phenomena relating cell death that I have experienced over that last several years. In its infancy, in the form of intuition, I applied the model and its assumptions to design the proper batch experiments that demonstrate the role of conditioned medium with and without insulin.

### 6.3.1 Proposed Model for "Conditioned Medium Hypothesis"

The proposed model is based on the survival or death of hybridoma cells in the absence of insulin. For this analysis, viable proliferation in the absence of insulin serves as an indication that deleterious conditioned medium factors have not accumulated to a level that would stimulate cell death. Sustained viable proliferation in the absence of insulin therefore is taken to imply that cellular metabolic function is intact, and thus, in this framework, that it is not being poisoned by high levels of conditioned medium factors. The presence of insulin supports a more robust metabolic function that allows cells to proliferate in the presence of higher levels of conditioned medium factors.

The production of conditioned medium factors is related to the integrated viable cell density of a culture and either the dilution rate or frequency of passaging. Factor production is assumed to be the result of viable cell proliferation and metabolism, as opposed to release of contents from dead cells. In this framework, the concept of production of conditioned medium factors can be expressed and manipulated similarly as other cellular rates, such as production of MAb. Furthermore, the production and

accumulation of the factors are taken to be the same, hence neglecting degradation or loss of factor activity.

A balance around a steady state chemostat for a quantity, F, the unknown factor that causes death, yields an equation for the production of "death factors" per cell as a function of the dilution rate, the level of factors F, and the number of viable cells in the reactor. The balance for conditioned medium factors in a chemostat is:

$$\frac{dF}{dt} = n_v q_F - DF$$

where F is the concentration of factors (unit/ml), $n_v$ is the viable cell density (cell/ml), $q_F$ is the specific production of F (unit/cell/hr), and D is the dilution rate ($hr^{-1}$). From such a balance at steady state, the specific production of factors for a chemostat is determined as:

$$q_F = \frac{DF}{n_v}$$

For batch and fed-batch cultures, the governing equation for accumulation of conditioned medium factors is given by:

$$F_2 - F_1 = \int_{t1}^{t2} n_v(t) \cdot q_F(t) dt = IVCD\Big|_{t1}^{t2} \cdot \bar{q}_F$$

To make use of the above equations, I must choose a relative reference point for the level of F below which viable proliferation is sustainable without insulin. This is akin to a toxicity level similar to those previously determined for ammonia and lactate, but in relation to the presence of insulin. In making this choice of reference, I am preliminarily assuming that the level of factor which stimulates cell death in insulin(-) medium is the same for all hybridoma cultures without insulin. I likewise make the same assumption for the insulin(+) F-level that I determine below. For now, such assumptions are sufficient to proceed and make sense of my own data.

Thus, I arbitrarily assign a value of 1.0 unit/ml for a F-level below which cells do not need insulin for viable proliferation and above which they do need insulin or else they die. From batches without insulin, cells can proliferate for about 25 hours at low density.

The corresponding IVCD up to the point of cell death is approximately 8.4 cell-hr/ml (an average of 3.5e5 cell/ml * 24 hr). Using the equation for accumulation of factors in batch, I can determine both the initial factor level for a typical experiment (which is inoculated using about 1/3 of inoculum conditioned medium) and the specific rate of factor production. Using an initial guess of 0.5 for $F_1$, I determine an initial value for $q_F$ = 0.63e-7. With this $q_F$ I then calculate F1 for an inoculum that is passaged daily. Using the new F1, I recalculate a new $q_F$. Iteration yields $F_1$ = 0.42 and **$q_F$ = 0.69e-7** unit/cell/hr. Taking this $q_F$, I calculate the F-level associated with the emergence of cell death for an insulin(+)-positive batch to be 2.82 unit/ml (48 hour culture at higher average density plus the initial 0.42 from inoculation). The following table summarizes the model for insulin(+/-) batch cultures.

**Analysis for Conditioned Medium Hypothesis in Batches**

| | **IVCD (cell-hr/ml) Viability > 90%** | **Specific Production of F (unit/cell/hr)** | **Threshold F-level for cell death (unit/ml)** |
|---|---|---|---|
| Insulin(-) | $0.84 \times 10^7$ | $0.69 \times 10^{-7}$ | 1.0 |
| Insulin(+) | $3.5 \times 10^7$ | $0.69 \times 10^{-7}$ | 2.8 |

The F-level associated with death in insulin(-) batches is used to analyze the survival or death of insulin-free chemostats. Using the equation for chemostats above, the dilution rate and the cell density of a steady state is used to calculate the minimum specific production rate of F in the cases that resulted in death and the maximum specific production rate of F for cases that resulted in survival. For instance, the culture at D=0.04 $hr^{-1}$ did not survive, and hence is taken to have an F-level greater than or equal to 1.0, and hence a $q_F$ that is at least 0.8e-7. The three scenarios for different dilution rates are summarized in the following table.

**Analysis for Conditioned Medium Hypothesis from Chemostats**

| Dilution Rate (hr-1) | Viable Cell Density (cell/ml) | Survival? | F at Steady State (unit/ml) | Specific Production of F (unit/cell/hr) |
|---|---|---|---|---|
| D = 0.04 | $5 \times 10^5$ | No | $\geq 1.0$ | $\geq 0.80 \times 10^{-7}$ |
| D = 0.03 | $5\text{-}7 \times 10^5$ | Yes | $\leq 1.0$ | $\leq 0.50 \times 10^{-7}$ |
| D = 0.02 | $6 \times 10^5$ | No | $\geq 1.0$ | $\geq 0.33 \times 10^{-7}$ |

The calculated $q_F$'s at each dilution rate demonstrate that the production rate for the factor cannot be constant within the context of this framework. The survival of the chemostat culture at D=0.03 is associated with a lesser production of conditioned medium factors relative to the cells in the chemostat at D=0.04. This drop in production is taken to be sufficient to allow for an F-level which is smaller at D=0.03 than at D=0.04. A further reduction in production rate for the chemostat at D=0.02 is possible, though the beneficial effect is not sufficient to outweigh the slower dilution and hence higher accumulation of conditioned medium factors.

The necessary variation of the production rate of conditioned medium factors to explain the insulin-free chemostat behavior is not unreasonable. Similar trends with growth rate exist for several metabolic rates, such as glucose and glutamine uptake and lactate and ammonia production. Variation of MAb production likewise can vary several-fold depending on culture physiology. If accumulation of death factors is truly a cause for cell death, then their dependence on culture growth rate, metabolism, and/or cell cycle distribution agrees with the coinciding link of such phenomena to the emergence of apoptotic cell death. With higher conditioning levels hypothesized to lead to metabolic impediment and cell cycle arrest, the robustness of cells that are growing slower (due to nutrient control or rapamycin) may, perhaps, be due to their lesser production of conditioned medium factors that stimulate apoptosis. Thus, as will be evident in the applications, I will use knowledge of culture metabolism and growth rate in specifying a high or low production rate for conditioned medium factors. Cultures under nutrient limitation (though perhaps of lower viability due to S-phase limitation) are assumed to

produce less factors. Likewise, cultures in the presence of rapamycin are also assumed to produce less factors as the result of their decreased growth rate and the preliminary evidence supporting their shift to more efficient metabolism.

## 6.3.2 Applications of the "Conditioned Medium Hypothesis"

### 6.3.2.1 Batch and Fed-batch Cultures

The analysis of many of my batch cultures, with and without insulin, is consistent with the F-levels I used in developing my model. Insulin-free cultures started with regular conditioned medium last only about 25 hours before the viability begins to decline. The F-levels for such insulin-free batches range from 0.5 to 1.0 depending on the type or absence of conditioned medium, whereas the F-levels prior to cell death for insulin(+) experiments range from 2.4 to 3.4. For insulin-free batches, the type of conditioned medium used is accounted for in the initial factor concentration at inoculation. The lower F-levels for special or no conditioned medium correlate well with lower death rates. Yet for the case of 100% fresh medium without insulin, death emerges at the lowest level of F. Positive factors in conditioned medium do play a role in supporting viability as well.

### 6.3.2.2 Re-fed Suspension Cell Cultures

During the investigation of the physiology of hybridomas grown within 3-mm polymer capsules, masters student Bettina Knorr developed a new protocol for her suspension cell culture control. For the encapsulated cells, eighty to ninety percent of the spent medium is exchanged for fresh medium on a daily (or 8 hour) basis. This exchange of medium is simple since the cells are encapsulated within the polymer beads. For a production process, such medium exchange is economically feasible since the product MAb is retained within the beads, whose polymer shell has a MW cutoff of around 10 kDa, which still allows nutrients and insulin to pass through. To achieve a better comparison between her encapsulated cells and the suspension cells, she decided to exchange 80-90% of the suspension conditioned medium on a daily (or 8 hour) basis as well, as opposed to fed-batch cultivation. She achieved this so-called "Re-fed Suspension" culture by spinning down the entire culture and resuspending it in 80% fresh, 20% conditioned medium.

The performance of the cells in the re-fed suspension culture is quite astonishing. As shown below, the re-fed culture dramatically outperformed control fed batches (my own data as a polynomial fit of 5 cultures) (Figure 6-3). The re-fed viable cell density reached 7.0 x $10^6$ cells/ml and maintained high viability (as measured by acridine orange and ethidium bromide, a harsher assay for viability than trypan blue) for around 100 hours. The average fed-batch control only reaches about 1.5-2.0 x $10^6$ cells/ml and lasts for only 50 hours with high viability. It appears that the exchange of most of the conditioned medium every eight hours by centrifugation and resuspension allows the cells to reach a viable cell density approximately four times that of a fed-batch. The re-fed density is a record for serum-free hybridomas.

The lactate and ammonia concentrations were shown to be high at the end of an 8 hour interval, but not above the established toxicity thresholds. The cause for the emergence of apoptosis for the re-fed cultures can be explained in terms of the conditioned medium hypothesis. The integrated viable cell densities at which death emerges is approximately 4.0 x $10^7$ cell-hr/ml (~ 5e6 cell/ml for 8 hours) for the re-fed culture, and 3.5 x $10^7$ cell-hr/ml (over 50 hours) for the fed-batch culture. Multiplying each IVCD by $q_F$ = 0.69 x $10^{-7}$ yields F = 2.8 and F = 2.4, respectively. Thus, in calculating similar levels of conditioning, the model helps me express the idea that the cells of the re-fed system reach higher densities as the result of removing conditioned medium rather than by some other cellular phenomenon. They both die at the similar F-levels. In addition to supplying fresh nutrients and insulin, the removal of conditioned medium factors allows the culture to proliferate viably to much higher densities.

This hypothesis was further applied to the improvement of culture performance within the polymer beads. Similar to the fed-batch performance, death emerged at around 50 hours for beads with the standard pore size. Yet viable cell density reached a density ten times that with standard beads when the pore size was made larger by the addition of PEG 3400 to the polymer shell solution during preparation. The increase in pore size would allow better transport for nutrients and insulin, though their transport was verified to be sufficient with the smaller pores. A possible explanation for the culture's improvement with the larger pore beads is the enhanced removal of conditioned medium factors that cause cell death.

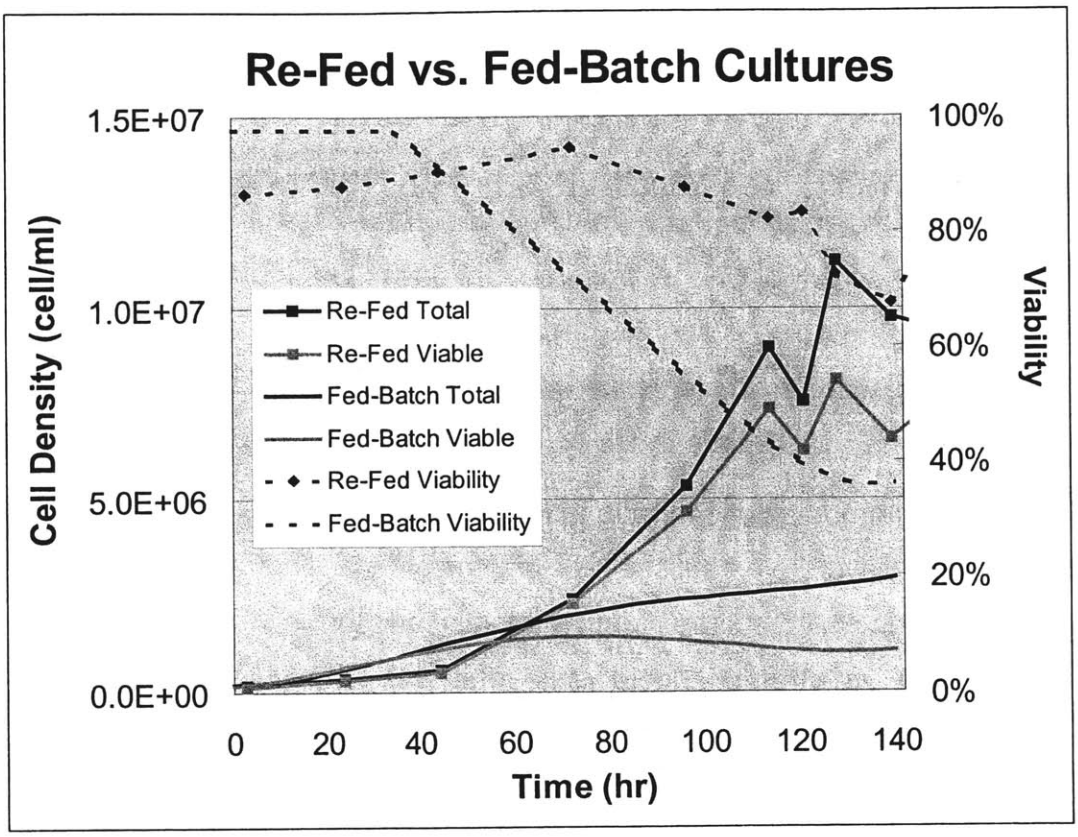


**Figure 6-3. Cell density and viability of re-fed vs fed-batch hybridoma cultures.**

### 6.3.2.3 Rapamycin-fed Fed-batch Cultures

Interestingly, cultures with rapamycin are observed to reach higher IVCD's than the control cultures before death emerges. A rap culture can persist for up to 80 hours before succumbing to cell death, reaching an IVCD of about $6.0 \times 10^7$ cell-hr/ml. This near doubling of the IVCD at which death emerges relative to control cultures signifies that (1) rapamycin cells are more resistant to whatever is causing the death or (2) that rap cultures, with their altered metabolism, are conditioning their environment at a slower rate or (3) a combination of both effects.

The conditioned medium model can be used to interpret rapamycin's effects on cell death in either of the above cases. For the first case, we assume the same $q_F$ as for control cells, namely $0.69 \times 10^{-7}$, and calculate the F at which death emerges to be 4.14. The higher F-value at which death emerges in the presence of rapamycin is analogous to the higher F-value at which death emerges in the presence of insulin. It is possible, like for insulin, that rapamycin leads to robustness by supporting cellular function. For the second case, we fix F at which death emerges constant at 2.8 for insulin(+) cultures, and calculate the associated $q_F$ to be 0.40e-7. Just as I explain the survival of cells in insulin-free chemostats as depending on the reduction of $q_F$, the extension of viability for rapamycin cells could be due to the reduction of $q_F$ as well. Either case is feasible to explain the reduction of death that rapamycin cultures exhibit. The rapamycin effect to alter a cells protein profile could render cells more resistant to apoptosis. Conversely, rapamycin's effects on cell cycle and metabolism could reduce the production rate of conditioned medium death factors.

### 6.3.2.4 Chemostat Phenomena

The Conditioned Medium Hypothesis can be used to explain the severe increase in apoptosis observed for the case of the lowest dilution rate chemostat. The associated F-levels for each of the steady states are 1.2, 1.3, 3.6, and 6.2 for steady states A, B, C, and D, respectively, where a constant $q_F$ =0.69e-7 is assumed. For C and D, the F-level is greater than the threshold for death established for insulin(+)-batches. Allowing for the fact that $q_F$ may be reduced for these growth arrested, metabolically shifted cultures, steady state D is still sufficiently high that even a halving of the production rate would

still result in a deleterious environment. Thus, the dramatic increase in cell death for the steady state at the slowest dilution rate may be due to the combined effects of nutrient depletion (causing S-phase related apoptosis) *and* the presence of high levels of conditioned medium factors.

The existence of a multiple steady state at 0.04 hr-1 can also be considered from the effect of conditioned medium factors for each steady state. Assuming similar specific productivity for $q_F$ for each state, the first one had an F-level of 1.2, whereas the second steady state, with nearly double the viable cell density, has an F-level of 2.3. Here it appears that the second steady state is robust despite (or perhaps as the result of) the higher F-level. However, if we assume that $q_F$ is decreased for the second steady state, as a result of its efficient metabolism, the F-level could be similar to the first. Thus, with the occurrence of multiple steady states being linked to metabolic phenomenon, the differences in F-levels or F-production may one day be useful for better understanding the multiplicity in cell density and specific productivity. Indeed, even though the dilution rate is the same, the different metabolism has effects on residual nutrient levels. Effects would also be expected in the extent of conditioning of the culture medium.

With regard to insulin-free chemostat experiments, I could use the conditioned medium model to re-design experiments at D=0.03 $hr^{-1}$ such that death should emerge. From the conditioned medium hypothesis, I can intuit that cell death will occur for cultures at D=0.03 $hr^{-1}$ if the steady state viable cell density was higher. The higher viable cell density could be obtained by increasing the glutamine feed concentration from 0.08 to 1.2 or 1.6 mM. Cell Death would be expected to occur for two complementary reasons: (1) higher residual glutamine would allow for higher cell densities and hence higher levels of the hypothesized death factors as calculated from the equation, and (2) a less substantial shift in metabolism that would result in a higher specific production rate for death factors than observed with a low glutamine feed medium. The proof of such an experimental design is in the works by several Stephanopoulos co-workers, as they study the role of insulin on cellular protein profiles (as measured by 2-D gels).

A final application of the model is in the explanation of the difficulty I experienced in chemostat startup with the hybridoma cell line. For the first two attempts at starting up

the chemostat, the cells were unable to reach a steady state due to severe growth arrest and the emergence of cell death. The initial protocol (just like the one I used with CHO cells) involved inoculating the reactor, letting cells grow in batch for two days, and then starting the dilution rate at the desired setpoint. Yet, despite healthy batch growth during the first two days, the hybridoma cells were unable to adapt smoothly to glutamine-limited growth. Part of the problem is now understood in terms of the defective cell cycle entry that I discovered for this cell line. As the glutamine concentration approaches the low residual level, the cell cycle entry is not reigned in by proper cell cycle control, resulting in even greater amounts of cell death within the S-phase. A second important aspect during startup may be the level of conditioned medium factors that are estimated to be present at the end of the two-day batch. Using a constant value of $0.69 \times 10^{-7}$ U/cell/hr for $q_F$, I calculate the net accumulation of factors during startup (Figure 6-4). As evident for the unsuccessful startup scenario below, the F-level for the unsuccessful protocol reaches and slightly surpasses the threshold for death for cells with insulin (F=2.8). Thus, in addition to improper entry to the cell cycle, it is possible that the severity of the death response is also due to high levels of death factors.

My solution to the problem at that time, based on simple intuition, was to start the dilution rate earlier (at 24 hours) and appropriately adjust the dilution in order to keep the cell density below 1.0e6 cell/ml. In the adjustment of the dilution rate, the growth rate of the culture was calculated and used in conjunction with the current density to set the appropriate dilution rate to stabilize the density below 1.0e6 cell/ml (I called this procedure "chasing the growth rate"). Thus, a successful start up consisted of my setting the dilution rate at 0.025-0.035 hr-1 on the first day, then increasing it to 0.04-0.05 hr-1 on the second day, and then gradually lowering it over several days to the final setpoint of 0.02-0.04 hr-1. For obvious reasons, keeping the culture at lower densities from the beginning helped when the glutamine-limitation was reached. Furthermore, as I plot below for the successful startup case, the avoidance of higher levels of conditioned medium by keeping cell density down may have also been beneficial. Startup may also have been improved by the use of conditioned medium from an inoculum that was passaged daily ($F_{initial}$ = 0.42 for the successful startup)

versus an inoculum that was passaged every other day ($F_{initial}$ = 0.89 for the unsuccessful startup).

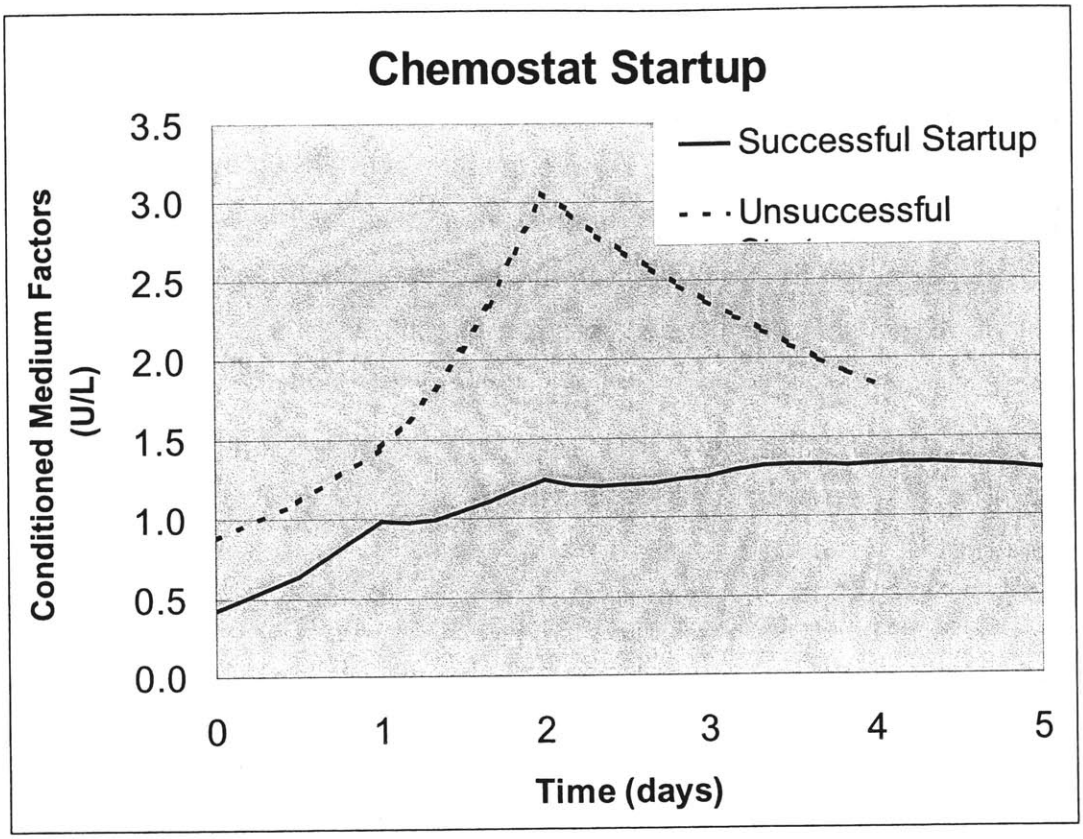


**Figure 6-4. Hypothesized death factor levels during successful and unsuccessful chemostat startup.**

### 6.3.2.5 Anchorage-dependent CHO Cultures

Anchorage-dependent cells lines are typically cultivated in 6-well plates (1-3 mL). Because the cells are attached, the culture medium is often replaced with fresh medium on a daily basis in order to replace nutrients and growth factors. Whether the medium is changed daily or not at all can have dramatic effects on the observed phenomena. From the point of view of the conditioned medium hypothesis, the experiment in which the medium is frequently replaced is more likely to be studying the effect of cellular phenomenon, whereas the true batch experiment provides an additional environmental effect, which in terms of cell death, may completely over-shadow the cellular phenomenon.

The model for medium conditioning may be helpful in the design such 6-well plate experiments. If the goal is to study the effect of conditioned medium factors, the medium should not be replaced. Feeding of additional nutrients and growth factors can be performed if necessary, as well as pH and osmolarity control. If the effects of conditioned medium factors are to be minimized, culture medium should be replaced frequently. The model can be used to estimate the necessary time interval between medium replacement, based on the viable cell density and an assumed specific production rate for conditioned medium factors, $q_F$. From my model for hybridomas, cultures containing insulin can proliferate up to a conditioned medium factor concentration of F = 2.8 units. If for growing cells we take $q_F$ = 0.69e-7 unit/cell/hr, the medium replacement interval will be equal to $F/(q_F*n_v)$. For an average of 1.0e6 cells/ml, the medium should be exchanged every 40 hours, and daily replacement would be adequate. For twice the density, however, medium exchange would need to occur within 20 hours, and hence daily feeding may not be capable of eliminating ill effects of conditioning.

Such effects of medium replacement are apparent in the works of Anna Sanfeliu. Below are cell density and viability profiles for anchorage CHO cells, switched from 5% serum to 1% serum, with or without insulin, on day 5 (Figure 6-5 a b). The first set of plots is for the case where medium is not exchanged at all; the second set of plots is the corresponding experiment during which she did exchange medium daily. A comparison

of the experiments reveals that insulin has a deleterious effect that is much more severe in the presence of conditioned medium factors. (Note: for the first set of experiments, measured levels of nutrients and waste metabolites verify that sufficient nutrients remain, and that lactate and ammonia, though quite high, are not past their toxic thresholds). A second observation is that cell densities are suppressed in the presence of conditioned medium factors. Both these observations are similar to the ones I have made for hybridomas. Conditioned medium factors may arrest cells uniformly throughout the cell cycle, leading to slower growth and cell death. For CHO cells, the presence of insulin has been shown to stimulate entry to the cell cycle. Thus, in the presence of conditioned medium factors that inhibit cell cycle progression, the increase in insulin's negative effect on CHO viability may be due to the arrestment that occurs within the cell cycle due to these factors. In the absence of conditioned medium factors, lesser inhibition through the cell cycle results in lesser cell death. Finally, as discussed above, it is interesting that the plateau in cell growth for the daily-fed cultures and the emergence of cell death in the insulin-experiment occurs at a density around 2e6 cell/ml. Though perhaps this density is that of confluence, another explanation is that daily replacement of the medium was too long an interval to avoid effects from conditioned medium factors.

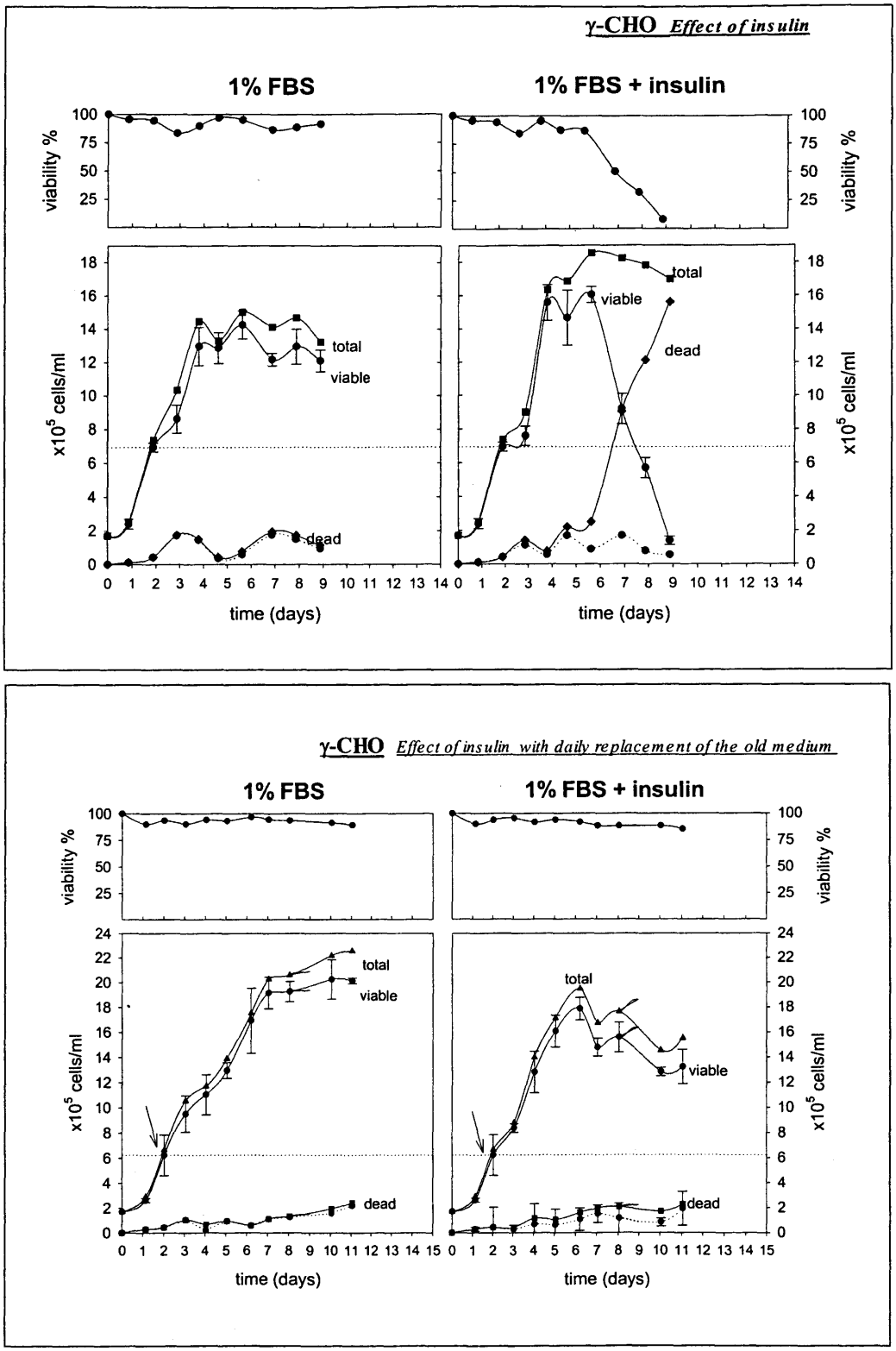


**Figure 6-5 a,b. Effect of insulin and conditioned medium on anchorage-dependent gamma-CHO cells.**

Data from Sanfeliu et. al. (Sanfeliu, Chung et al. 1999 in press)

# 6.4 OPPORTUNITIES FOR FURTHER IMPROVEMENT OF FED-BATCH CULTURES

## 6.4.1 Reduce Apoptosis by Supporting Cellular Function

### 6.4.1.1 Membrane Integrity and Energy Potentials

Part of the improvement of fed-batch cultures for the production of therapeutic proteins described within this thesis stems from the reduction of apoptotic cell death through the support of metabolism. It was shown that cells can shift their metabolism to accommodate a limitation in a key nutrient (Chapter 3) and that metabolic robustness depends on insulin stimulation (Chapter 5). Furthermore, there is sufficient evidence to hypothesize that the conditioning of culture medium impedes metabolic function. Thus, in the presence of insulin and in the absence of nutrient limitation and high levels of conditioned medium factors, a shift in metabolism towards efficiency is associated with better culture performance. In the chemostat experiments, a higher viable cell density steady state (the multiple steady state for D=0.04 hr-1) is achieved for a hybridoma culture upon lifting the nutrient limitation by increasing the dilution rate back up to D=0.04 hr-1 from 0.01 hr-1. Similarly, cells of a culture that are grown on a leaner medium exhibit extended viable proliferation during fed-batches (Brian Follstad's Thesis). Brian Follstad has linked differences in metabolic behavior to shifts in central carbon metabolism towards oxidative phosphorylation for cells with greater mitochondrial activity and potentials. Hence, the importance of metabolic function for culture robustness stems from the reduction in cell death that is observed for cultures exhibiting more efficient metabolism. Additional research to further protect metabolic function during fed-batches is warranted. The degradation of cellular membranes by environmental factors, and the resulting decrease in ion potentials, could very well be the principle cause for metabolic impediment. Brian Follstad's experiments with mitochondrion decoupling agents demonstrate that loss of mitochondrial potential results in apoptosis via the release of apoptosis inducing mitochondrial factors such as cytochrome C and reactive oxygen species.

The "strengthening" of the mitochondrial membrane is a possible explanation for the benefits of Bcl-2 overexpression. Bcl-2 is an anti-apoptotic protein that is found predominantly in mitochondrial membranes. It has been shown that part of its protective role is associated with decreasing the release of cytochrome C from the inner mitochondrial space. In light of the role that mitochondrial potential has in enhancing culture performance, it is not unreasonable to suggest that genetic factor like Bcl-2 is actually a "pro-mitochondrial" protein first and foremost, rather than anti-apoptotic protein. The relationship between Bcl-2's role in reducing apoptosis and its presence within mitochondrial membranes agrees very well with the evidence for metabolic function and apoptosis presented in this thesis and that of Brian Follstad.

#### 6.4.1.2 Robustness within Cell Cycle

For the hybridoma cell line, the support of proper cell cycle progression initially takes the form of addressing the defective cycle entry that leads to cell death under suboptimal conditions. Observation of defective cell cycle control in chemostats, and the measurement of high levels of c-Myc, led to the use of cell cycle arresting agent rapamycin to mildly slow the progression of cells through the G1-phase. This was remarkably successful in delaying the emergence of apoptosis and minimizing it thereafter. As is discussed earlier, possible explanations for the anti-apoptotic effect include allowing cells to complete production of intracellular factors, minimizing cell death due to S-phase arrestment, shifting metabolism towards efficiency, reducing the production of detrimental conditioned medium factors, and/or affecting the regulation of any of a number of other intracellular proteins.

There are ample opportunities for additional research into the role of cell cycle progression and apoptosis. To dissect the current handful of explanations for rapamycin's effect, other means for arrestment should be investigated. For example, it may be of interest to see if the knockout and reintroduction of *c-myc* on an inducible promoter would yield a hybridoma that is much less prone to apoptosis. Another interesting pursuit would be to investigate the induction of apoptosis within the S-phase and G2/M-phases. Cell biology contains much information about checkpoint-mediated arrests, and the ensuing apoptosis that can occur if the checkpoint is not passed.

Perhaps, given that delays are bound to occur within the cell cycle due to nutrient or conditioned medium factor limitation, it would be useful to learn how to fortify cells against apoptosis when such delays occur. The arrestment is probably best left intact to prevent serious genetic problems, but a delay in apoptosis upon checkpoint arrest may grant cultures another level of robustness against suboptimal conditions.

Interestingly, the protein Bcl-2 protein has also been implicated in delaying progression of cells from the G1-phase into the S-phase. Knowledge herein pertaining to the interaction between metabolic function and cell cycle progression may imply that Bcl-2's effects on cell cycle could be due to its primary support of metabolism. In the absence of Bcl-2 (and for cell lines showing normal cell cycle entry regulation), lesser metabolic capacity under suboptimal conditions may perhaps compromise the control of cell cycle entry, which depends on phosphorylation events requiring energy.

#### 6.4.1.3 Three-Prong Strategy

In the direct pursuit of high-density fed-batch cultures that yield record-high therapeutic protein titers, I believe that a combination of pro-cellular function / anti-apoptotic strategies could achieve the best fed-batch performance to date, even in serum-free medium. The three strategies that have each been proven to enhance culture viability are Bcl-2 overexpression (Joydeep Goswami and others), adaptation of inocula to metabolic efficiency and increased mitochondrial potential (Brian Follstad), and medium supplementation with rapamycin (myself). Since each strategy may be unique in its mechanism to reduce apoptosis, I would expect some synergy among the effects. Hence, a conservative estimate of the possible process improvement would be that the effects combine to delay the emergence of apoptosis up through 96 hours (4 days), one more day beyond rapamycin alone. Similar to my analysis of previous fed-batch performances in Chapter 2, I estimate that the expected titer for such a 10-day fed-batch process would be 1.3 g/L, up 0.35 g/L from the expected 0.95 g/L for a serum-free rapamycin-fed fed-batch.

### 6.4.2 Search for Conditioned Medium Death Factors

#### 6.4.2.1 The Presumed Nature of the Death Factor

The observed effects of conditioned medium on insulin-free batches point to both positive and negative factors that influence proliferation and apoptosis. Being that insulin supplementation appears to provide sufficient stimulation, that even over-rides the effects of other positive factors such as Il-6 (data not shown), the determination of the negative factors and the prevention of their negative effects is a promising hypothetical direction to pursue for the improvement of fed-batch processes.

As discussed in earlier sections, I am hypothesizing that the conditioning of culture medium negatively affects cellular metabolism. Under this hypothesis, the effects of both insulin and rapamycin on metabolism lead to protection against the accumulating conditioned medium factors. Furthermore, as calculated for insulin-free chemostats, the cellular metabolism may play a role in the rate that medium is conditioned. Cultures under sufficient glutamine-limitation and cultures in the presence of rapamycin may survive longer as a result of the shift in their metabolism, that in turn possibly reduces the rate of contamination of the culture environment.

Relying on the findings in this thesis, as well as those from Brian Follstad, concerning the effect of metabolism on apoptosis, I imagine that the conditioned medium factors impede metabolic function by interfering with the integrity of cellular membranes (such as in the mitochondria) and the energy potentials that they maintain.

#### 6.4.2.2 Preliminary Findings

The hypothesis of other unknown factors that stimulate cell death stems from the partial success of rapamycin in delaying apoptosis. If rapamycin's effect were purely of a cellular nature, I would have to hypothesize why that nature succumbs to cell death eventually anyway. It seems more plausible to imagine that rapamycin protects cells against the conditioning of the medium, in the same fashion that we imagine how insulin protects cells.

The differences in metabolism between rapamycin and control batches and fed-batches were first observed in the difference in the color of T-flask cultures. With phenol red as a pH indicator, it was evident that the rap culture pH at day 2 was around 7.1 (red color still), whereas the control culture at day 2 was around 6.8-7.0 (more orange). This observation raised the question of whether the difference in pH was simply the result of

differences in cell proliferation (rap ~ 0.8-1.2e6 cell/ml vs. control ~ 1.0-1.5e6 cell/ml) or if it were also an indication of differences in cellular metabolism. As shown in Chapter 4, measurements of metabolites indicate that rapamycin culture metabolism appears to produce less lactate. Knowing that cell death occurring in the absence of excessive lactate accumulation or suboptimal pH, the difference in T-flask lactate and pH was taken to be evidence that other unknown factors that affect cell death could also be different. In this sense, it was rapamycin's effect on the conditioning of medium that could possibly explain its anti-apoptotic effect.

At the same time I was trying to hypothesize why rapamycin cultures still were being compromised by cell death, phenomena from the polymer-encapsulated hybridoma cultures provided possible evidence of negative factors that may accumulate within the beads. The increase in pore size, discussed earlier, allowed the cells to reach densities 10 times greater. Knowledge of the estimated pore sizes for standard polymer beads and those with peg 3400 provide a rough indication of the size of the hypothesized death factor. Cells in standard polymer beads had pores large enough to allow insulin (5 kDa) to pass through, but small enough to keep the MAb product (150 kDa) within the beads. The larger pore size had an estimated cutoff of roughly 150 kDa up to 2000 kDa, yet still retained a significant amount of MAb's within the beads. Thus it seems plausible that a "death factor" could be a compound about 10 to 100 kDa in size. However, transport across a pore also depends on the shape and charge of the compound, hence the compound could be outside of this range nevertheless.

The search for the unknown cause of apoptotic cell death in hybridoma cultures began by screening two well known environmental protein death factors, FAS ligand (FAS-L) and Tumor Necrosis Factor Alpha (TNF-$\alpha$). These substances have been shown to directly trigger apoptosis in susceptible cell lines. However, neither of these substances triggered cell death during 22 hours of incubation, as would have been expected in FAS or TNF-$\alpha$ sensitive cell lines.

To determine if death activity could be contained within conditioned medium, I ran batch cultures in the presence of concentrated fractions of highly conditioned medium. The fractions were obtained from the conditioned medium of a viable culture grown up to 1.8

x $10^6$ cell/ml during 48 hours of culture (F = 2.6) for this conditioned medium). This highly conditioned medium was concentrated by ultrafiltration using filters with molecular weight cutoffs of 10k, 50k, and 100k. 15 ml of the conditioned medium was placed in each type of ultrafiltration device, and spun down at room temperature for one hour, at which time the retentate volumes were about 1-2 ml (7.5x-15x concentrates). The retentate was then spiked into 18 hr old, high viability, 5.0 ml cultures. Assuming the death factor was retained by the ultrafilters and completely transmitted, the recipient culture would have received more than twice as much death factor as that present just before death typically emerges in batch experiments (the F-level of the conditioned medium would have risen from 0.8 to 6.1-7.2). Such a dose of death factors should have killed the culture immediately. However, no such cell death was observed and all the recipient cultures grew normally with high viability until 45 hours, after which they began to die similar to the control. Taken at face value, this experiment tells me that the death factor is not necessarily a very large molecule. Perhaps it is around 5k MW, yet of shape and charge that a change in the pore size of the polymer beads was still influential in its transport. Indeed, an autoinhibitor for murine hybridomas was isolated previously – by hollow fiber filtration it is estimated to be around 3000 Da (Lee, Yap et al. 1995) but remained unidentified.

#### 6.4.2.3 Possible Candidates for Future Investigation

So the question remains as to what compound or quality of the conditioned medium the death factor may be. From experience I can rule out pH, temperature, lactate accumulation, ammonia accumulation, and dissolved oxygen limitation. Possible culture parameters that I have not measured myself include dissolved $CO_2$ levels, osmolarity, ionic strength (salt balance) of the medium, and reducing power of the medium. It is known that these factors can be detrimental to cultures, though cells can typically grow within wide ranges of each such factor. The emergence of death during batches *and* chemostats (where pH, DO, are controlled, and where fresh medium maintains CO2 levels and osmolarity and reducing power within healthful levels) detracts from these being good candidates to pursue. Based on my preliminary observations, my best guess for the nature of the death factor at the moment is that it may be a low-level metabolite associated with excessive and/or inefficient glycolytic metabolism. Such a

metabolic (methyl glyoxal) has been reported for CHO cells and is linked to catabolism of amino acids and glucose (Chaplen, Fahl et al. 1996; Chaplen, Fahl et al. 1998). This type of factor fits my preliminary findings in terms of size. It also satisfies my hypothesis that metabolic profile of a culture can influence production of the unknown factor.

A good opportunity for identifying a death factor lies in the comparison of rapamycin-conditioned and regular conditioned medium. To prove that the rap conditioned medium is actually the key to understanding how rap cultures have delayed apoptosis in batch cultures, one would need to demonstrate that 50hr-rapamycin-conditioned medium (Rap-CM) *can* support viable proliferation, whereas 50hr-control-conditioned medium (C-CM) *cannot*. This could be shown by exchanging the conditioned medium of two parallel cultures, one grown with and one grown without rapamycin, at approx. 50 hrs of culture, when both cultures still have high viabilities. According to the conditioned medium hypothesis, I would predict that the rap-cells will die when placed in C-CM, while the control cells will actually live in the Rap-CM. Several variations of such experiments could be run to vary the effects of culture age (24-hr cells versus 48-hr cells) and the strength of the conditioned medium (40 versus 50 versus 60-hr CM) if necessary. It would also be wise to measure and compare pH, osmolarity, $pCO_2$, and any other possible measurable parameters, for clues concerning the emergence of cell death.

# 7 CONCLUSION

## 7.1 BRIEF SUMMARY

The study of hybridoma culture physiology in terms of metabolism, cell cycle progression, and apoptosis has in the end provided a better phenomenological description of the changes in some essential cellular functions that are associated with the emergence of apoptosis. The emergence of death was examined in response to several distinct environmental factors: under nutrient limitation, following insulin withdrawal, with rapamycin supplementation, and in the presence or absence of different types of conditioned medium. In all cases it appears that cellular metabolism shifts towards greater efficiency and (assuming a constant P/O ratio) greater energy production. As the result of deregulation of cell cycle entry, the cell cycle is delayed in the S-phase for nutrient limitation and in all the phases for insulin depletion and accumulation of higher levels of conditioned medium factors.

These characterizations for metabolic and cell cycle changes associated with apoptosis have yielded fruitful insights and strategies for the improvement of fed-batch cell culture. I have successfully applied rapamycin supplementation to minimize the negative effects of the faulty control for cell cycle entry. For reasons unknown, a delay within the G1-phase of the cell cycle serves to postpone the emergence of apoptosis. Brian Follstad has identified that metabolically robust cells are ones that survive longer during fed-batches or yield multiple steady states of higher cell density in continuous culture. He has linked such metabolic robustness to increased mitochondrial activity and potential. The prominence of metabolic phenomena in the emergence of apoptotic cell death leads me to believe that insulin's protective role for hybridomas is based on its support for metabolism. Likewise, even though it is better known as a G1-phase arresting agent, rapamycin's protective role may also be linked with a metabolic shift supported by preliminary evidence. Lastly, the effect of conditioned medium on hybridoma cultures is made evident in survival or death in the absence of insulin. Survival factors such as

insulin and rapamycin (as well as possibly Bcl-2) appear to offer protection only in the midst of high levels of conditioning.

In terms of further enhancing fed-batch technology, there remains plenty of room for improvement of culture performance in regard to extending viable proliferation and maximizing productivity. Such advances will come from the understanding and exploitation of the cells own resources for survival. The basis on which we choose further environmental and genetic modifications to cellular behavior will depend on continued advances in our understanding of the cellular constraints (such as those that have been observed herein for metabolism and cell cycle progression) that determine whether a cell lives or dies by apoptosis.

## 7.2 LIST OF OVERALL FINDINGS

The following list serves to itemize my findings in this thesis concerning the effects of rapamycin and insulin on the cell cycle and apoptosis of hybridoma cells. Due to its prominence in the discussion, such a list must also include findings regarding metabolism to be complete, and so I also include some overlapping findings from Brian Follstad. In the following statements I also try to be clear about stating what has been determined as fact and for which there is proof, and what I have hypothesized and therefore is open to interpretation and future investigation.

1. Glutamine-limitation by the reduction of dilution rate in continuous culture steady states is associated with increases in apoptotic cell death of hybridomas.

    1a. Cell cycle progression through the S-phase is delayed from 10 hours up to 20 hours, while progression through other phases remains relatively constant. The increase in S-phase progression correlates with the emergence of apoptotic cell death. The shortage of precursors for DNA synthesis may be the cause for the delay of progression. Cell cycle regulation for DNA synthesis (perhaps by p53) may be the trigger for apoptotic cell death.

1b. The emergence of cell death within the S-phase is very likely exacerbated by the lack of regulation of cell cycle entry, that is due at least in part by the measured high expression levels of c-Myc. Inappropriate entry to the cell cycle under sub-optimal conditions (such as nutrient limitation) may lead to higher levels of apoptotic cell death than would otherwise occur. This has been indirectly demonstrated by inhibiting cell cycle entry with rapamycin, though the mechanism for rapamycin's anti-apoptotic effect is unclear.

1c. Metabolism for steady states at lower dilution rates shifts towards greater efficiency for the consumption of metabolites yet higher consumption of oxygen. The shift in metabolism is deemed a robust response since the efficient metabolic state is shown to yield a multiple steady culture of higher viable cell density upon returning to a fast dilution rate and is associated with enhanced viability for fed-batches inoculated with cells of greater metabolic efficiency.

2. Cell death in fed-batch cultures occurs by apoptosis and is associated with the inhibition of growth rate.

2a. When apoptosis emerges, cell cycle progression is delayed for all phases of the cell cycle. It is therefore presumed not to be the primary cause for growth inhibition.

2b. Metabolism is observed to shift towards increased efficiency, as if to meet increased energy requirements. This increase in energy demand is taken to be reflective of an increase in maintenance energy required as the culture medium becomes more conditioned. Thus, as culture progress in fed-batch, energy supply may be a limiting factor and may be the cause for growth inhibition, delays in cell cycle, and apoptosis.

3. Rapamycin postpones the emergence of apoptotic cell death in insulin(+)-hybridoma cultures.

3a. Rapamycin, a known G1-phase arresting agent, delays the progression of hybridoma cells through G1 by 2 hours during optimal conditions early in a batch or fed-batch.

3b. Rapamycin postpones the emergence of cell death from 48 to 72 hours after the start of a fed-batch. This anti-apoptotic effect of rapamycin has not been previously described. This phenomenon is explained by the known effect of rapamycin on the control of mRNA translation and ribosome function, and hence the overall protein profile of the cells. Proteins reported to be affected by rapamycin include some related to the regulation of cell cycle entry. Preliminary evidence regarding a shift in metabolism may reflect changes in proteins related to metabolic function as well.

3c. The optimum concentration for the hybridomas of this study was determined to be 50-100 nM. For extended cultures, the poor stability of rapamycin is managed by feeding 50 nM daily (starting at day 2), in addition to the 100 nM added initially. Loss of rapamycin activity is observed in a decreased fraction of cells in G1 and higher cell death. 200-nM rapamycin is observed to be suboptimal due to the increased suppression of growth rate, and hence lower cell density, without the benefit of the decreased death rate.

4. Indirect evidence demonstrates that insulin acts as a protective factor for serum-free hybridomas through its effect on metabolism rather than cell cycle.

4a. Insulin's protective role is demonstrated by its support in extending viable proliferation from 25 to 50 hours in batch cultures. High viability of batches passaged daily and the survival of three of five insulin-free chemostats implicate insulin as protecting against environmental factors found in conditioned medium.

4b. The presence of insulin for cell cycle progression is not absolutely required. Cell cycle progression in the absence of insulin implies that either other autocrine factors are present to stimulate signal transduction or that such circuits are even more deregulated beyond that observed for *c-myc* expression. The positive effect of insulin-free chemostat conditioned medium on proliferation makes the explanation of autocrine factors more plausible.

Furthermore, cell cycle phenomena associated with cell death are similar with and without insulin and are not associated with an arrest in any particular phase of the cell cycle

4c. Metabolic support by insulin is implicated by the requirement for insulin in environments that are more conditioned. With cell death in fed-batches possibly being due to energy limitation (a limitation possibly arising as the result of increased conditioning of culture medium), insulin is thought to confer additional metabolic capacity for the cells as they are observed to shift their metabolism toward TCA cycle as they die.

5. Unknown conditioned medium factors are hypothesized to play a role in the emergence of apoptosis.

5a. Insulin-free experiments revealed that the environment plays a key role in the survival of cultures in insulin-free environments. Cells surviving without insulin at steady state in a chemostat still required insulin for viable proliferation past 24 hours in batches. Furthermore, the insulin-free chemostat-conditioned medium was able to support viable proliferation for up to 48 hours for both insulin-free chemostat cells and a fresh stock of cells.

5b. The production and accumulation of conditioned medium factors that stimulate apoptosis is modeled. Calculations for insulin(-), insulin(+), and insulin(+)-rapamycin. experiments reveal consistent thresholds for each case. Moreover, insulin-free chemostats point to the production of such factors as being variable, perhaps depending on metabolism and cell cycle.

5c. Within this framework, the hypothesized conditioned medium factor that stimulates cell death is attributed to be one of the unknown causes for cell death in fed-batch cultures. If indeed a principle cause for apoptosis, accumulation of the factor could be imagined to result in a metabolic impediment, which insulin and rapamycin allow cells to combat more effectively.

6. Monoclonal antibody production is observed to be a function of metabolism, cell cycle progression, and cell death.

6a. An optimum in specific productivity for MAb production of 2.3e-9 mg/cell/hr is observed at an intermediate dilution rate of 0.03 $hr^{-1}$ in steady state chemostats. Such an optimum is believed to reflect differences in metabolism rather than cell cycle progression. A lower productivity from the multiple steady state may support this belief.

6a. The combined effects of rapamycin on cell cycle distribution and metabolism lead to an increase in the apparent specific productivity of MAb's from 1.5e-9 up to 3.2e-9 mg/cell/hr. This physiological change is the dominant effect for the achievement of higher MAb titer.

6b. For the 6-day fed-batches performed, rapamycin addition resulted in an increase of titer from 0.25 to 0.55 g/L. On a ten-day basis for comparison of fed-batch titers, a fed-batch process with rapamycin is expected to yield a record titer of 0.95 g/L for a serum-free process, which is competitive to previous processes with this cell line, where 2.4 g/L was obtained from a three week fed-batch using serum.

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