Characterization of the Down Regulation of Antibody Production in High Cell Density Perfusion Culture

by

Joyce Catherine Morrill

B.S. Chemical Engineering, Massachusetts Institute of Technology (1984)
M.S. Mechanical Engineering, Duke University (1987)

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Signature of Author _____
Department of Applied Biological Sciences
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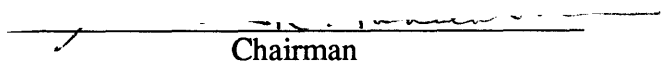
Certified by _____
William G. Thilly
Professor of Applied Biology and Civil Engineering
Thesis Supervisor

Accepted by _____
Steven R. Tannenbaum
Chairman, Departmental Committee on Graduate Students

"This doctoral thesis has been examined by a Committee of the Department of Applied Biological Sciences as follows:"

Professor Steven R. Tannenbaum



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Professor William G. Thilly

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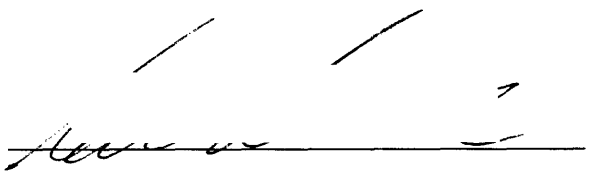

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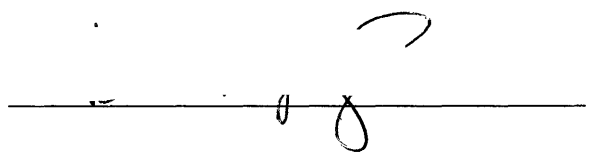

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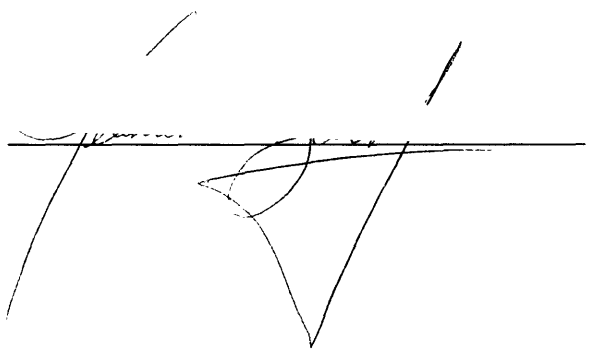

Characterization of the Down Regulation of Antibody Production in High Cell Density Perfusion Culture

by Joyce Morrill
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Abstract

A marked drop of secretory protein production with increasing cell density is a common problem with industrial scale bioproduction. Research in this laboratory with a high density perfusion reactor has reproduced this phenomenon at the bench scale using an antibody producing cell line. As the cell density increased from 10^6 to 10^7 c/ml, the antibody secretion rate declined 10 fold from about 0.2 to 0.02 pg/cell-h. The reversibility of the down regulation was demonstrated by removing cells from high density and observing that at lower density these cells regain their secretion ability. A concomitant but unexplained metabolic change is that the overall uptake rate of amino acids declines by 5 fold (Tyo, 1991).

This research answers two questions. The first question is, "What is the signal for the reversible decrease in antibody production ?" Three hypotheses have been examined: nutrient limitation, cell-to-cell contact inhibition, and a diffusible inhibitor. The nutrient limitation theory was eliminated because amino acids and saccharides were not depleted at high cell density (Tyo, 1991), and antibody production was not restored by increases in serum components and sulfur containing compounds. The cell to cell contact theory was eliminated as a primary down regulator because high density medium (without cells) inhibited antibody production, and membrane fractions did not inhibit production. Diffusible inhibitors are the external signal for the decrease in antibody production. Lactate and ammonia are the primary inhibitors, accounting for about 2/3 of the inhibition of antibody production. Experiments with fractionated high density, low productivity media showed that the additional factors inhibiting antibody production are of 10,000 MW or less.

The second question is, "What do cells do with the excess of amino acid uptake at low density ?" Cells at low density in the bioreactor take up amino acids at a rate greatly exceeding requirements for protein and nucleotides. Most of this "excess" can now be attributed to de novo lipid synthesis. I have investigated several major changes in cell biochemistry coincident with the decrease in antibody production and increase in cell density. With the experimental data for growth rates, cellular content, and amino acid uptake rates, the mass balance on amino acid uptake at both high and low cell density is now closed. By flow cytometry, I demonstrated that the cells continue to grow in the reactor and that cell cycle time lengthens by 25% (at high density). Between 1 and 10 million cells/ml, cellular content declines: (a) 66% decline in total lipid per cell, (b) 33% decline in total protein, and (c) 85% decline in the free amino acid pool. This correlates with a 50% decline in cell volume. I found that the uptake of radioactive leucine into lipid is a function of cell density. The changes in lipid are, however, not a function of the antibody inhibitors lactate and ammonia. Because amino acid uptake into lipid declines as a function of cell density, and because of the decline in total lipid per cell, the amino acid requirements for lipid synthesis becomes much less than protein and nucleotides at high density.

Thesis Supervisor: Dr. William G. Thilly
Title: Professor of Applied Biology and Civil Engineering

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The technical assistance of others is gratefully acknowledged. Lori Tsurida isolated the the high producing clone that we used. Peter Becker contributed to the second bioreactor experiments. Jackie Griffith helped with many experiments over the years. Thanks to the members of the Langer and Grodzinskey groups for the use of the plate readers. I would like to thank Stuart Conner and Glen Paridis in the flow cytometry lab for sorting samples, for helping me to work out experimental procedures and for teaching me how to run samples.

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List of Symbols and Abbreviations

Ala	alanine
Arg	arginine
Asp	aspartate
Asn	asparagine
c	cells
Ci	Curie
cpm	counts per minute
D	dilution rate in bioreactor (h^{-1})
DMEM	Dulbecco's Modified Eagles Medium
DMEM*	Dulbecco's Modified Eagles Medium supplemented with 6 mM glutamine, 2 mM pyruvate, and 50 μM β-mercaptoethanol
E_h	redox potential (mV)
E'_0	standard potential of redox couple (mV)
F	flow rate (ml/h)
F	Faraday constant (96,406 joule/volt)
FBS	fetal bovine serum
fmole	femtomole (10^{-15} mole)
G_o	resting phase of cell cycle
G_1	gap 1 phase of cell cycle
G_2	gap 2 phase of cell cycle
Gln	glutamine
Glu	glutamate
Gly	glycine
h	time (hours)
HDLP	high density, low productivity
His	histidine
IgG	immunoglobulin G
Ile	isoleucine
l	liter
Leu	leucine
M	mitosis phase of cell cycle
Met	methionine
nm	nanometers

mg	milligram
ml	milliliter (10^{-3} mole)
mM	millimolar (10^{-3} mole/l)
μM	micromolar (10^{-6} mole/l)
mV	millivolt (10^{-3} volt)
n.d.	not determined
PBS	phosphate buffered saline
pg	picogram (10^{-12} gram)
Phe	phenylalanine
pmol	picomole (10^{-12} mole)
Pro	proline
Q	volumetric flow rate (ml/min)
Q_p	volumetric rate of product formation (mole/l-h or g/l-h)
q_p	specific rate of product formation (mole/cell-h or g/cell-h)
Q_S	volumetric rate of substrate utilization (mole/l-h)
q_S	specific rate if substrate utilization (mole/c-h)
R	gas constant (8.31 joule/^{o}K-mole)
s	time (seconds)
S	synthesis phase of cell cycle
	or
S	substrate concentration (mole/l)
S_i	inlet substrate concentration (mole/l)
S_o	initial substrate concentration (mole/l)
Ser	serine
t	time (seconds, minutes or hours)
td	doubling time of cells (hours)
T	temperature (^{o}K)
The	threonine
Trp	trptophan
Tyr	tyrosine
V	bioreactor volume (ml)
Val	valine
X	cell concentration (cells/ml)
X_o	initial cell concentration (cell/ml)
X_D	dead cell concentration (cell/ml)
X_V	viable cell concentration (cell/ml)

α	specific death rate (h^{-1})
ε	separation ratio of cells removed from reactor (dimensionless)
ε_D	separation ratio of dead cells (dimensionless)
ε_V	separation ratio of viable cells(dimensionless)
μ	specific growth rate (h^{-1})
μ_{app}	apparent growth rate (h^{-1})

I. Introduction

A. Background

1. Antibodies for Human Use

The recent advances in biotechnology have provided the possibility for the diagnosis and treatment of human diseases with large quantities of proteins. Instead of purifying proteins from animal sources, cells which produce the desired product are grown in a regulated cell culture system of either bacteria, yeast, or mammalian cells. Currently marketed proteins produced from bacteria cells (*E. coli*) are recombinant human insulin, human growth hormone and interferon-alpha 2a. The hepatitis B vaccine is produced in yeast cells. Marketed proteins produced from mammalian cells are tissue plasminogen activator, Factor VIII, erythropoietin, beta-interferon and monoclonal antibodies. Mammalian cells are chosen over yeast or bacterial cells when the protein requires post-translational modifications, such as glycosylation, proteolytic cleavage, or disulfide bond formation. Another advantage of mammalian cells is that the protein is secreted in an active form. The sales from these biotechnology products about 50% from mammalian cells (Spier, 1991). The sales of monoclonal antibodies for health care in the US is projected to increase to one sixth of the market share of recombinant proteins (Frost and Sullivan, 1989).

Antibodies, as found in blood serum, are a heterogeneous mixture of proteins which bind with great specificity to a foreign substance called an antigen. The antigen is often a protein or polysaccharide. The antibodies bind specifically to the three dimensional structure of the antigen in a "lock and key" fashion. B Lymphocyte cells secrete antibodies in response to an antigen. *In vivo,* many antibody proteins may bind to each antigen, and many antigens are present.

An antibody (or an immunoglobulin) protein consists of four amino acid chains, two heavy chains and two light chains. The four chains are joined by inter and intrachain disulfide bonds. There are five classes of antibodies that play different roles in the body. One portion of each chain is variable in sequence, the rest of the chain is constant for the class. The antigen binding regions are composed of the variable parts of each heavy and light chain. The variable sequences form unique three-dimensional pockets in which an

antigen binds. Each immunoglobulin has two identical antigen binding regions. A binding region is specific to a single region of an antigen, such as 5 or 6 amino acids or sugar residues of a protein.

The five classes of human antibodies proteins are designated IgG, IgA, IgM, IgD and IgE, and vary in the constant regions of the heavy chains. The corresponding heavy chains are calls γ, α, μ, δ and ε. The molecular weights of the heavy chains range from 50,000 to 70,000. The light chains in any class are of either two types (κ of Λ) with molecular weight of 23,000. IgG, IgD and IgE are composed of two heavy (H) and two light chains (L) for H_2L_2. IgM is composed of five H_2L_2 units joined by five joining (J) chains. IgG consists of ~75% of all the antibody in the blood. IgA is in body secretions as a dimer $(H_2L_2)_2$ with a secretory component and a J chain, and as either the dimer (without the secretory component) or H_2L_2 in the blood. Subclasses of H chains also divide the classes IgG, IgM and IgA. Characteristics of the IgG class include ability to signal other cells by activating the complement cascade and cytotoxic activity through receptor interactions. The four subclasses of IgG differ in their cytotoxic activity and complement activity. IgG is the only class that crosses the placenta. IgM is expressed first during an immune response, and has antibacterial activity. and the highest complement fixation activity. In secretions, IgA blocks antigens and has antivirial and antibacterial activity. Both IgG and IgA boost the immune system of infants through mothers milk. IgD is the class predominantly present on the surface of B lymphocytes. IgE is present in the lowest concentrations in blood and is associated with allergic type reactions. In the production of antibodies, cell lines secreting IgG or IgM are most likely to be produced by the fusion technique of Köhler and Milstein (1975), described below.

Köhler and Milstein (1975) developed a methodology to fuse a mortal antibody-producing B lymphocyte cell with an immortal B lymphocyte tumor cell, resulting in a hybrid cell line (or hybridoma) producing identical antibody molecules. Before then, in order to isolate antibody one had to immunize animals and purify blood serum, resulting in a heterogeneous (or polyclonal) mixture of antibodies. The technique of Köhler and Milstein allowed for isolation and expansion of single hybridoma cells into a population of identical cells (or clones) producing a homogeneous (or monoclonal) solution of antibody. By this method, an animal is injected with the antigenic substance and after the animal has had time to produce an immune response, the spleen (containing B lymphocytes) is removed from the animal. The spleen cells and the tumor cells are mixed and fused with inactivated Sendai virus or chemically with polyethylene glycol. The

immortal tumor cells are deficient in an enzyme required for the salvage pathway of nucleotides and will not grow in the selective media. The selective media, HAT, blocks the de novo pathway for nucleotide synthesis with aminopterin and supplies precursors to the salvage pathways, hypoxanthine and thymidine. The spleen cells do not grow in culture, and only fused cells survive in the HAT media. The population of fused cells are plated as single cells into multiwell plates and allowed to grow as clones of cells. Each clones produces one antibody protein. The media from the wells are tested for presence of the desired antibody, and selected clones of cells are expanded for production of the monoclonal antibody. Monoclonal antibodies have been produced in the past *in vivo* in the ascities fluid of rodents, and currently *in vitro* in cell culture.

The practical significance of improved antibody production is to reduce the production costs of proteins for health care for applications ranging from cancer therapy to medical imaging to diagnostics. Antibodies find many applications because they bind with great specificity to antigens such as on the surface of cancer cells and other disease-related antigens. A summary of the US demand for antibodies is presented in the following table. The demand for monoclonal antibodies is predicted to reach $18 billion by 2000 (The Freedona Group, 1991). The home medical testing market is dominated by pregnancy and ovulation prediction kits. In vivo imaging products use radioactivity labeled antibodies to locate cancers and cardiovascular disease. The largest growing application in the in vitro diagnostics market is testing for AIDS. Antibodies for therapeutic uses have been developed for the treatment of cancers (or immunotherapeutics), such as breast and colon, and for autoimmune diseases, such as rheumatoid arthritis.

Table 1: US Market for Monoclonal Antibodies ($ Millions)

year	1990	1995	2000
Home Medical Testing	125	310	530
In Vitro Diagnostics	930	3100	7800
Therapeutic	75	700	7500
In Vivo Imaging	-	250	1560
Other	105	240	410
Total	1235	4600	17800

By the year 2000, the anticipated therapeutics market will require thousands of kilograms of antibody protein per year. Monoclonal antibodies used for human use sell for as much as 10 to 100 million dollars per kilogram due to the high cost associated with clinical trials, purification and quality assurance testing required for human injections (Leist, 1990). Using hundreds of milligrams per patient per year for 10,000 patients would require kilograms of antibody per year in order to treat a single form of cancer. For example, Sears *et. al.* (1985) used single doses of 200 to 250 mg of the murine 17-1A antibody in phase II clinical trials to treat gastrointestinal tumors. Antibody products are being developed to treat such cancers as: colorectal, prostate, lung, ovarian, breast, lymphatic, leukemia and melanoma.

2. Reactor Systems for High Density Production of Antibodies

Research in the field of monoclonal antibody production is driven by the needs for simple, practical and economic methods for producing antibody protein. A variety of reactor systems have been developed to produce antibody protein. The simplistic view of optimizing production is that by increasing the cell density by a factor of ten, ten times the nutrients are required and ten times the product is excreted. In reality, there is a density dependence of the metabolism of the cells and the production kinetics. The goal of this work is to characterize the effects of high cell density on one antibody producing cell line.

To reduce costs of purification, high density culture with a high antibody concentration is favored over a low density, low antibody concentration culture. Antibody producing cells do not attach to surfaces and grow freely in suspension. Antibody producing cells can be grown in any of the following methods of culture:

- batch -- cells are grown in a bioreactor without replenishing nutrients or removing metabolic products
- continuous culture -- cells are grown in a bioreactor with continuous supply of nutrients and continuous removal of cells and metabolic products
- perfusion culture -- like continuous culture, cells are grown in a bioreactor with continuous supply of nutrients and removal of metabolic products and antibody, but most or all of the cells are prevented from exiting the bioreactor

Both batch and continuous cultures give relatively low, maximum cell density on the order of 10^6 cells/ml. By retaining cells in a reactor and continually feeding nutrients and removing metabolites, perfusion systems can achieve cell densities of 10^7 to 10^8 cells/ml.

High densities of suspension type, antibody producing cells, have been achieved in both homogeneous (well-mixed) and heterogeneous perfusion systems. Some recent examples of heterogeneous perfusion systems used for antibody production include entrapment in hollow fibers (Altshuler *et. al.*, 1986a; Klerx *et. al.*, 1988), immobilization in packed beds of foam (Karkare *et. al.*, 1985) or glass (Bliem *et. al.*, 1992), and encapsulation in beads of alginate (Sinacore *et. al.*, 1989) or capsules of chitosan - carboxymethyl-cellulose (Yoshioka *et. al.*, 1990). Although these systems achieve high densities of cells (10^8 cells/ml), and sometimes high concentrations of antibody protein, they are not well mixed for nutrients or oxygen. Therefore the conditions of the bioreactor are not uniform and some conditions are suboptimal. During the operation of hollow fiber systems flow patterns must be alternated to prevent regions were oxygen and other nutrients are depleted or pH is decreased. Similarly, encapsulated cells in the center of beads have a different environment then cells at the surface of beads. Densities of immobilized systems are often limited by oxygen transfer. In a homogenous perfusion system, in contrast, all or most of the culture is retained in a stirred tank, and optimal conditions can be maintained uniformly throughout the bioreactor.

To study the effects of high cell density on metabolism and antibody production a continuous suspension, perfusion culture was selected. To maintain a high cell density a cell separation method is required, and a conical cell settling device was developed for this research (Tyo, 1991). This device separates cells by inclined sedimentation without moving parts. Against an upward flow of media, the cell separator operates on the principle of gravity settling cells down an inclined plane. (The bioreactor system is described further in section II.A).

This novel, conical cell settler design was selected over previously existing devices. Some researchers have used systems which require the continual pumping of cells out of the reactor, into the separation device, and returning the cells back to the reactor. Examples of such designs used to study antibody production include tangential flow cartridges (Reuveny *et. al.*, 1986), hollow fibers (Flickinger *et. al.*, 1990; Seamans and Hu, 1990; Smith *et. al.*, 1991; Hiller *et. al.*, 1993), and continuous centrifuges (Hamamoto *et. al.*, 1989; Tokashiki *et. al.*, 1990). Eliminating the passage of cells

through pumps, Shirai *et. al.* (1991) placed a .45 micron membrane at the bottom of a stirred reactor and achieved densities up to $7x10^6$ c/ml. Another method is to place the separation device, such a rotating spin filter (Himmelfarb, 1969) in the reactor. Some disadvantages of these devices include loss of cell viability and cell number as cells pass through pumps and clog filters.

Cell settling devices separate cells based on gravimetric settling of cells. These devices have the advantages of no moving parts or membranes that can clog. Various designs have been used to with hybridoma cultures to reach maximum densities up to 10^7 cells/ml. For example, Takazawa and Tokashiki, (1989) used an inner settling tube and Batt *et. al.*, (1990) used an external, inclined rectangular channel. The external, single cone design (Sato *et. al.*, 1983) has been used by many to reach densities of $1x10^7$ cells/ml (Kitano *et. al.*, 1991; Shintani *et. al.*, 1991; Hülsher *et. al.*, 1992). The settler design used in this work has two conical channels enclosed with an inverted cone and has been used to achieve densities of $2x10^7$ cells/ml (Buser, 1992; Tyo, 1991). The higher cell density may be a function of cell type, the number of channels or the angle of incline of the channels.

B. Problems in Achieving High Density Production

In theory, perfusion culture systems can maintain a culture indefinitely at high density. Nutrients such as amino acids, vitamins, and saccharides need to be continuously supplied to the bioreactor in amounts required for cell growth and in concentrations that can easily be taken up by the cells. Also, metabolic by-products need to be continuously removed and kept at concentrations low enough to prevent cell death. Oxygen must be supplied in amounts necessary for growth, and in a manner that does not cause cell death by foaming of the media or bubble bursting. The pH and redox can be held in a physiological range by buffering of the media and reducing lactic acid concentrations. Damage due to hydrodynamic forces is minimized by choice of stirring speed and bioreactor configuration.

For cell lines that secrete antibody best at slow growth rates, such as many mouse-mouse hybridomas, very slow growth and high density are desired for high production. High density perfusion culture of mouse hybridomas often increases production rates (e.g. Seaver *et. al.*, 1987; Batt *et. al.*, 1990) or does not change the antibody production rate (e.g. Hamamoto *et. al.*, 1990; Tokashiki *et. al.*, 1990; de la Broise *et. al.*, 1992). For cell lines that produce best at high growth rates, such as some human hybridomas (e.g. Shintaini *et. al.*, 1991; Kitano *et. al.*, 1991) and transfectomas (Robinson and Memmert, 1991), good growth and high density are desired for high production.

Even with all of the parameters optimized to achieve fastest cell growth and highest cell density, the secretion of the product can decline over time. Some loss of production over time is inevitable. Researchers usually resort to periodically restarting a culture with a frozen vial of high producing stocks. Sometimes the loss of production is reversible, and production of the population is rescued by removing the cells from unproductive bioreactor conditions. Research presented previously (Buser, 1992) and in this thesis, demonstrated both the irreversible loss of production over time and the reversible loss of production due to reactor conditions.

1. Irreversible Loss of Antibody Production

In general, the rate of production of antibodies from a population of cells decreases irreversibly with time in culture (Scharff *et. al.*, 1973). The rate of loss of production of myeloma and hybridoma cells has been reported in the range of 10^{-2} to 10^{-5} per cell per generation (Scharff *et. al.*, 1973, Galfré *et. al.*, 1980; Gardner *et. al.*, 1985). The mechanisms for an irreversible loss of production include:

- deletion of the entire gene
- small genetic alterations such as point mutations
- a decrease in transcription (DNA to RNA)
- a decrease in translation (RNA to protein)
- a decrease in protein transport out of the cell

These effects are thoroughly documented in the biological literature (James and Bell, 1987).

The overall irreversible loss of production of a cell line has been attributed to the appearance of a non-producer cell population (Frame and Hu, 1990; Ozturk and Palsson, 1990). A mass balance model including the rate of loss of antibody production and difference in growth rates of the populations of producers and non-producers has been analyzed by Lee *et. al.* (1991). When the non-producers grow slower than the producers then a stable population balance occurs at any rate of loss. When the non-producing subpopulation grows at a slightly faster rate than the producing cells, then over time, the population of nonproducers takes over the culture and the average production rate decreases. For example, at a rate of loss of production of about 10^{-3} per cell per generation, and a growth advantage of 10% for the non-producers, the culture is 50% non-producers in 100 generations.

The irreversible loss of production is a problem in the manufacturing of monoclonal antibodies. For example, Dean (1989) noted production of a murine hybridoma declined in a low density, continuous culture but was stabilized in a high density, immobilized culture of microsponges. Similarly, Lee and Palsson (1990) found immobilization in alginate beads to stabilize a culture. In continuous culture maximum growth rate can be obtained while in immobilized cultures very slow growth rates occur. The growth advantage of non-producers over producers is reduced at slower growth rates for mouse-mouse hybridomas. In the manufacturing of monoclonal antibodies, no serum low serum concentrations are desired to reduce purification costs. The irreversible loss of

production has been shown to be faster in low serum and serum free conditions than in high serum for one mouse hybridoma (Ozturk and Palsson, 1990, Chuck and Palsson, 1992).

2. Reversible Loss of Antibody Production

A reversible loss of production is defined here as a situation where high density per-cell protein production declines and the loss is reversed by returning the cells to low cell density.

The phenomenon of a reversible decline in antibody production is a common industrial problem, though it is rarely mentioned in the literature. Nevertheless, Kidwell (1989) has noted this in hollow fiber culture of two mouse hybridomas, COl 12 (IgG) and END (IgM). In continuous, suspension perfusion cultures, the mouse hybridoma AFP-27 (IgG) (Seamans and Hu, 1990) and the human hybridoma TFC-7 (IgM) (Kitano *et. al.*, 1991; Shintani *et. al.*, 1991) decreased production as cell density increased.

One cause for the decrease in the production can be nutrient limitation. Nutrients supplied to a culture include glucose, amino acids, vitamins, inorganic ions, and growth factors. In batch culture, rapidly consumed amino acid compounds, such as glutamine or isoleucine, are completely consumed. Research by Jo *et. al.* (1990) assumes that nutrient limitation is more important than waste product limitation and they have designed various nutrient-fortified media for high density culture of hybridoma cells. To maintain a continuous culture, we have used the strategy of perfusion culture to supply nutrients at a rate needed for cell growth.

Another cause can be an increase in concentration of diffusible inhibitors. The metabolism of glucose and glutamine lead to an accumulation of lactate and ammonia. Lactate and ammonia are inhibitors of growth and antibody production of hybridoma cells (Glacken *et. al.*, 1988, Reuveny *et. al.*, 1986.) The uptake of pyruvate and the metabolism of glucose to lactate alters the major redox pair, and the redox state of the culture (Imamura *et. al.*, 1982). Cells may secrete factors to regulate product production. Merten *et. al.* (1985) suggested that the antibody protein is a feedback inhibitor.

A third cause may be the physical interaction of cells at high cell density. Some cell lines that adhere to surfaces will stop growing when they cover the surface and are therefore called contact inhibited. Lieberman and Glaser (1981) reviewed the contact inhibition phenomena and the experiments that demonstrate that the plasma membranes of such cells inhibit growth. Cells that grow in suspension may also require interaction to signal high density and to slow growth. For example, Stallcup *et. al.* (1984a) demonstrated that cell membrane factors decrease the growth rate of lymphoid cells.

C. Reversible Decrease of Antibody Production in CLC Cells

Research in this lab has encountered the loss of antibody production in perfusion culture with a genetically engineered mammalian cell line, CLC, designed to produce a chimeric mouse/human antibody protein for colorectal cancer therapy (Sun *et. al.*, 1987). We have shown that the phenomenon is reversible by subsequently lowering the cell density. Experiments have shown that the phenomenon is accompanied by a molecular shift characterized by a decline in the steady-state mRNA and a decrease in transcription and translation rates (Buser, 1992). Additionally, there is a metabolic shift characterized by a decline in some amino acid uptake rates (Tyo, 1991).

Experiments to investigate the molecular cascade (Buser, 1992) comparing high production to low production with a second set of bioreactors demonstrated that a 10 fold drop in productivity can be accounted for by the following:

- no instability of the antibody protein or proteases in cell free bioreactor medium,
- no change in the copy number of heavy or light chain genes,
- the steady state heavy and light chain mRNA levels dropped three fold,
- a decrease in the immunoglobulin translation rate estimated to be 2.4 fold, and
- a general decrease in translation, as shown with α–tubulin, by 1.4 fold.

Buser (1992) demonstrated that the cells removed from the bioreactor return to low density secretion rate within two days. Live cells from the bioreactor were inoculated into low density spinner cultures, and the extracellular antibody secretion rate was determined by ELISA and intracellular antibody content by FACS. The population analysis of intracellular antibody showed return to low density, high production in two days. Therefore the effect of a condition in the reactor (nutrient limitation, cell to cell contact, or diffusible inhibitors) can be reversed, or up-regulated, in a short time.

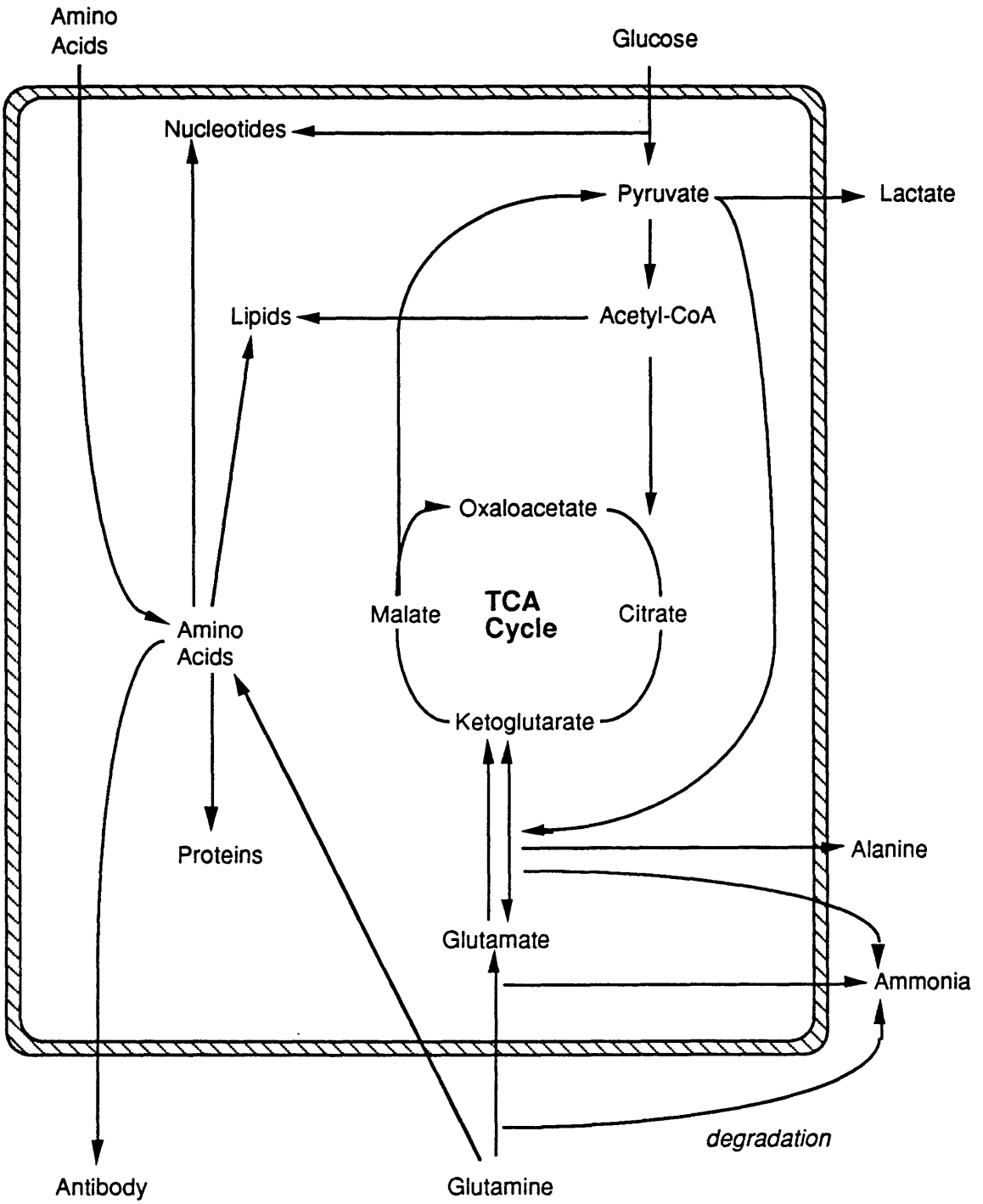
D. Changes in Hybridoma Metabolism at High Density

Although most of the published research on perfusion culture of hybridomas has focused on reactor design, ability to reach and maintain a high cell density, and maintain antibody secretion, a few research groups have also looked at metabolism.

The metabolic pathways of hybridoma cells are summarized in Figure 1 (redrawn from Batt and Kompala, 1989; Gaertner and Dhurjati, 1993a). The major substrates for cell growth are glucose, glutamine and other amino acids. Glucose is metabolized via the pentose phosphate pathway to nucleotides or via glycolysis to pyruvate. Through pyruvate, glucose is converted to the acetyl-CoA, and through the TCA cycle into lipids, amino acids, or energy. Most of the glucose is excreted as lactate. The glycolysis efficiency, defined as twice the percentage rates of lactate secretion to glucose uptake, can approach 100% for cells in culture. Glutamine enters the TCA cycle via an analogous pathway of glycolysis, called glutaminolysis (McKeehan, 1982). Glutamine is a carbon and nitrogen source for amino acids for proteins, nucleic acids, and lipids. Glutamine supplies more energy than glucose, as was determined by Reitzer *et. al.* (1979) with HeLa cells. Since animal cells in culture cannot synthesize amino acids, and amino acids must be transported into the cell from the cell culture media (Eagle, 1955). Hybridomas secrete antibody protein, as well as ammonia, lactate and alanine. Ammonia and lactate are inhibitors of growth and antibody production (Reuveny *et. al.,* 1986; Glacken *et. al.*, 1988).

Research by Tyo (1991) focused on the analysis of the metabolic changes occurring as the cell density increased and the antibody production declined. A metabolic shift in glycolysis efficiency and amino acid uptake occurred. At low density the glycolysis efficiency was 65 %, and for the other reactor conditions the efficiency was nearly 100%. The uptake rate of pyruvate also declined from 2.5 to 1.5 fmole/cell-hour. A metabolic shift in the amino acid uptake paralleled the decline in cell density. The total uptake of the amino acids (produced plus consumed rates) declined 5 fold from about 250 to 50 fmole/cell-hour. The uptake rate or production rate of each amino acid declined. For example, the uptake rate of glutamine and the secretion rate of alanine decreased coordinately 3 fold (from 60 to 20 fmole/cell-h).

Figure 1: Metabolic Pathways



All these changes at high density suggest fewer precursors available for cellular protein, lipid and nucleotide synthesis as well as secreted antibody. At high density the amino acid supply is nearly equal to the requirements for proteins and nucleotides, while at low density amino acids are taken up in excess of these requirements (Tyo, 1991).

A few studies with hybridoma cell lines in perfusion have looked at density effects on specific uptake rates of glucose and glutamine. A similar case of antibody production declining with increasing cell density were observed by Seamans and Hu (1990) in the perfusion culture of the AFP-27 hybridoma. They found that glycolysis was constant. In contrast, Shirai *et. al.* (1991) perfused a mouse-mouse hybridoma 4C10B6 with a .45 micron membrane filter at the bottom of the reactor, and observed a 2 fold drop in glucose consumption and lactate production and a 3 fold drop in glutamine consumption. They did not report antibody concentration or production data. Hülscher *et. al.* (1992) used the combination of airlift for suspension and a single cone for separation of the mouse-mouse hybridoma XR6-G10-B3, and found specific antibody production constant but about a four fold drop in uptake rates of glucose and glutamine and production of lactate.

There have been no studies on the density effects on specific uptake rates of amino acids. However, comparison of amino acid uptake rates at different growth rates have been made by Hiller *et. al.* (1991, 1993) for the mouse hybridoma X-D. They fixed growth rate by controlling dilution rate in continuous culture and in perfusion culture. Comparison of the specific amino acid uptake rates of fast growth versus low growth rates also shows similar trends as observed from low density to high density. A comparison of bioreactor conditions was made by Büntenmeyer *et. al.* (1991). They found that the uptake rates of the hybridoma VIN2 was faster in chemostat culture than in batch, presumably because the growth rate was faster. They also observed that the total uptake rates of the GL3 hybridoma were lower at a steady state in a perfusion culture (with cell harvest) than in batch. They did not investigate the transient behavior in perfusion culture. Common to all of these studies, the growth rate declined as the cell density increased.

E. Goals of Research

This research aimed at two questions posed by the reversible phenomena. First, "What is the cause of the decrease in antibody production at high cell density?". Second, "What do cells do with the high rate of amino acids taken-up at low density?"

This thesis sets forth three theories regarding the cause of the observed down regulation of protein production and then addresses each theory:

- nutrient limitation
- cell-to-cell contact inhibition
- diffusible inhibitor

In summary, I have found as a result of this work that the cause is a combination of diffusible inhibitors. Lactate and ammonia can account for about two-thirds of the observed inhibition. A third diffusible inhibitor of a small molecular weight (less than 10 kDa) exists, though its identity is unknown.

The inhibition of the antibody protein production is coincident with the decrease in amino acid uptake. Tyo (1991) concluded that cells are taking up amino acids at low density at a rate exceeding requirements for protein and nucleotides. This thesis investigates the utilization of amino acids at different densities. I present evidence that this low-density "excess" is used primarily for lipid synthesis.

II. Experimental Techniques

A. Cell Culture

Cell Line

The cell line chosen for this research was a Sp2/0 transfectoma called C46 which produces a chimeric (mouse/human) antibody of the class IgG_1K (Shaw *et. al.*, 1988b). A high producing sub-clone designated CLC LT 14 (Tsuruda, 1990) was selected. Experiments were performed with the sub-clone, and these cells will be referred to as CLC cells. The specific antibody production rate in batch culture is about .2 pg/cell-h and the final antibody concentrations reaches 10-15 μg/ml.

The antibody protein was cloned from a combination of mouse and human genomic genes. The antibody binding portion, the variable region, was cloned from mouse (murine) sequences from a mouse hybridoma (Herlyn *et. al.*, 1979). The murine variable region binds to a cell surface antigen of colon carcinoma cells (Sun *et. al.* 1987). The murine antibody was tested in phase I and II clinical trials with some success for reducing tumors, but the foreign antibody elicited an immune response (Sears *et. al.*, 1982; Sears *et. al.* 1985). The constant region portions were cloned from human sequences, with the class one IgG chosen for its cytotoxic characteristics (Shaw *et. al.*, 1988a). The non-secreting hybridoma Sp2/0 was transfected with two genes, one for the chimeric heavy chain and one for the chimeric light chain. The chimeric antibody did not cause an allergic reaction in patients and circulated in the body longer than the mouse counterpart (LoBuglio *et. al.*, 1989). Other cell lines secreting chimeric antibodies towards tumor associated antigens, such as the human common acute lymphocytic leukemia antigen, have been constructed by the same genetic engineering methods (Morrison and Oi, 1989).

Stock Culture

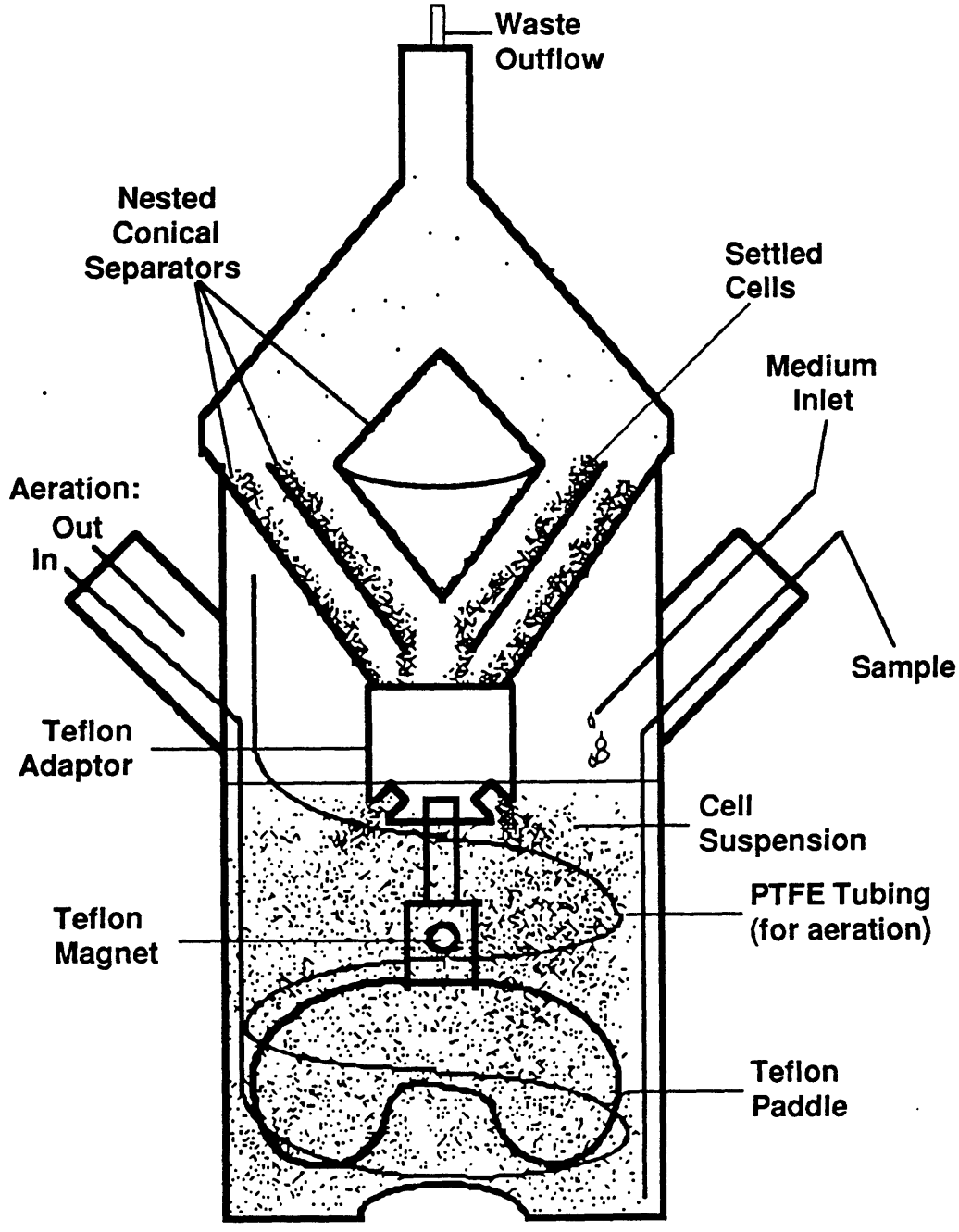
Cells were grown in Dulbecco's Modified Eagle's Medium (Gibco, Grand Island, New York or JRH Biosciences, Lenexa, KS) supplemented to final concentrations of 2 mM pyruvate, 6 mM glutamine, 50 μM β-mercaptoethanol (called DMEM*) and 4% heat treated, fetal bovine serum (FBS) (Hyclone, Logan, UT). Stock cultures were maintained in exponential growth in 75 cm^2 T-flask culture by passaging every 2-3 days. Cultures were grown at 37 ^oC in a humidified incubator with CO_2 maintained at 8%. Cell culture grade chemicals were obtained from Sigma (St. Louis, MO) unless otherwise indicated.

Bioreactor Design

A perfusion system was designed in order to study the effects of high cell density. The bioreactor design is shown in Figure 2. A 250 ml microcarrier flask with two side arms was modified. Fresh media was pumped continuously at one port, and spent media with some cells are removed through the top of a novel conical cell separator (Tyo, 1991). The culture was stirred with a magnetic blade suspended from the cell separator. Humidified air or an enriched oxygen mixture was passed through a .22 micron filter, and diffuses through a coil of porous polytetrafluoroethylene (PTFE) tubing (W.L. Gore & Associates, Elkton, MD).

The conical separator operates by inclined sedimentation in an upward flow of cells and media. The device uses no moving parts, such as pumps, which can damage cells. The separator consists of three nested glass cones enclosed by one inverted cone. The separation ratio, the concentration of cell leaving compared to the concentration of cells in the reactor, is a function of the flow rate. In the extreme case, at a flow rate of one reactor volume per hour the separator would allow all the cells to leave. In reactor conditions studied here, the flow rates were of 1 to 6 reactor volumes per day, and the cell separator retained about 95% of the cells. Maximum cell densities of 2 $x10^7$ cells/ml were achieved in this 250 ml bioreactor, with perfusion rates of 3 reactor volumes per day.

Figure 2: Schematic of Perfusion Bioreactor



High Density Experiments

Two sets of triplicate bioreactor experiments were inoculated with about 5 x 10^5 cells/ml. Perfusion of medium was increased from 1 to 3 volumes per day for the first 3 to 5 days. In the first set, flow rates were increased to 6 and 9 volumes per day. Maximum total cell densities of 1-2 x10^7 cells/ml were achieved in this 250 ml bioreactor, with perfusion rates of 3 reactor volumes per day.

Through the sampling port, sterile samples were withdrawn at least once daily. Cell density was determination with a Coulter Counter Model ZF (Coulter, Hialeah, FL) and percent viability was measured by trypan blue exclusion. Cells removed from the bioreactor were sometimes used immediately for flow cytometric analysis for antibody content or for cell cycle. Some cells were frozen as live cells for later experiments in a solution of 10% dimethylsulfoxide, 20% FBS and 70% DMEM*. Cells were removed by centrifugation at 500g, and the supernatant was frozen at -20°C for subsequent metabolic studies and for measurement of excreted antibody concentration.

Low Density Experiments

Growth and antibody production was tested in the high density, low productivity (HDLP) media removed from the bioreactor. Experiments were performed at "low density" with cells that had been carried as stock cultures. Cells were inoculated at 2 - 5 x 10^4 cells/ml in 24 wells plates, T-flasks or spinner cultures and permitted to grow to final density of less than 2 million cells/ml. Total or viable cell density was measured after three days. The specific antibody production rate was measured as the change in amount of antibody per cell per time interval. Amount of antibody was measured as either 1) extracellular antibody secreted into the media by ELISA or 2) intracellular antibody content by flow cytometry. Details of methods follow in analytical methods.

B. Analytical Techniques

1. Antibody Measurements

Antibody Concentrations by ELISA

Extracellular antibody concentrations were measured with a sandwich type ELISA. Flat bottomed, 96 well microtitration plates (cat. 86-381-04) were obtained from Flow Laboratories (McClean, VA). Plates were coated with 100 µl/well goat anti-human $F(ab')_2$ fragment (Jackson Immunoresearch, West Grove, PA, cat. #109-005-097) diluted to 10 mg/ml in .05 M sodium carbonate buffer, pH 9.8. After a two hour incubation the plates were washed with wash buffer consisting of phosphate buffered saline (PBS) with .05% Tween 20. PBS prepared with 0.2 g/l KCl, 0.2 g/l KH_2PO_4, 8 g/l NaCl and 1.15 g/l Na_2HPO_4, pH 7.1. The plates were blocked with 200 µl/well with a solution of 3% bovine serum albumin (BSA) and 1% normal goat serum in PBS. After a one hour incubation plates were rewashed. Plates were stored at 4°C until samples were assayed. Samples were diluted in two fold serial dilution in complete media at room temperature. The plates were incubated for two hours and then rewashed. The second antibody was an alkaline phosphatase conjugated goat antihuman IgG (Tago, Inc. Burlington, CA, cat. #2490). The second antibody was diluted about 1:5000 in a solution of 1% BSA in PBS and added at 100 µl/well. After a two hour incubation, the plates were rewashed. The substrate (Sigma, St. Louis MO, cat. #104-105) was diluted at 1 mg/ml in the following buffer: 24.5 mg MCl_2 in 400ml plus 48 ml diethanolamine, pH 9.8. The substrate was plated at 100 µl/well. The reaction was stopped after 30 minutes with 100 µl/well of 2 N NaOH. The conversion of the substrate was measured at 450 nm. Controls of human IgG (Jackson Immunoresearch, West Grove, PA, cat. #009-000-003) were run with each plate.

Intracellular Antibody Distributions by Flow Cytometry

Intracellular antibody content was determined by staining permeabilized cells with a fluorescent tagged antibody. Live cell samples of $2\text{-}4 \times 10^6$ cells were washed three times with PBS to remove extracellular antibody. All centrifugation steps were done at 500g, 4°C for 5 minutes. The washed cells were permeabilized with 70% ethanol in PBS on ice for 30 minutes. After the incubation the cells were rewashed with PBS to remove ethanol. The permeabilized cells were stained with a fluorescein isothiocynate (FITC) conjugated goat F(ab) fragment which binds to human IgG (heavy + light chain) (Jackson Immunoresearch, West Grove, PA, cat #109-096-088). After the 30 minute room

temperature incubation, the cells were washed again and placed on ice. The cells were analyzed by flow cytometry with a Coulter EPICS@ C System (Coulter, Hialeah, FL). The fluorescein tag was excited at 488 nm and emission monitored at 530 nm. The negative controls were the non-secreting parental cell line, Sp2/0.

Conversion of the log mean fluorescent value reported by the EPICS@ C System to linear means was accomplished by the method of Schmid *et. al.* (1988). Since the EPICS@ C System collects data in 256 channels, a theoretical base number is calculated as below:

$$\text{theoretical base} = 1000^{(1/256)} = 1.02735$$

The linear mean of a sample then equals:

$$\text{linear mean} = 1.02735^{(\text{channel \# of log mean})}.$$

A cut off for the percent positive staining cells are set so that 1% of the positive controls are "negative staining". Antibody content equals the linear mean of the positive staining cell population multiplied by the percent of positive staining cells. Relative antibody content is normalized to the low density, fresh media control.

Intracellular Antibody Concentrations

Cellular antibody content was also determined by lysing cells by the procedure of Meilhoc *et. al.* (1989) and measuring antibody content by the ELISA procedure. Cells are lysed in a low osmotic buffer (5 mM KH_2PO_4) and stabilized with an equal volume of high osmotic buffer (15 mM KH_2PO_4 with 270 mM NaCl). For the protein extraction, added to the .4 ml of lysed cells were 50 µl 1% Triton X-100 and 12 µl of 2 M CHAPS. The soluble proteins were separated by centrifugation at 13,600 g for 5 min. As done by Meilhoc *et. al.* (1989), the completeness of the extraction procedure was tested by assaying for antibody concentrations in higher concentrations of detergents and in second extraction steps.

2. Cell Cycle Analysis

Cell cycle was determined by measuring DNA content of permeabilized cells stained with a nucleotide binding dye. Cells were removed from the bioreactor, washed twice with PBS, and permeabilized with 70% ethanol for 30 minutes. The permeabilized cells were washed again with PBS and stained for DNA with 20 µg/ml propidium iodide (Sigma, St. Louis, MO, cat. #P-4170) and 40 µg/ml RNAse (Sigma, St. Louis, MO, cat. #R-5503)

(Crissman and Steinkamp, 1982). After a 30 minute incubation period the samples were analyzed by flow cytometry with a Coulter FACStar System (Coulter, Hialeah, FL). The excitation wave length was 488 nm and the emission wave length was 590 nm. The bimodal DNA profile was fit to three populations by the program Modfit™.

3. Extracellular Metabolic Concentrations

Metabolic Concentrations by Enzymatic Assays

Extracellular concentrations of glucose, lactate, pyruvate and ammonia were determined with diagnostic kits numbered 16-UV, 828-UV, 726-UV and 170-UV, respectively (Sigma, St. Louis MO).

Amino Acid Analysis

Amino acid concentrations of bioreactor samples were determined by Tyo (1991). A brief description follows. Extracellular amino acid analysis was analyzed by HPLC. Bioreactor samples were deproteinized with 1000 MW cutoff ultrafiltration membranes (Amicon Centrifree, Beverly, MA). The amino acids were derivatized with DABS-Cl method (Change *et. al.*, 1983) and ornithine was used as the internal standard. The samples were injected into a reverse phase column, Beckman Ultrasphere ODS at room temperature. The amino acids were eluted with an increasing gradient of acetonitrile vs. citrate buffer at pH 6.5. Peaks were detected with a Hewlett-Packard Model 1090 diode array detector.

4. Cellular Membrane Preparation

Crude membranes of either the transfectoma CLC or the parental Sp2/0 cells were isolated by differential centrifugation based on published procedures (Stallcup et. al., 1984a; Lemonnier *et. al.*, 1978). All centrifugation steps and handling procedures were done at 0-4°C. Cells were harvested from "low" density batch spinners (1-2 $x10^6$ c/ml) by 15 minute centrifugations at 500g, pooled, washed with PBS and resuspended at ~10^8 cells/ml in Earle's Balanced Salt Solution (Gibco, Grand Island, New York). Cells were homogenized by sonication. The whole cell homogenate was centrifuged 15 minutes at 3600g and the resulting low speed pellet contained nuclei, mitochondria, etc. The low speed supernatant was then centrifuged 30 minutes at 22,000 g. The resulting high speed pellet was the crude membrane fraction used for growth and antibody secretion assays.

Purification characteristics of two batches are summarized in the table below. Total membrane activity was determined with freshly prepared samples by the marker 5'nucleotidase (5'-ND Sigma kit 265 UV). Total protein content was measured by a modified Lowry method (Sigma kit P 5656). Specific 5'ND activity is expressed per mg protein. Yield is percent of total 5'-nucleotidase activity of the homogenate. Fold purification is the ratio of the specific activity of a fraction to the specific activity homogenate.

Table 2: Purification of Crude Membranes

Sample	Total Protein (mg)	Specific Activity (mmol/mg-min)	5'ND Total Activity (mmol/min)	Yield 5'ND (Percent)	Fold Purification
Prepared from 2.3 x 10^9 CLC cells					
Homogenate	186	7.2	1341	100	1.0
Low Speed Pellet	64	3.0	196	15	0.4
Low Speed Supernatant	118.	7.7	910	68	1.1
High Speed Supernatant	120	4.9	585	44	0.7
High Speed Pellet	27	11.0	296	22	1.5
Prepared from 2.3 x 10^9 Sp2/0 cells					
Homogenate	174	5.1	879	100	1.0
Low Speed Pellet	71	2.5	175	20	0.5
Low Speed Supernatant	114	6.7	762	87	1.3
High Speed Supernatant	105	3.8	400	45	0.8
High Speed Pellet	19	12.6	236	27	2.5

5. Fractionation of Inhibitors

High molecular weight components of the media from the bioreactor outlet were separated with a size exclusion column. The gel filtration media used was sephacryl S-300 (Pharmacia, Piscataway, NJ). This hydrophobic gel is of 25-75 μm particles of cross linked alkyl dextran and N,N'-methylene bisacrylamide. The S-300 gel filtration material separates globular proteins of $1x10^4$ to $1.5 x10^6$ MW. The gel was packed into a column of 2.5 x 50 cm with PBS at 4 ml/minute. The 10 ml bioreactor samples of high density, low productivity (HDLP) media were loaded on the 200 ml bed and eluted with DMEM*

(with glutamine, pyruvate and β-mercaptoethanol and no serum) at 2.5 ml/minute. Fractions of 10 ml were collected and sterile filtered (.2 μ Millex, Millipore, Bedford, MA). Fractions were assayed for protein by adsorbance at OD 280, and concentrations of lactate, pyruvate, ammonia and antibody with enzymatic kits (Sigma, St. Louis, MO).

6. Leucine Uptake

Incorporation of leucine into protein and lipid was measured in 5 ml cultures after two or four hours at various densities. Cells were passaged at low density in fresh or high density, low productivity (HDLP) media for four days before the experiment, and then suspended in fresh or HDLP media. Cultures at densities ranging from $2x10^4$ to $3x10^6$ cells/ml were placed into 15 ml centrifuge tubes and mixed in a roller rack. Radioactive leucine (^{3}H- 3,4,5-leucine NET-460 or ^{14}C [U]-leucine NEC-279E, New England Nuclear) was added and time zero samples were removed to later check for the amount of hot leucine added. After the labeling period, cells were centrifuged and the supernatants were set aside. For the ^{3}H-leucine experiments, parallel cells samples were washed with DMEM* media without serum. One sample was precipitated with 10% TCA for total protein and lipid and the other sample was extracted with chloroform: methanol for total lipid (Folch *et. al.*, 1957). For the ^{14}C-leucine experiments, samples were prepared in sequential steps. First cells were washed with DMEM*, and then protein and lipid were precipitated with cold 30% TCA. The lipid was separated from the protein precipitate in a series of extraction steps. The precipitate washed twice with ethanol, twice with acetone, twice ethanol-ether, (3:1, v/v) and once with ether (Bhargava *et. al.*, 1959). The final protein precipitate was dissolved in 0.6 N NaOH and samples were scintillation counted. The lipid containing solvents were pooled into scintillation vials, dried, and scintillation fluid was added for counting.

The concentrations in the free amino acid pool were determined by HPLC analysis of the TCA soluble extracts (MIT Biopolymers Lab). This method gives information for all amino acids except cystine and tryptophan. The hydrolysis and derivatization procedures results in one combined value each for a) glutamate and glutamine, and b) asparagine and aspartate. The combined values for asparagine and aspartate were below the level of sensitivity of the assay.

7. Lipid and Protein per Cell

Lipid Content

For total lipid measurement, samples were extracted by the Folch method (Folch *et. al.*, 1957). Frozen cell samples of ~ $5x10^7$ cells were washed with PBS to remove serum lipids and resuspended in 1 ml distilled water. To the 1 ml cell sample, 12.5 ml chloroform: methanol (2:1) was added. This mixture was sonicated and then set aside overnight. The lipid extracts were filtered through general purpose filter paper (Scheicher and Schull #595) using chloroform to rinse paper. The filtered extracts were backwashed with 4 ml 0.05% calcium chloride. The phases were separated by one 15 minutes centrifugation at 400g. The bottom organic phase was removed, dried (by speed vacuum and drying oven) and weighed to determine picograms lipid per cell.

Protein Content

Portions of the cell samples for lipid determination were set aside for protein measurement. These samples were assayed for protein content with a modified Lowry method (Sigma Assay Kit, P 5656). This procedure uses deoxycholate to solubilize proteins.

C. Kinetic Equations

In this section, the kinetic equations for growth, product production and substrate consumption will be presented for batch, continuous, and perfusion culture. These equations are used to calculate rates of growth, death, antibody production and substrate consumption. Input values from experimental data including cell density, viability, dilution rate, and concentrations of antibody and nutrients.

1. Growth and Death

The production of cells is a function of the growth and death rates of the cells and a function of the method of cell culture. In batch culture, the rate of change in time t of the viable cell concentration X_V (viable cells/ml) equals the rate of growth minus the rate of death of cells:

$$\frac{dX_V}{dt} = \mu X_V - \alpha X_V \qquad (1)$$

where:

μ = specific growth rate constant (h^{-1})

α = specific death rate constant (h^{-1})

In chemostat culture, the flow rate of fresh medium into the reactor equals the flow rate of spent medium and cells out of the reactor. The dilution rate is defined as:

$$D = \text{dilution rate } (h^{-1}) = \frac{\text{Flow (ml/h)}}{\text{Reactor Volume (ml)}}$$

Therefore, the equation for the rate of change in viable cell concentration contains three terms, one each for the growth, death and removal of cells by dilution.

$$\frac{dX_V}{dt} = \mu X_V - \alpha X_V - DX_V \qquad (2)$$

In continuous perfusion culture, the flow rate into the reactor equals the flow rate out of the reactor, but most or all of the cells are retained in the bioreactor. With the conical cell separator (Tyo and Thilly, 1989), some of the cells exit the bioreactor. Since the cell separator is a settling device, the distribution of cells exiting the bioreactor contains a higher ratio of dead cells (X_D) to live cells than the distribution of cells remaining in the bioreactor (Tyo and Thilly, 1989). Therefore two separation ratios have been defined:

ε_V = separation ratio for viable cells = $X_{V,out}/X_{V,in}$

ε_D = separation ratio for dead cells = $X_{D,out}/X_{D,in}$

The separation ratio of viable cells ε_V is not equal to the separation ratio of dead cells ε_D in this reactor.

The rate of change of the viable cell concentration (X_V) equals the accumulation of cells due to cell growth minus the removal of cells by death and washout. Similarly, the rate of change of the dead cell concentration X_D (cells/ml) equals the accumulation of dead cells minus the removal of dead cells. The differential equations describing the rate of change of X_V and X_D in the perfusion bioreactors are shown below:

$$\frac{dX_V}{dt} = \mu X_V - \alpha X_V - (\varepsilon_V X_V D) \qquad (3)$$

$$\frac{dX_D}{dt} = \alpha X_V - (\varepsilon_D X_D D) \qquad (4)$$

where D (h^{-1}) is the term for the dilution or the perfusion rate. Solving the differential equation for the viable cell concentration X_V:

$$X_V = X_{V_0} e^{(\mu_{app} - \varepsilon_V D)\Delta t} \qquad (5)$$

where the apparent growth rate is defined as the actual growth rate minus the death rate:

$$\mu_{app} = \mu - \alpha \qquad (6)$$

Rearranging, the solution for the apparent growth rate is:

$$\mu_{app} = \frac{1}{\Delta t} \ln \left[\frac{X_V}{X_{V_0}}\right] + \varepsilon_V D \qquad (7)$$

Substituting the solution for X_V into the differential equation of the dead cell concentration X_D the solution for the death rate α is:

$$\alpha = \frac{B\left(X_D - X_{D_0} e^{-\varepsilon_D D t}\right)}{X_{V_0} e^{-\varepsilon_D D t}\left(e^{Bt} - 1\right)} \qquad (8)$$

where the term B is:

$$B = \mu_{app} + D(\varepsilon_D - \varepsilon_V). \qquad (9)$$

The actual growth rate μ is calculated from the sum of the apparent growth rate μ_{app} and the death rate α. These equations reduce to the equations for chemostat culture when ε_V and ε_D are both set equal 1.

2. Antibody Production

The production of antibody is modeled as a function of the viable cell density. In batch culture, assuming there is no degradation of antibody, the equation describing the rate of change in antibody concentration is:

$$\frac{dAb}{dt} = q_P X_V \qquad (10)$$

The specific rate of production q_P (pg/cell-h) is assumed to be constant during the time interval measured. Solving for the specific production rate:

$$q_P = \left[\frac{Ab_t - Ab_o}{X_{V_t} - X_{V_o}}\right] \ln\left[\frac{X_{V_t}}{X_{V_o}}\right] \qquad (11)$$

During exponential growth, the viability is high ($X_V \approx 95\%$ X) and the specific production rate q_P may be calculated from the total cell density X.

With this bioreactor design the secreted antibody protein is homogeneously mixed in the reactor and the concentration inside the reactor equals the concentration exiting the reactor. The equations describing antibody production are the same for continuous chemostat and continuous perfusion culture. The differential equation for antibody concentration Ab (μg/ml) is a function of the production by viable cells and the removal by dilution.

$$\frac{dAb}{dt} = q_P X_V - DAb \qquad (12)$$

Solving for the specific rate of production q_P (pg/cell - hr) gives:

$$q_P = \frac{Q_P}{X_{V_t} - X_{V_o}} \ln\left[\frac{X_{V_t}}{X_{V_o}}\right] \qquad (13)$$

where the volumetric antibody production rate Q_P is:

$$Q_P = \frac{D[Ab_t - Ab_o e^{-Dt}]}{1 - e^{-Dt}} \qquad (14)$$

3. Substrate Consumption

The consumption of substrates are modeled as a function of the viable cell density. In batch culture, the equation describing the rate of change in substrate S concentration is:

$$\frac{dS}{dt} = q_S X_V \qquad (15)$$

The specific rate of consumption q_S (mole/cell-h) is assumed to be constant during the time interval measured. Solving for the specific consumption rate:

$$q_S = \left[\frac{S_t - S_o}{X_{V_t} - X_{V_o}}\right] \ln\left[\frac{X_{V_t}}{X_{V_o}}\right] \qquad (16)$$

In either a continuous chemostat or a homogeneous perfusion culture system, the differential equation for the rate of change of a consumed substrate S is a function of the input rate from the inlet stream, the removal rate in the outlet stream (D) and the consumption by viable cells (X_V).

$$\frac{dS}{dt} = D(S_i - S) - q_S X_v \qquad (17)$$

where:

q_S = specific uptake rate of substrate (mole/cell-h),
S_i = inlet substrate concentration (mole/liter), and
S = substrate concentration in reactor and outlet stream (mole/liter).

The uptake rate at for a given interval is:

$$q_s = \frac{K\left(S - S_o e^{-e_D Dt} - S_i(1 - e^{-Dt})\right)}{X_{v_o} e^{-Dt}\left(1 - e^{Kt}\right)} \qquad (18)$$

where the term K is a combination of the growth rate, separation ratio of viable cells and dilution rate.

$$K = (\mu + D - \varepsilon_V D) \qquad (19)$$

In order to estimate the concentration of an unmeasured substrate at various dilution rates and cell densities, the following assumptions were made. The substrate consumption rate is assumed to be constant. Also, at steady state the effect washout of the initial supply is

negligible, and the equation can be simplified by setting $S_o e^{-Dt}$ equal to zero. Rearranging, the amount required is:

$$(S_i - S) = S_i e^{-Dt} + \frac{q_S X_{V_o} e^{-Dt}}{K} \qquad (20)$$

where the term K is calculated from equation 19.

III. Results

In this chapter, results are presented on the following topics:

- Decrease in Antibody Production in High Density - In perfusion bioreactors runs, the cell density and doubling time increase and the antibody production decreases with time in culture. The decrease in antibody production is reversible.
- Nutrient Limitation - Nutrients were not depleted during the bioreactor runs.
- Cell to Cell Contact - Incubation of media removed from the bioreactor, but not membranes of the cells, caused a decrease in production.
- Analysis of Inhibitors - Inhibition by lactate, ammonia, redox potential and secreted amino acids were tested. Unknown inhibitors are characterized by fractionation of media removed from the bioreactor.
- Mass Balance - Density dependence of the uptake of leucine into proteins and lipids was tested. Amino acid pools as a function of cell density was determined. Total protein and lipid content of bioreactor cells declined as cell density increased.

A. Decrease in Antibody Production in High Density

In order to study the various effects of high cell density, two sets of triplicate bioreactor experiments were performed. Conditions of the first set of bioreactors are presented here, and conditions of the second set will be presented in section III.B.4. We observed a decrease in antibody production in the reactor as the cell density increased and the doubling time increased. This decrease in production was shown to be reversible in a series of experiments. A characterization of the phenomena is presented below.

1. Bioreactor Experiments

Triplicate bioreactors were inoculated at about 5 x 10^5 cell/ml, and perfused with the commercial media supplemented glutamine, and β-mercaptoethanol (DMEM*) with 4% fetal bovine serum. The perfusion rate was increased from 1 to 3 reactor volumes per day from day 0 to day 3, and increased to 6 volumes per day at day 19. At day 33 the inlet media of reactor #2 was diluted with a salt solution for part of the metabolic studies. As shown in the next figure, the viable cell density of bioreactors #1-3 increases from $5x10^5$ to $1\text{-}2x10^7$ cells/ml over the first sixteen days, and then remains essentially constant. Viability of the cells (not shown) ranged from 60-80%.

The cell separator operates on the principle of gravity settling cells down in inclined plane in an upward flow rate. The design therefore operates more efficiently at lower flow rates. In testing the design Tyo (1991) found the viable separation ratio to increase exponentially with flow rate. Over the course of the bioreactor runs, the cell separator retained more than 90% of the total cells. The separator was more efficient at removing smaller and therefore dead cells. At a flow rate of 3 volume per day, about 5% of the viable cells were removed and about 20% dead cells were removed. At a flow rate of 6 volumes per day, 6% of the viable cells and 30% of the dead cells were lost.

The decrease in antibody production with increasing cell density is demonstrated with bioreactor #1 in Figure 4. During the first five days of culture, the cell density increased to $2x10^6$ c/ml while the specific antibody production rate remains at the maximum rate of about .2 pg/cell-hour. Over the first three weeks, as the cell density increased to $1\text{-}2x10^7$ c/ml the antibody production rate decreased from 0.2 to 0.002 pg/cell-hour. Fitting the decline in production from $2x10^6$ to $2x10^7$ cells/ml, to an exponential equation of:

$$q_p = q_{po} * \exp(-a_o t)$$

results in a hypothetical secretion rate (q_{po}) of .37 pg/cell-h at time (t) zero, and an exponential rate of decline (a_o) in production of .21 per day. This reversible rate of decline in production is about 50% every 3 days.

The phenomenon has been shown to be reversible. Removing the cells from high density conditions returns the secretion rate to its nominal state. Cells were harvested at various time points and frozen. Sample removed from days 2, 3, 7, 10, 20 and 21 were thawed and grown at lower density in spinner cultures. Plotted in Figure 4 are the secretion rates of control cultures which were continuously carried at lower density (0.5 to 10 x 10^5 cells/ml) and the samples grown after the reactor runs at lower density. Cells maintained at low density show an irreversible decline in production in six weeks from .2 to .05 pg/cell-hour. Fitting the irreversible decline in time t to an exponential of the form:

$$q_p = q_{p1} * \exp(-a_1 t)$$

gives a maximal secretion rate (q_{p1}) of .23 pg/cell-hour, and an exponential decline rate (a) of .035 per day. The irreversible rate of decline in production is 50% every 20 days.

Figure 3: Viable Cell Density

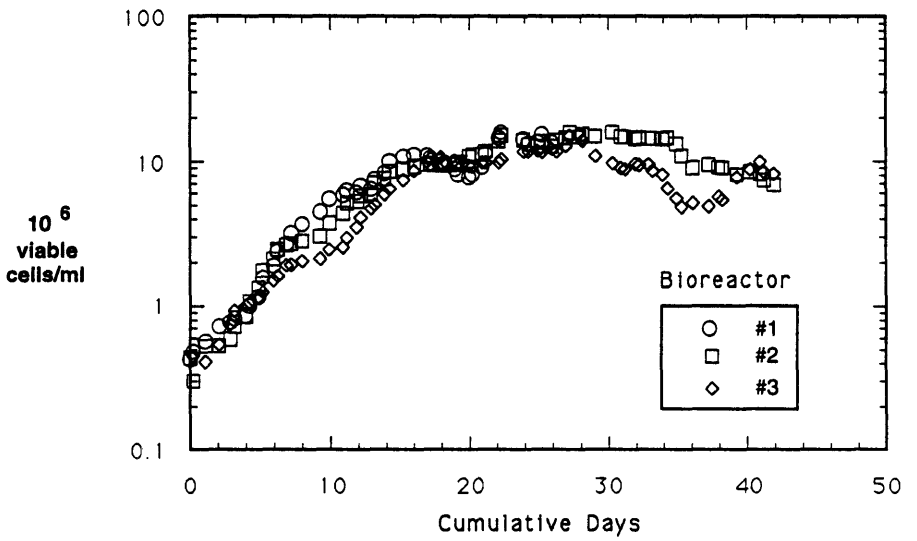
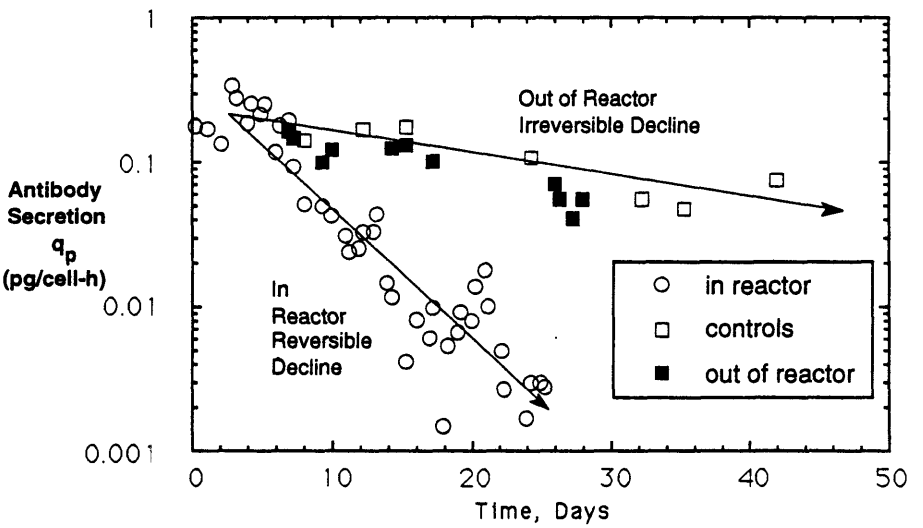


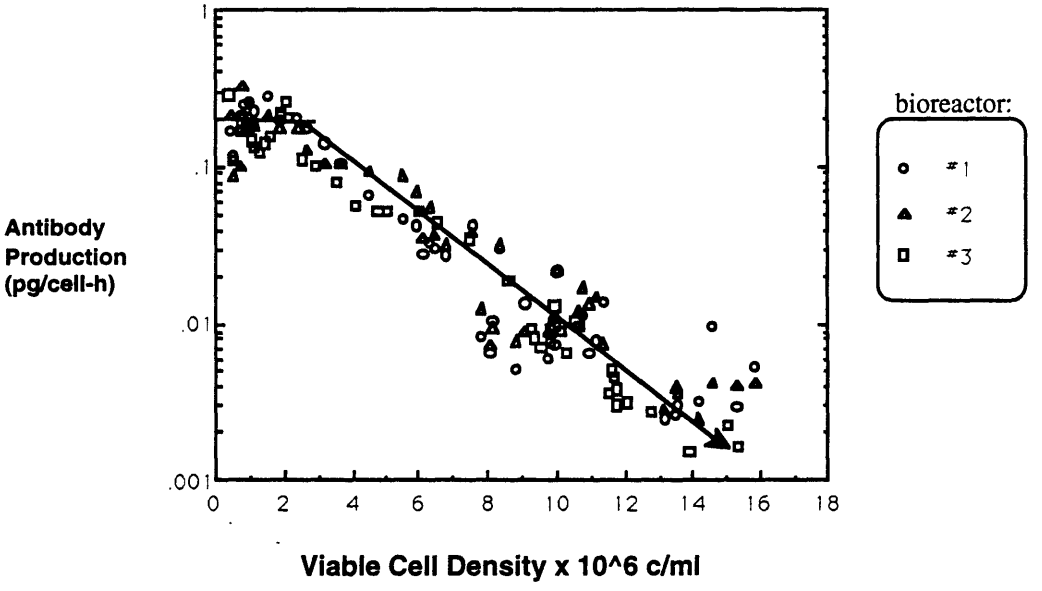
Figure 4: Reversible Decline of Antibody Secretion



The decrease in antibody secretion clearly occurred at the same time as the increase in cell density. Even in bioreactor #3, cell density and antibody secretion paralleled. From days 33 to 42, the cell density transiently deceased to $6x10^6$ c/ml, and the antibody secretion rate transiently increased to .05 pg/cell-h. These results supports to the hypothesis that antibody production is a function of cell density.

Plotted in Figure 3 is antibody production vs. viable cell density for the first set of bioreactor runs. The antibody production rate declined at day 5, the same time as the cell density exceeded the maximum density attained in batch conditions. In the reactor at viable cell densities less than 2 x 10^6 c/ml, the antibody secretion rate did not appear to vary with cell density. As the viable density increased from 2 x 10^6 to 11 x 10^6 cells/ml, the antibody production rate decreased 20 fold from .2 to .01 pg/cell-h.

Figure 5: Specific Antibody Production vs. Viable Cell Density



This decrease in production vs. density is fitted to an exponential trend of the form:

$$q_p = q_{p2} * \exp (- a_2 X_v).$$

With the data for all three reactors, this equation gives the maximal secretion rate (q_{p2}) as .225 pg/cell-hour, and the rate of decline (a_2) as .327 per million viable cells/ml. This relationship is consistent with the idea that antibody production is inversely related to cell density.

2. Growth and Death Rate Analysis

2.1 Doubling Time Estimates by Kinetic Modeling

Growth and death rates were estimated with the kinetic model presented in section II.C. A shown previously in Figure 3, viable cell concentrations increase log-linearly until about day 15 and then reaches a maximum. Although the viable cell density reaches a maximum of $1x10^7$ cells/ml, the cells have not stopped growing. Growth is balanced by death and the removal of cells through the cell separator. Comparing values at low to high density, doubling times increase by a few hours from about 17 - 18 to 19 - 23 hours.

The growth and death rates were estimated by the kinetics equations 7-9 and are listed in the following table. The growth and death terms are functions of the flow rate, total and viable cell density in the bioreactor, and the fractions of total and viable cell density exiting the bioreactors. The apparent growth rate equals the actual growth rate minus the death rate. The doubling time equals, by definition, ln(2) times the inverse of the growth rate. Since these values are based on many experimental measurements, error is unavoidably large and these values are considered to be estimates.

The low density condition represents data at an interval where the average cell density was about 2 million cells per ml and the antibody secretion rate averaged .02 pg/cell-h. Values at high density are taken 3 volumes per day and at an average cell density of 9 - 10 million cells per ml and antibody secretion rate of .01 pg/cell-h. The cell density vs. time graphs show in increase in density in the first case and a constant value in the second case.

Comparing low to high cell density, the apparent growth rate decreased from .15 - .02 h-1 to .01 h^{-1}. The death rate observed in the bioreactors is much higher than the death rate observed in batch spinner culture. Death rates in the reactors were about .02 - .03 hours $^{-1}$ vs. 0.001 hours $^{-1}$ at exponential growth in spinners cultures. The estimated growth rates in the bioreactors ranged from .030 to .041 hours $^{-1}$ (doubling times 17 to 24 hours). For comparison, typical growth rates in batch culture are .043 to .039 hours $^{-1}$ (doubling times 16 to 18 hours). The growth rate decreases in each reactor, or in other words, the doubling time increases by a few hours in each reactor as the antibody production declines.

Table 3: Growth and Death Rates from Kinetic Modeling

Bioreactor Number	Viable Cell Density (10^6 cells/ml)	Apparent Growth Rate (h^{-1})	Growth Rate (h^{-1})	Death Rate (h^{-1})	Doubling Time (hour)
low density					
1	2.2	.022	.040	.018	17.3
2	2.1	.021	.038	.017	18.1
3	2.2	.015	.041	.036	16.9
high density					
1	10.6	.011	.030	.019	23.4
2	9.4	.008	.030	.022	23.2
3	10.0	.010	.037	.027	18.7

In reviewing the data, I find that the estimation of doubling times depends on the choice of the values to hold constant, such as flow rate or cell density. The same kinetic model has been used previously with this data set to obtain doubling time rates. In estimating of the doubling times, Buser (1991) obtained values in the 16 to 24 hour range. She concluded that the doubling times are similar to those of spinner cultures and may remain constant in the reactor. In reviewing her values I find they increased from low to high density in each reactor by a few hours. In contrast Tyo (1992), concluded that the doubling times became longer, increasing from 21 to 42 hours, from the low to high density 3 volume/day case. Together these values support the hypothesis that the cells are growing in the reactor at high density, and the doubling time has increased.

2.2 Doubling Time Estimates by Flow Cytometry

To confirm the doubling time calculations based on kinetic equations, the doubling time was determined by use of flow cytometry. Comparing low to high cell density, doubling times increases by a few hours from 16 - 17 hours to 18 - 21 hours. The two method confirms the conclusions that coincident with the cell density increasing and the antibody production decreasing, the doubling time increases.

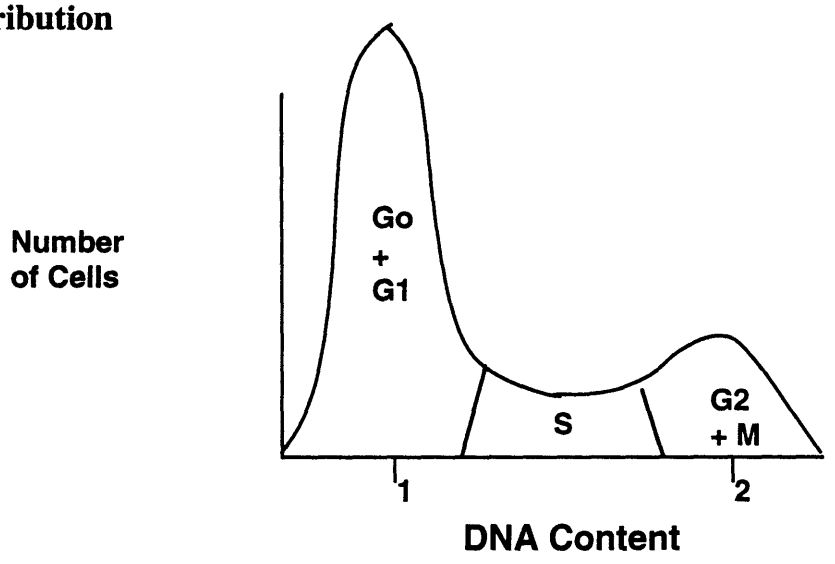
I stained live cell samples from the second set of bioreactors at day 3 and day 10 (Figure 6) for DNA content with propidium iodide. The distribution of DNA content was analyzed by flow cytometry, and the bimodal DNA profile was fit to three populations by the program Modfit™, as shown in Figure 6a. As a cell passes through a cell cycle it doubles its DNA content. Cells in the resting or arrested cell phases (G_0 or G_1) have half of the DNA content of cells about to enter (G_2) or in the mitosis (M) phases. Cells in the synthesis phase (S) have DNA content between one and two copies.

The doubling time was calculated with the following two assumptions. First, the time a cell spends in the S + G_2+ M cell cycle phases is considered fixed and the time spent in G_1/G_0 varies. Second, the cell number vs. cell age distribution follows the function $f(t)= 2^{(t/td)}$, where td is the doubling time (Figure 6b). Since the observed doubling time of the controls was 18 hours and the average fraction of cells in S + G_2 + M was 63%, the calculated time the population spends in S + G2 + M phases was 12.5 hours. For the day 3 and day 10 samples, the percent of cells in Go/G1 increased from 30 to 46 percent. A summary of the doubling time data appears in the table below.

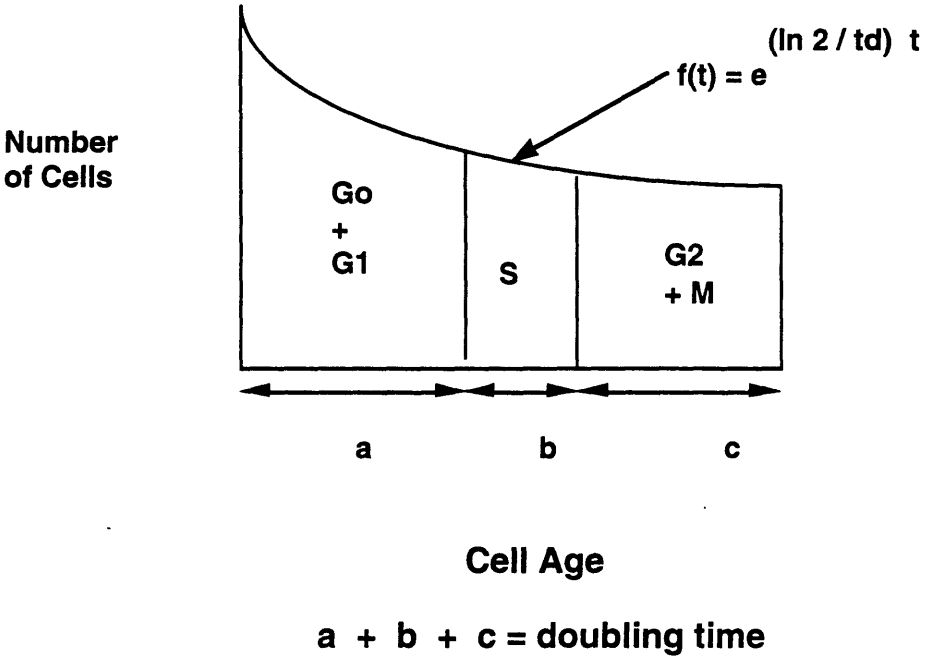
The size distribution was analyzed by flow cytometry by forward light scattering (FLS). Relative cell size was estimated by comparing the linear mean values of each population to a control culture. Cells at day 3 are larger than cells at day 10 by 30 - 60%; cells at day 10 are smaller than at day 3 by 23 -34 %..

Figure 6: Distribution of Cells by DNA Content and by Cell Age

a) DNA Distribution



b) Cell Age Distribution



a + b + c = doubling time

Table 4: Doubling Time Values from FACS Analysis

Day in reactor	Reactor #	Total Cell Density (10^6 c/ml)	Secretion qp (pg/c-h)	Size %	G1/Go %	G2 + M %	S %	doubling time (hours)
Day 3	4	2.8	0.07	131	28	11	60	16.3
	5	3.1	0.04	130	32	8	60	17.0
	6	2.2	0.09	168	31	16	55	16.9
Day 10	4	16.9	0.03	101	48	14	39	21.0
	5	18.3	0.02	102	45	14	40	20.2
	6	17.0	0.04	110	39	17	44	18.4
Controls	C-a	0.6	0.18	n.d.	36	20	44	17.9
	C-b	1.2	0.18	n.d.	37	14	49	18.1
	C-c	0.9	0.12	100	37	9	55	17.9

Control cultures were passaged at low density in parallel to the bioreactor experiments. Controls C-a and C-b correspond to the same time in culture as the day 3 sample, and culture C-c corresponds to time in culture as the day 10 sample. The doubling time at day 3 was slightly faster than the doubling time for the controls, but the antibody production was suppressed to a level 2-4 fold below the corresponding controls. The doubling time at day 10 was equal or slower than the control (18-21 hours), and antibody production was suppressed to 4-6 fold of the corresponding control. Between day 3 and day 10 the doubling time for each reactor lengthened by 2-3 hours. Therefore, the estimated doubling time increased from about 16-17 to 18-21 hours as the cell density increased 7-8 fold (from 2-3 to 17-18 x 10^6 c/ml) and as the antibody production dropped 2 fold in the reactor.

The doubling times estimated by the FACS analysis (Table 4) are in good agreement with the calculation for the average doubling time from the kinetic equations (Table 3). The values in Table 3 and 4 are quite similar. The values at low density range from 16 to 18 hours and at high density ranges from 18 to 23 hours. During the transient phase of increased growth and decreased antibody production, both methods of calculation show the doubling time increased 2 to 4 hours.

The pattern of antibody production declining with increasing doubling time is consistent with growth associated production kinetics. The exact relationship of growth rate to

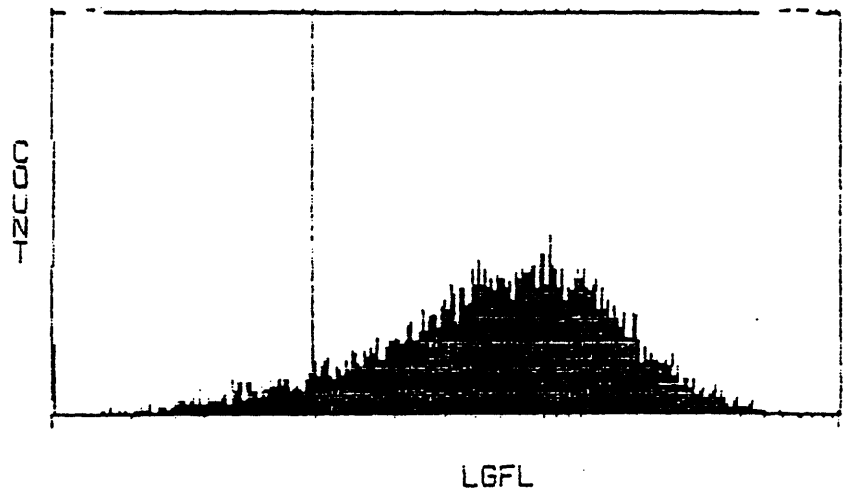
antibody production for this cell line is unknown. Robinson and Memmert (1991) found that a similar cell line had growth associated production. This cell line was also a Sp2/0 myeloma that was transfected with two chimeric genes. They varied the growth rate by nutrient limitation (chemostat culture) and found a linear relationship; antibody production declined linearly with decreasing growth rate (increasing doubling time).

3. Population Shifts to Lower Producers

The reversible decline in production has been studied in detail in this research group and full details are presented in the thesis of Buser (1991). The experiments presented here demonstrate that the population of antibody producing cells was not taken over by non-producing cells.

The reversible decrease in production was hypothesized to occur by either 1) a switch of production from producers to non-producers or 2) a shift in production to lower producers. The first hypothesis would result in two populations and the second would result in one population changing its average per-cell antibody secretion rate with time. Using the cell samples frozen during the bioreactor run, and later grown at low density, the distribution of production ability was found the be unimodal by two methods: a) intracellular fluorescent staining of antibody and b) cloning and screening for secretion. The majority of the cells produced antibody, and the average production ability irreversibly decreases in time. Figure 7 demonstrates the unimodal distribution for cells removed from the reactor at day 20, grown at low density conditions, and stained for intracellular heavy chain. The number of cells staining is plotted vs. the log of the green fluorescence (LGFL).

Figure 7: Histogram of LGFL to Heavy Chain of Sample Removed at Day 20 and Passaged Two Weeks at Low Density



Secretion of antibody by clones is summarized in Figure 8 (also Figure III-58 of Buser, 1992). Cells were removed from bioreactor #1 at days 3, 7 and 21, frozen and later grown for two weeks at low density. Cells were plated at .2 cells/well, and after two weeks the antibody concentrations were determined by ELISA assay. Plotted is a histogram of antibody concentrations of the supernatants from clones. The distributions of antibody concentrations did not separate into two groups. Most of the clones were producers with less than 5% of the clones being non-producers. The following table showed that both the mean and median antibody secretion decreased 3 fold with time in culture.

Figure 8 : Analysis of Clonal Populations Isolated from Bioreactor: Samples were Carried Two Weeks at Low Density

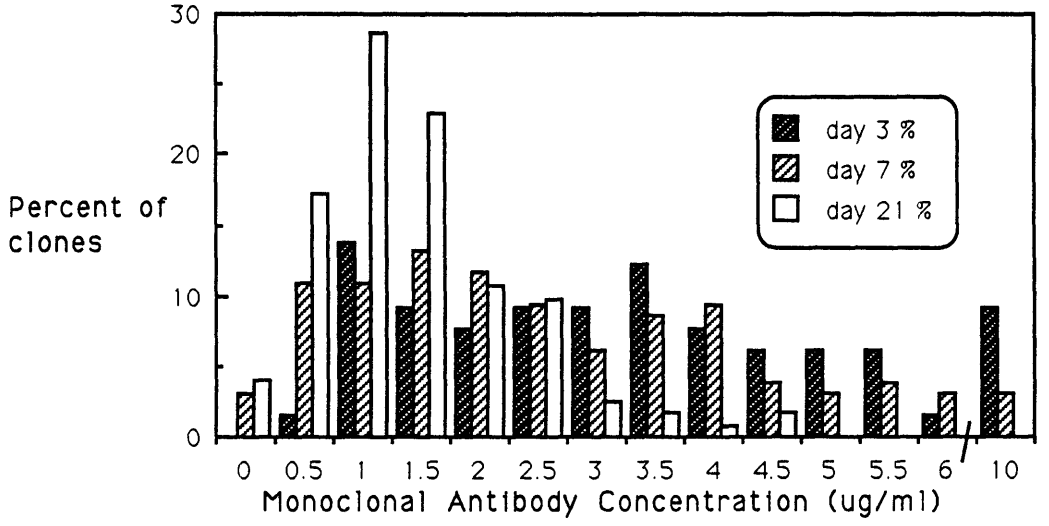


Table 5: Mean and Median Antibody Concentrations for Clonal Populations

Days in Bioreactor	Days in culture	# of clones	Mean IgG (μg/ml)	Median IgG (μg/ml)
3	14	65	3.3	2.9
7	18	129	2.44	2.0
21	32	122	1.2	1.0
Control	24	97	2.0	1.7

Further experiments by Buser (1991) confirmed that the low production at high density was reversible. The conclusion of the these experiments include the following:

- A non-producer population was not lost by freezing the cells. Plating efficiency of cells removed from the reactor was the same as cells maintained outside of the reactors.
- The decrease in secretion rate was reversed in two days. Cells removed directly from the bioreactor and carried at lower density in spinner cultures returned to the out of reactor secretion rate by three days. By flow cytometry, the intracellular antibody distribution returned to the out of reactor level within two days.

In the analysis of the molecular changes in the decrease in production in the reactor, Buser (1991) further confirmed that an irreversible loss of the gene copy number had not occurred. By determining the steady state mRNA levels and the translation rate by polysome analysis, she concluded that the loss in production in the bioreactors was a decrease in transcription and translation rates.

In the reactor, I observed a decrease in cell size of about 25%. Cell size, in general, is not a characteristic of producer vs non-producer hybridoma populations. Altshuler *et. al.* (1986b) and Ozturk and Palsson (1991) compared non producer to producer hybridoma cell lines and showed that cell size (by forward light scatter) does not change. Ozturk and Palsson (1991) also showed that DNA, RNA and protein content did not change.

B. Nutrient Limitation

One of the three hypothesized mechanisms for the reversible decline in antibody production was nutrient limitation in the bioreactors. In the metabolic studies of the first three bioreactor experiments, Tyo (1991) concluded that the cultures were limited by waste products and not nutrient limitation. These studies did not rule out limitation of components of the serum, vitamins, the sulfur containing amino acids or β-mercaptoethanol. The experiments and calculations of nutrient utilization are presented here which eliminate nutrient utilization hypothesis.

1. Utilization of Amino Acids, Glucose and Pyruvate

In the metabolic studies (Tyo, 1991) with the first set of bioreactor runs, concentrations of nutrients, glucose, pyruvate and amino acids, were measured by HPLC, and the rates of consumption or production were calculated. Presented in the table below is a comparison of the measured concentrations of nutrients which were supplied to the bioreactors (after two day incubation at 37°C) and the concentrations observed in bioreactor #1 at a constant flow of 3 volumes per day when antibody production was down regulated by a factor of 10.

Table 6:

Extracellular Nutrient Concentrations at High Density

Compound	Medium Concentration (μM)	High Density Low Productivity (μM)	Percent Remaining
GLUCOSE	24000	9000	38
PYRUVATE	700	540	77
LACTATE	2100	21500	1023
AMMONIA	1000	3500	350
Medium Amino Acids:			
GLN	3111	1294	42
ILE	835	705	84
LEU	850	640	75
LYS	760	648	85
THR	776	530	68
VAL	800	712	89
ARG	288	169	59
GLY	427	519	122
PHE	416	360	86
SER	413	182	44
TYR	430	521	121
CYS	113	61	53
HIS	127	137	108
MET	179	148	83
TRP	78	61	78
Serum Amino Acids:			
ALA	58	1295	2233
ASP	16	15	94
ASN	75	75	100
PRO	15	174	1160
GLU	43	12	25

Initial concentrations of amino acids in DMEM are as follows: 1) isoleucine, leucine, lysine, threonine, and valine at 800 μM, 2) arginine, glycine, phenylalanine, serine, and tyrosine at 400 μM, 3) cysteine, histidine, and methionine at 200 μM, and 4) tryptophan at 78 μM. Changes in aspartate and glutamate values were difficult to determine, since their concentrations approached the sensitivity of the measurement. Some of the amino acids concentrations decreased due to heating of the medium for sterile testing. Glutamine was initially supplied at 6 mM, and 2.5 mM typically remained, with

breakdown to pyrrolidone carboxylate and ammonia (Tyo; 1991). Also, arginine is unstable in tissue culture media (Lambert and Pirt, 1975).

At the constant flow rate of 3 volumes per day, nutrients were not exhausted and some compounds were excreted. Lactate and ammonia levels increased due to glucose and glutamine and other amino acid metabolism. The amino acids that were produced were alanine, proline, and glycine. (Experiments to test the inhibitory effects of these produced chemicals are discussed in section III.D). Among the consumed amino acids, cysteine and methionine dropped to the lowest concentrations. None of the consumed compounds measured were shown to be completely depleted at this or any other time point.

2. Adding Serum, Vitamins and Amino Acids in Low Density Experiments

I attempted to identify any nutrient limitation by looking for a media component that, when added to high density, low productivity (HDLP) medium, returned antibody production to the high level. I performed many groups of experiments with the supernatants of the reactor samples or outflow medium from the six bioreactor experiments. In general the supplementation of serum, vitamins, or amino acids to outlet media removed at later time points from the bioreactors did not relieve the suppression in antibody production of low density cells. The doubling time in the high density, low productivity media experiments was typically 25 - 40 hours while the doubling time of the fresh media was 17-20 hours. These experiments did not identify a limiting nutrient. The observations that cells can grow in the outlet media of the bioreactors, shows that nutrients were not depleted, and supports the conclusion that nutrient limitation did not occur in the bioreactors. However, at high cell density, a higher concentration of a nutrient may be required than at low cell density for transport into the cells.

3. Supply of Vitamins Exceeds Calculated Requirements

Although nutrient limitation by a vitamin supplied by the serum has been eliminated, nutrient limitation supplied by the defined medium (DMEM) has not been directly tested. The indirect tests of growing cells in the conditioned outlet medium indicate that an essential vitamin was not depleted. Since DMEM supplies 4 times the vitamin

concentrations than some of the other commercial media (eg. RPMI), this hypothesis is unlikely. Measurements of the vitamin concentrations or uptake rates have not been done with this cell line. I have estimated amounts of vitamins required at various reactor conditions and examples of these calculations are presented in the following table. Amount of vitamins supplied by DMEM and the 4% fetal bovine serum are listed. The vitamin consumption rates reported by Lambert and Pirt (1975) for MRC-5 cells have been converted to a per cell value (q_{vit}) with units pg/cell-hour. The amount of vitamin required for a batch culture is estimated as a 3 day culture inoculated at $5x10^4$ c/ml with a doubling time of 18 hours. The amount required for perfusion culture was calculated using the equation 20 inputting the total concentration of vitamins supplied for S_i. The other values were obtained from bioreactor #1 at a viable density of 10^7 c/ml, growth rate of .031 h^{-1}, separation ratio of .03, and a dilution rate of three volumes per day. At higher dilution rates the amount of vitamins is lower than listed below.

Table 7: Estimated Vitamin Utilization: Vitamins are not Depleted

Vitamin	Amount Supplied by DMEM (mg/L)	Amount Supplied by Serum 4% (mg/L)	Uptake rate q_{vit} (pg/cell-h)	Amount Required Batch 10^6 c/ml (mg/L)	Amount Required Perfusion 10^7 c/ml (mg/L)
biotin	0	1.6	.002	0.1	0.3
choline	4.0	4.2	.043	2.2	6.6
folic acid	4.0	0.1	.007	0.4	1.1
inositol	7.2	0.9	.040	2.0	6.1
nicotinamide	4.0	0.04	.007	0.34	1.1
pantothenic acid	4.0		.004	0.2	0.6
riboflavin	0.4	0.01	n.d.		
pyridoxine	4.0	0.004	.014	0.7	2.2
thiamine	4.0	0.005	.004	0.2	0.6

For all vitamins, the amount required for batch or perfusion culture is less than supplied by the defined media DMEM plus serum. The highest vitamin requirements are for choline and inositol, and the DMEM plus serum supplies about 8 mg/L of each. The estimated amount required in perfusion culture is over 6 mg/L, or 3/4 of the amount supplied. Overall, these calculations do not indicate a possible vitamin limitation during perfusion culture.

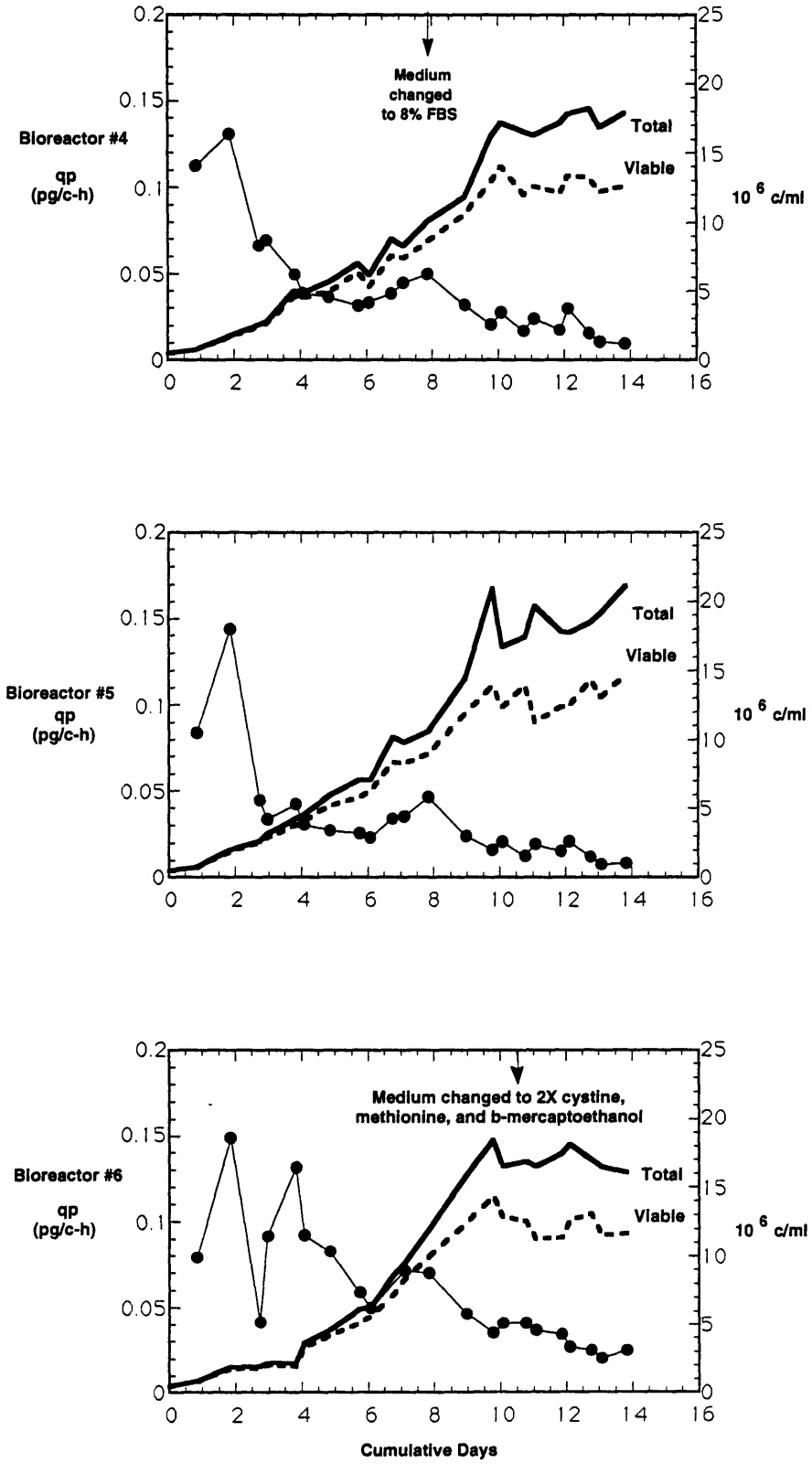
4. Adding Serum and Sulfur-containing Compounds during Bioreactor Experiments

Although the research by Tyo (1991) did not find a nutrient that reached zero concentration, the hypothesis that a nutrient limitation may have occurred was not entirely ruled out. First, nutrient limitation may occur at a low, non zero, level of a nutrient. Secondly, not all of the medium components were measured by these studies. The media supplied to the bioreactors was partly defined (Dulbecco modified eagle's medium (DMEM) supplemented with 50 μM β-mercaptoethanol, 6 mM glutamine and 2 mM pyruvate) and partly undefined (4% fetal bovine serum). From the undefined serum, glucose and amino acids were measured, but other essential nutrients, such as vitamins, hormones, and growth factors, were not measured. Therefore a first hypothesis is that a component supplied only by the serum, such as biotin, or vitamin B_{12}, was limiting.

One of the defined components of the medium that was not measured was β-mercaptoethanol, which is required for the growth of these cells and may have a role in the formation of disulfide bonds of the antibody protein. Since the measurement of cystine and methionine had the largest error, it was possible that one or both of these two amino acids transiently reached a near zero level. Therefore, a second hypothesis is that a sulfur containing compound became limiting for the production of the disulfide rich immunoglobulin product

In order to test the two hypotheses, the concentrations of nutrients were doubled in the inlet to different bioreactors. Shown in the figure below are the second set of perfused bioreactor runs numbered 4, 5, and 6. To test for serum limitation, the serum concentration was increased to 8% on the eighth day in bioreactor #4. To test for sulfur limitation, the inlet concentrations of cystine, methionine and β-mercaptoethanol were doubled at the eleventh day in bioreactor #6. In neither case did the antibody production return to the initial level of about 0.15 pg/cell-hour. Instead antibody production continued to decline from ~0.05 pg/cell-hour to 0.01 - 0.02 pg/cell-hour. In conclusion, the theories of nutrient limitation by either (a) serum, or (b) combination of methionine, cystine and β-mercaptoethanol have been eliminated.

Figure 9: Nutrient Limitation Experiments in Three Bioreactor Runs



5. Oxygen Limitation

Nutrient limitation of oxygen was prevented by the operation of the reactor system. To all three reactors, 40% oxygen was supplied to the stirred portion of the bioreactor, since it was previously shown that this cell line can tolerate this oxygen concentration and the oxygen supply would exceed oxygen uptake rates of .1 $x10^{-6}$ μM/cell-h (Oller *et. al.*, 1989). To bioreactor #5, silicone tubing was added to the cell separator and oxygen was supplied throughout the time of the run. On the last day of bioreactor #4-6, the oxygen concentration was determined to be about 120 μM (Buser, 1992). The trend of decline in antibody secretion was similar among the reactors, and the hypothesis of oxygen limitation in the cell separator was eliminated.

C. Cell to Cell Contact

One of the three possible mechanisms hypothesized for the down regulation of antibody production with increasing cell density is cell to cell contact. In the reactor system used, not only are the cells constantly stirred in the reactor but also the cells are concentrated in the conical separator and returned to the reactor. The question "Is cell to cell contact the signal?" was addressed by two approaches.

In the first approach, supernatants were taken from the down regulated phase of the bioreactors and tested for ability to suppress of antibody production of high producing, low density cells. From the data of the amounts of nutrient available, I calculated that cells had enough nutrients for three days growth when inoculated at $5x10^4$ cells/ml. In the initial experiments antibody secretion was measured by ELISA and by intracellular antibody content. I observed a suppression of antibody production, and concluded that the signal for the down regulation was conveyed in the medium and not by the cells.

Since the growth rate of cells in the conditioned medium was slower than the controls, and growth rate and antibody production appear linked, I was not convinced that the cell to cell contact theory was ruled out. It was possible that cells were nutrient limited, or that the accumulation of metabolic inhibitors slowed growth during the conditioned medium experiments.

In the second approach, I wanted to mimic high cell density without the presence of metabolizing cells. I chose to prepare crude membranes from both the high producing cells and the non secreting parent cell line and add them to fresh medium. Therefore nutrient limitation or diffusible inhibition differences between the test cultures and the controls was eliminated. In these experiments antibody production was the same as controls when the viability was > 85% and the doubling time was less than 40 hours. Therefore, membranes prepared from low density cells, do not convey a signal to shut down antibody production of high producing, low density cells.

1. Medium from the Bioreactor Inhibits Growth and Antibody Production

1.1 Growth and Antibody Secretion in Microwell Cultures

In the first experiments, conditioned medium removed from the first set of bioreactors down regulated the antibody production. In these experiments samples were drawn from the reactor, cells were removed by centrifugation and the supernatant was stored frozen (~2 ml). These supernatants were defrosted, sterile filtered (.2 micro Millex GV), and mixed with high producing cells in microwell plates. In these experiments, overall antibody secretion of initially high producing cells was determined after three days. From calculation of nutrients available and maximal nutrient uptake, cells would not be nutrient limited during the three day experiments. Plotted below are the observed secretion rates (Figure 10) and the apparent doubling times (Figure 11). Antibody production in the samples decreases with time in culture. Antibody production increased by mixing the samples 1:1 with fresh medium. But, since the apparent doubling time was 35 to 60 hours, it was likely that the cells stopped growing or changed growth rate during the test period.

Figure 10: Antibody Secretion of Low Density Cells in Test Supernatants

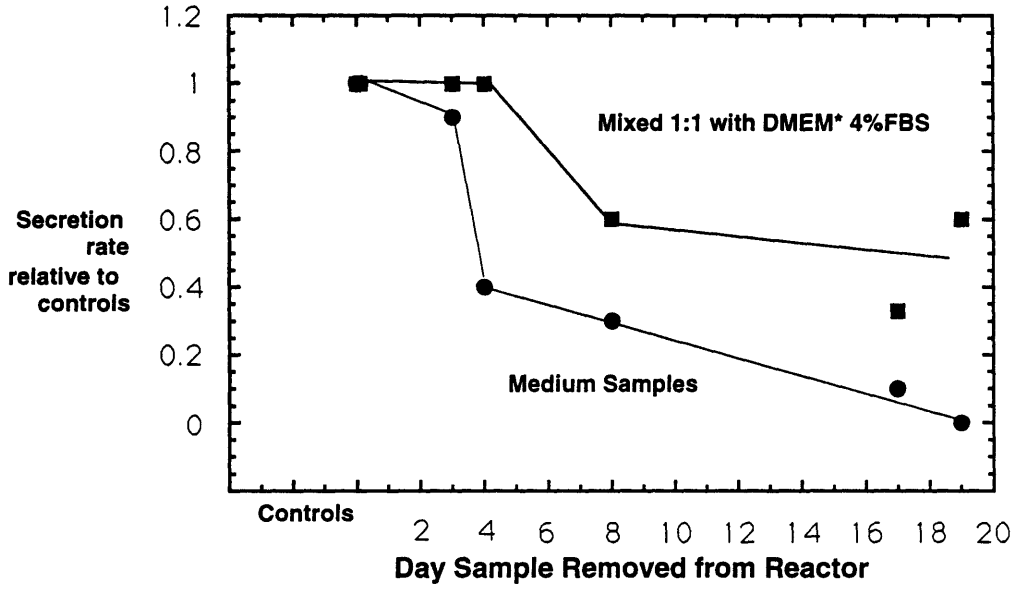
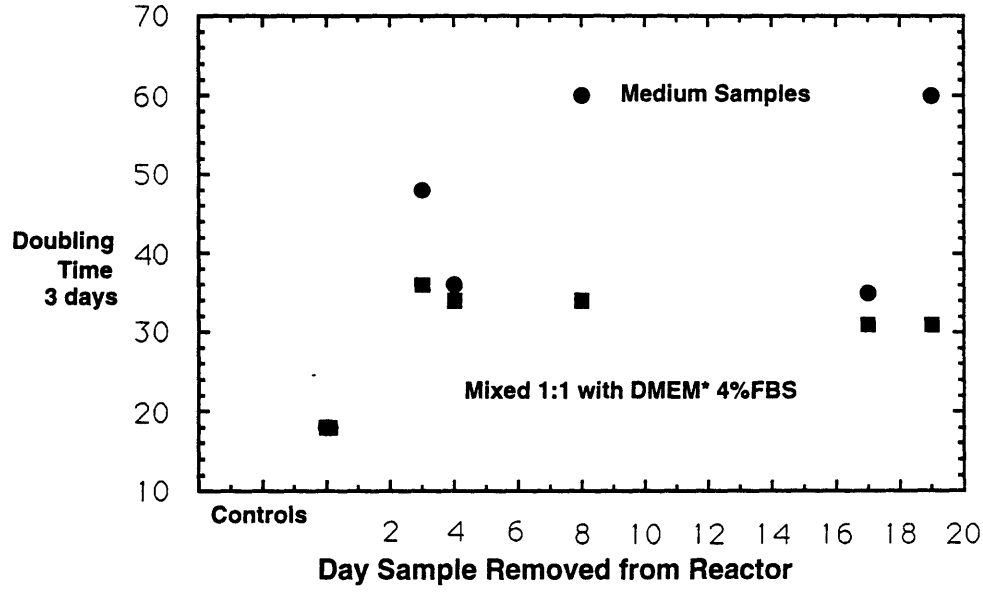


Figure 11: Doubling Time of Low Density Cells in Test Supernatants

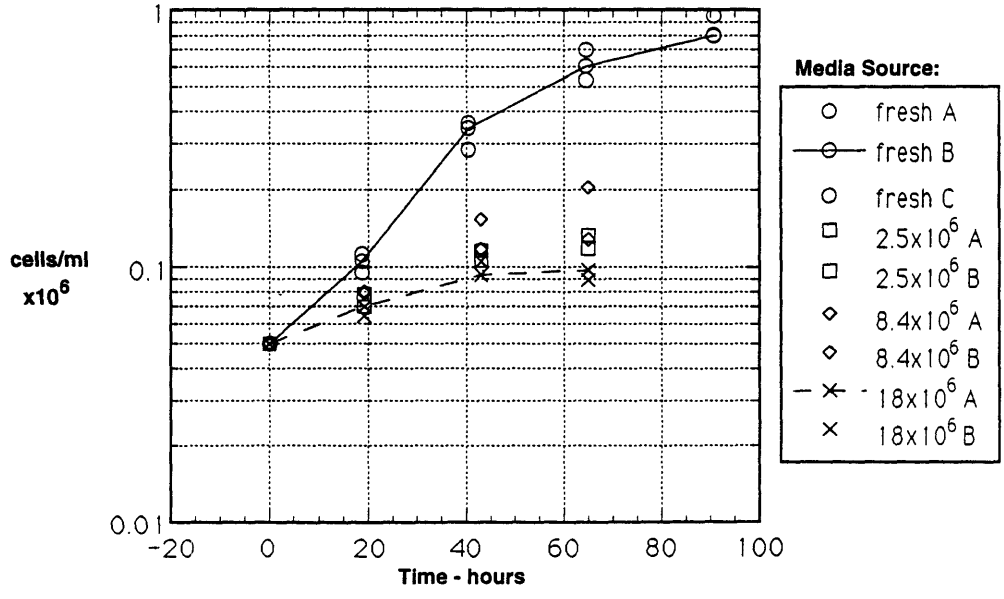


1.2 Growth Inhibition in T Flask Cultures

Conditioned media was collected from the outflow of bioreactors #4, #5 and #6 after cells were removed by one centrifugation spin at 500 g. The high density, low productivity media were stored at -20°C for later experiments. All cell debris was removed before testing the media by sterile filtration through a low protein binding 0.2 micron filter.

Cell density and antibody concentrations were measured daily for three days. High producing cells were inoculated at 5 x10^4 c/ml in medium removed from bioreactor #4 and #5 at days 3, 7 and 13 into 12 ml in 25 cm^2 T flasks. Plotted below in Figure 12 are the cell density in bioreactor #4 conditioned media. The cell density curves show that the growth rate in the test media is slower during the first two days than the fresh media cultures. For example the doubling time in fresh media was 15 hours, and the doubling time of cells grown in media from day 13 was 44 hours. Between the second and third day little or no increase in cell density was observed. Therefore the maximum density in fresh media is nearly 10 times the density reached in the day 13 (high density, low productivity) media. Antibody concentrations did not increase significantly as detected by the ELISA method.

Figure 12: Growth in Bioreactor #4 High Density, Low Productivity Media



1.3 Intracellular Antibody Contents in Spinner Cultures

The average intracellular antibody content correlates with the antibody secretion rate and can be measured with a few million cells by FACS analysis. Permeabilized cells are stained with a fluorescent conjugated goat antibody which binds human IgG(H+L) . With this method, Buser (1992) observed a decline in intracellular antibody content over time to 10% with cells removed directly from bioreactors #4, #5 or #6. When the down regulated cells were grown for two days in spinner culture at low density in fresh medium, she observed intracellular antibody content equal to the controls.

In order to compare results, I inoculated high producing cells at 5 x 10^4 c/ml into duplicate spinner cultures with the conditioned outlet medium of reactor #5. I stained for intracellular antibody after two days growth. I also lysed some samples by the procedure of Meilhoc *et. al.* (1989) and determined total cellular antibody content by ELISA. Results are listed in the table below.

Table 8: Intracellular Antibody Content after Growth in High Density, Low Productivity Media

Sample	10^6 cells/ml in bioreactor	Percent positive fluorescent staining	Percent Relative Intracellular Antibody by FACS	Percent Relative Cellular Content by ELISA
Controls		99 99	97 103	98 102
Day 3	2.6	93 91	54 49	39 27
Day 6	7.1	96 68	55 72	49 57
Day 10	16.7	88 98	57 74	29 20
Day 13	21.1	57 68	22 28	n.d. n.d.
Sp2/0		0 0	2 6	0 n.d.

n.d. not determined.

By intracellular antibody content, the antibody production was partially down regulated after two days in the conditioned media, but not to the extent observed during the bioreactor run. At days 3, 6, 10 and 13 in the bioreactor, the relative mean intracellular antibody content by FACS was approximately 65, 30, 15, and 10 percent (Buser, 1992 Figure III-48); but out of the reactor cells in the media gave averaged values of 51, 48, 68, and 25 percent. At day 13 in bioreactor #5, when the secretion rate was 0.01 pg/cell-h, the intracellular content by FACS was 10% of controls. In the day 13 the secretion rate was down to 0.07 pg/cell-h and the intracellular content by FACS to about 25%.

2. Membranes from the Secreting and Nonsecreting Cell Lines Do No Inhibit

The first goal of these experiments was to test the effects of addition of the crude membrane in a range which decreases the doubling time similar to the bioreactor condition. The second goal of these experiments was to test the theory that the membrane bound antibody was the signal to decrease growth and possibly antibody production.

I prepared side by side batches of crude membrane from the secreting CLC cells and the non-secreting parental Sp2/0 cells. With four batches I estimated an 18 - 27% yield (by 5' nucleotidase activity) and 1.5 - 2.5 fold purification. As a comparison, Lemonnier *et. al.* (1978) reported a 40% yield and 5.2 fold purification with a batch of cytotoxic T cells homogenized by nitrogen cavitation.

In two sets of experiments, the range of doses of crude membranes spanned from 0.2 - 12 $x10^7$ equivalent cells/ml. Crude membrane preparations were added to duplicate 1 ml cell cultures in 24 well plates. Cells were inoculated at $4x10^4$ cells/ml, and viable cell densities were determined by trypan blue exclusion after three days. No growth was observed in some cultures at a dose 3×10^7 equivalent cells/ml and cell lysis was observed at doses 6, 8 and 12×10^7 equivalent cells/ml.

Antibody secretion was determined by ELISA, accounting for the initial contribution by the crude membranes. Antibody contribution from the CLC crude membranes was linear with amount of CLC crude membrane. No degradation of the standard IgG compared to controls was observed after a 3 day incubation at 37^o C with the CLC crude membranes. And as expected, no antibody was detected in the crude membranes prepared from the Sp2/0 cells.

Plotted in the next four figures is the data for the doses ranging up to $1.5x10^7$. The crude membrane fraction decreased the final cell density and at 1.5×10^7 dose the final cell number declined to ~ 20% of the control. Up to dose of $0.75\ x10^7$, viability was generally high at ≥ 95%, and at the 1.5×10^7 dose the viability declined slightly to 88-93%. The doubling time increased from 16-18 to 30-35 hours. Although the antibody secretion rates are noisy, they do not indicate a trend in inhibition. The data shows no obvious difference between the crude membranes from either secreting CLC or the non-secreting Sp2/0 cells, and therefore the membrane bound antibody is not a signal.

Figure 13: Growth with Addition of Crude Membranes

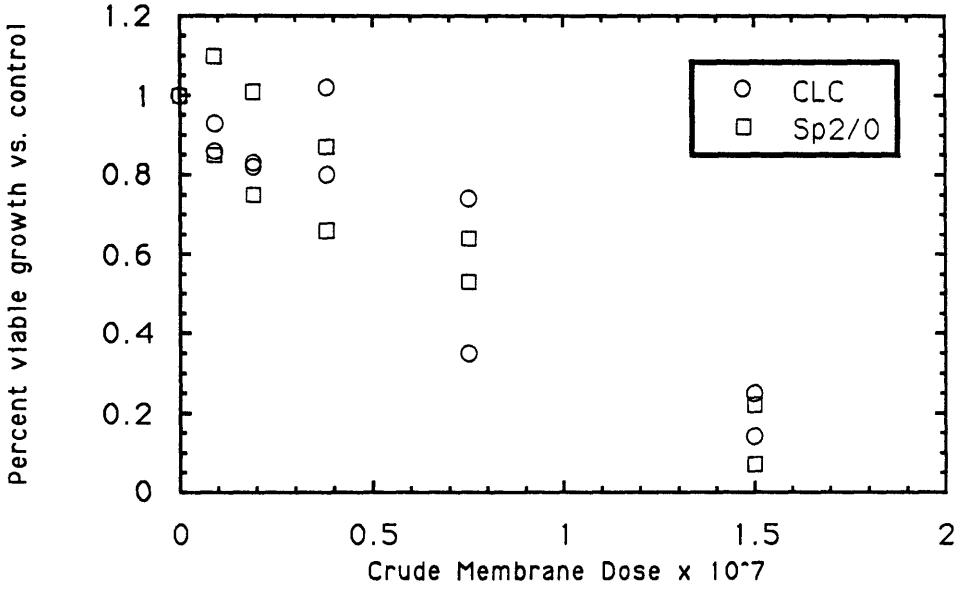


Figure 14: Viability with Addition of Crude Membranes

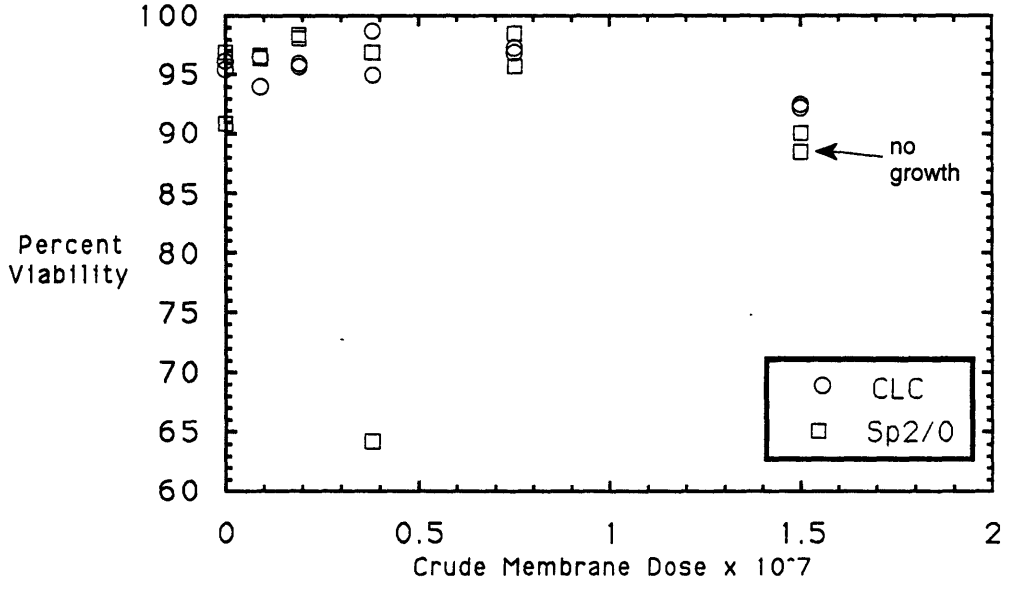


Figure 15: Doubling Time with Addition of Crude Membranes

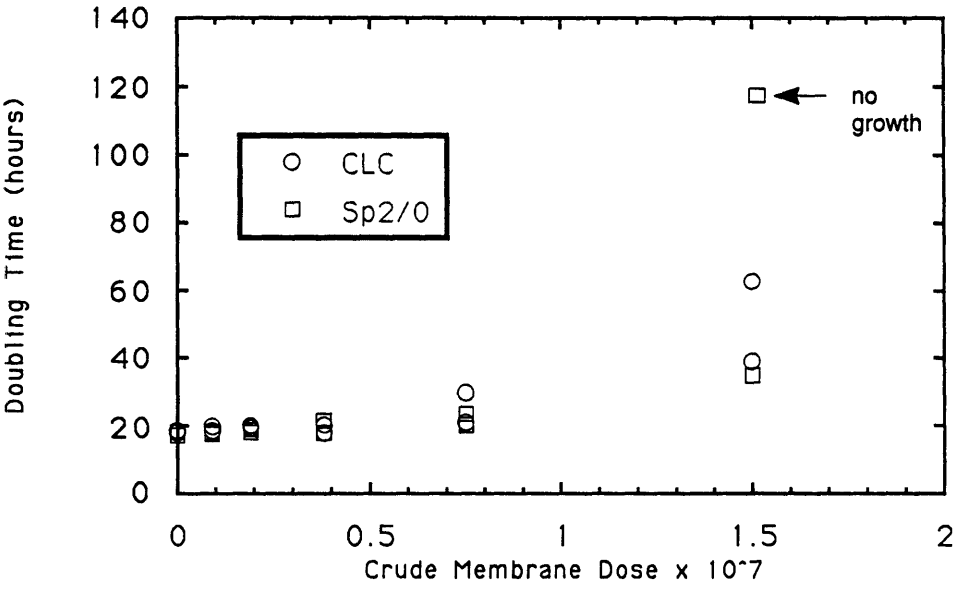
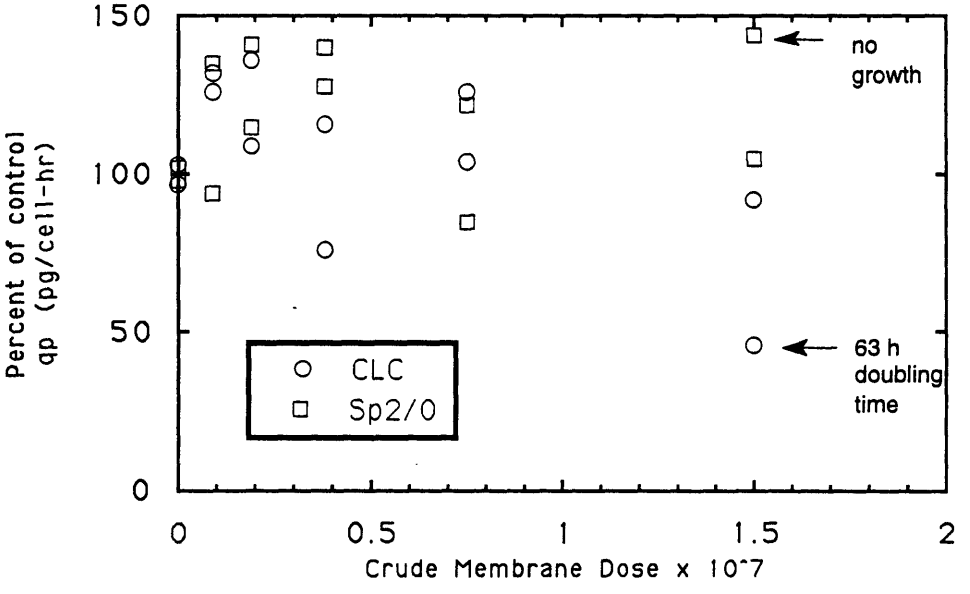


Figure 16: Antibody Secretion with Addition of Crude Membranes



D. Diffusible Inhibitors

The third hypothesized mechanism for the reversible decrease in antibody production was diffusible inhibitors. The separate and combined effects of various inhibitors of antibody production were tested in low density cultures. Results presented below demonstrated that secreted amino acids did not inhibit antibody production. Redox potential alone, a function of lactate and pyruvate concentrations, did not decrease antibody production. Lactate and ammonia accounted for part of the inhibition of antibody production. To characterize the unknown inhibitors, the HDLP medium was fractionated by gel filtration.

1. Secreted Amino Acids Do Not Inhibit Antibody Production

The concept of a diffusible inhibitor includes any compound secreted by the cell including amino acids. In the bioreactor medium amino acids are supplied in the 0.2 - 0.8 mM range. Since alanine concentrations rose to over 1 mM in the reactor, it was hypothesized that alanine was a competitive inhibitor for other neutral amino acids. Another possible signal was proline. Proline concentrations increased from about 100 to 500 mM as the cell density increased from ~ $1x10^6$ to 6 $x10^6$ c/ml. Similarly, glycine concentrations increase 0.2 mM over in the input of 0.4 mM. The hypothesis that the elevated concentrations of amino acids observed at high cell density was a signal for the decreased antibody production was tested in lower density cultures. Fresh medium was supplemented with 1.4 mM alanine, 0.5 mM proline an 0.2 mM glycine. Cells were inoculated at 5 x 10^4 cells/ml in duplicate 12 ml cultures, and growth and production was determined over four days.

Table 9: Amino Acid Imbalance, Addition of Amino Acids to Fresh Medium

Medium	Doubling time (hours)	Secretion rate (pg/c-h)
Control	20	0.15
with alanine	20	0.14
with proline	21	0.18
with glycine	20	0.14
with Alanine, proline and glycine	21	0.18

The addition of alanine, proline and glycine had no large effect on growth or antibody production. The final average antibody concentrations for all the cultures ranged from 4.4-5.0 μg/ml. With the typical errors of measurement of cell density and antibody concentration of ± 5% and ±10%, respectively, (Phillips *et. al.*, 1991) the rate of secretion is not signifantly different in this experiment. Another researcher (Duval *et. al.*, 1991) tested the addition of 10 mM alanine to a murine hybridoma and also found no decrease in antibody secretion. Therefore, neither alanine, proline or glycine signal the decreases antibody production.

2. Lactate and Ammonium Decrease Antibody Production

In the reactor samples at high cell density, lactate and ammonium concentrations reached 28 mM and 4 mM, respectively. Since lactate concentrations of 20 mM and ammonium concentrations of 5 mM had been reported to decrease antibody production in other hybridoma cells (Glacken *et al.*, 1987; Reuveny *et al.*, 1986), I tested the effects of these two metabolic waste products. Sodium lactate and ammonium chloride were added to batch spinner cultures inoculated at 5 $x10^4$ c/ml and antibody secretion over three days was measured. The data for the two experiments are presented below. In the first experiment 30 mM sodium lactate was tested. The doubling time increased from about 17 to 19 hours and the antibody production decreased by one half. In the second experiment four conditions were compared to controls. The addition of 2 and 4 mM ammonium chloride was tested alone. Also, tested were two combinations observed in the bioreactor : 2 mM ammonium and 5 mM lactate (high productivity condition); and 4 mM ammonium and 20 mM lactate (low productivity condition). The first combination increased the doubling time from 17 hours to 19 hours but had no effect on antibody production. The higher combination increased the doubling time to 21 hours and decreased antibody production 33% compared to controls. Therefore, these two metabolic inhibitors, when were tested at lower cell density, did not account for the 10 fold reduction in antibody production as observed at high cell density in bioreactor #1-3.

Table 10: Effect of Added Lactate and/or Ammonium to Spinner Cultures

Additions to Medium	doubling time (hours)	qp antibody (pg/cell-h)
None	17	0.14
30 mM sodium lactate	19	0.08
None	17	0.21
2 mM ammonium chloride	18	0.17
4 mM ammonium chloride	21	0.13
2 mM ammonium chloride and 5 mM sodium lactate	19	0.19
4 mM ammonium chloride and 20 mM sodium lactate	21	0.14

3. Redox Potential Alone Does Not Inhibit Antibody Production

During the bioreactor runs a number of conditions changed at the same time as the antibody production declined, including the redox potential. The observed redox potential was calculated with the Nernst equations below:

$$E'_h = E'_o + \frac{RT}{F} \log \frac{[\text{electron acceptor}]}{[\text{electron donor}]}$$

where E'_o is the standard redox potential (mVolts), R is the gas constant (8.31 joules/^oK-mole), T is the temperature (^oK), and F is the faraday constant (96,406 joules/volt). Since the redox pair of lactate and pyruvate are in excess over other pairs, the redox potential (m Volt) can be estimated by the following equation.

$$E'_h = -0.215 + 0.03 \log \frac{[\text{pyruvate}]}{[\text{lactate}]}$$

As demonstrated in the plots below with data from bioreactor #1, the specific secretion rate (q_p) and the viable cell density (X_V) show clear trends with redox potential.

Figure 17: Antibody Productivity vs. Calculated Redox Potential in Bioreactor #1

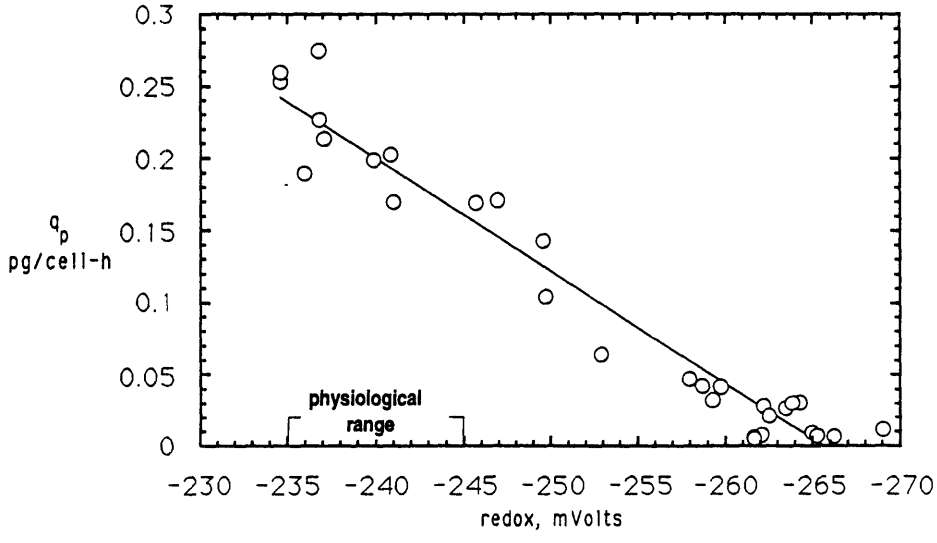
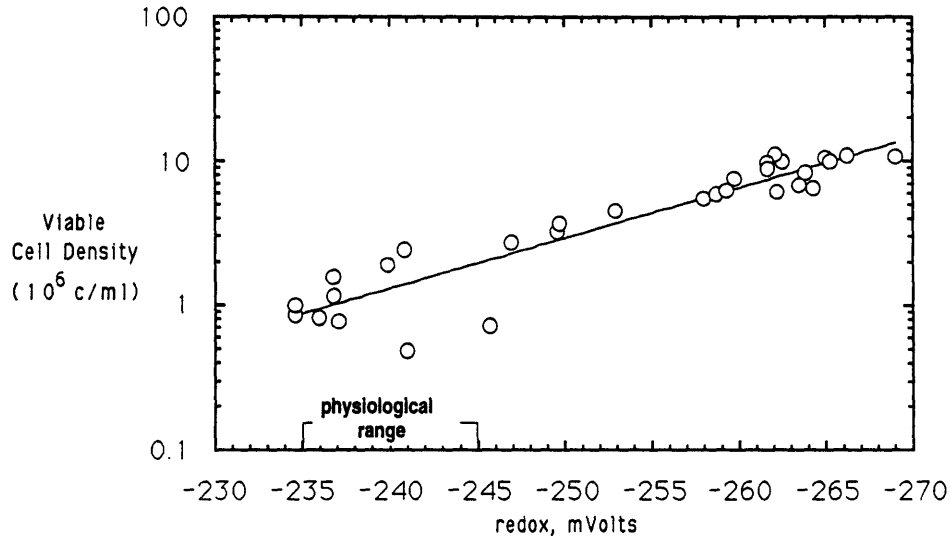


Figure 18: Viable Cell Density vs. Calculated Redox Potential in Bioreactor #1



For antibody production, the trend of redox potentials was a linear decline from -235 to -265 mVolts, where:

$$q_p = a_1 + a_2 \text{ (redox)}$$

and for data in Figure 17:

$$q_p = 2.1 + 0.008 \text{ (redox)}.$$

Since the specific secretion rate (q_p) is a function of viable cell density above ~2 million cells/ml (X_{VO}) where:

$$q_p = q_{po} * \exp(-a_o X_V) = \exp(-a_o X_{VO}) * \exp(-a_o X_V)$$

then the viable cell density increases exponentially with redox from -235 to -265 as:

$$X_V = a_3 * \exp(-a_4 * \text{redox}).$$

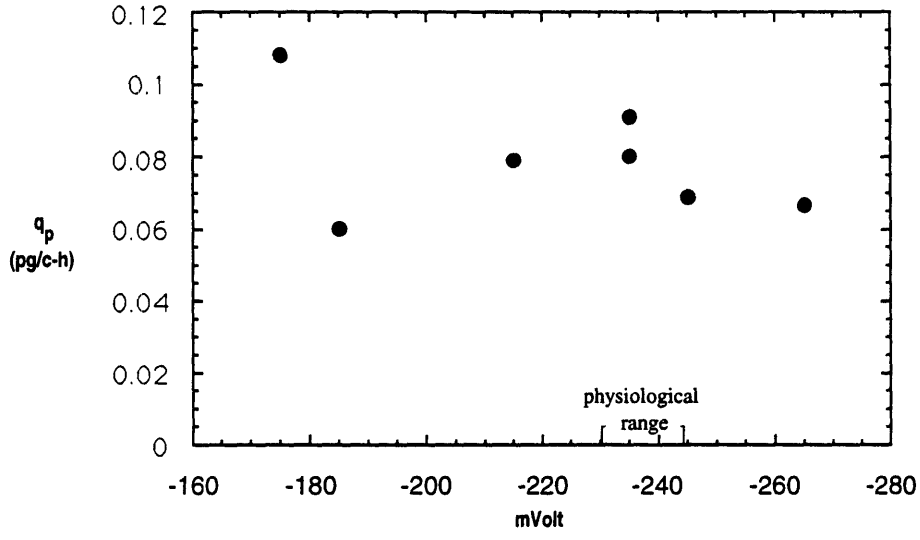
For the data in Figure 18:

$$X_V = 5.7 \times 10^{-9} * \exp(-.08 * \text{redox}).$$

One hypothesis that was proposed early in this project was that the redox potential was driving the metabolic and antibody production changes. The physiological range for perfused rat liver cells was observed by Brunengraber *et. al.* (1973) to be in the -236 to -244 mVolt range. I tested the hypothesis that the redox potential was the signal for the decrease in antibody synthesis by changing the initial pyruvate to lactate ratios of lower density cultures. In order to reduce effects of lactate inhibition, total lactate plus pyruvate concentrations equaled 10 mM, except for the control which contained 2 mM pyruvate and no lactate. In this experiment, -265 mVolt condition was set with 9.8 mM lactate and

0.22 mM pyruvate. The antibody production at ~10 mM lactate in bioreactor #1 was decreased to ~20 % and bioreactor #4 was decreased to 75%. For this lower density test, cells were inoculated at $6.6x10^4$ cells/ml and grown for three days. Plotted in Figure 19 are the antibody production rates.

Figure 19: Antibody Production at Lower Density in Redox Media



Within the range of -215 to -265 mV no change in the doubling time was observed. At an initial value of -180 mV, which is outside the range observed in the reactor, the doubling time was 47 hours. Within the -235 to -265 mV range, antibody production declined about 90% from 0.2 to 0.02 pg/cell-h, in the reactor, but in lower density cultures the antibody production is not significantly different. Although the depression of antibody production was shown to be partially reversed within one day (Buser, 1992), the external signal for this change may require more than three days. Therefore as observed in the above 3 day experiment, the redox potential condition does not by itself decrease antibody production.

4. The Combined Effects of Lactate, Ammonium and Redox

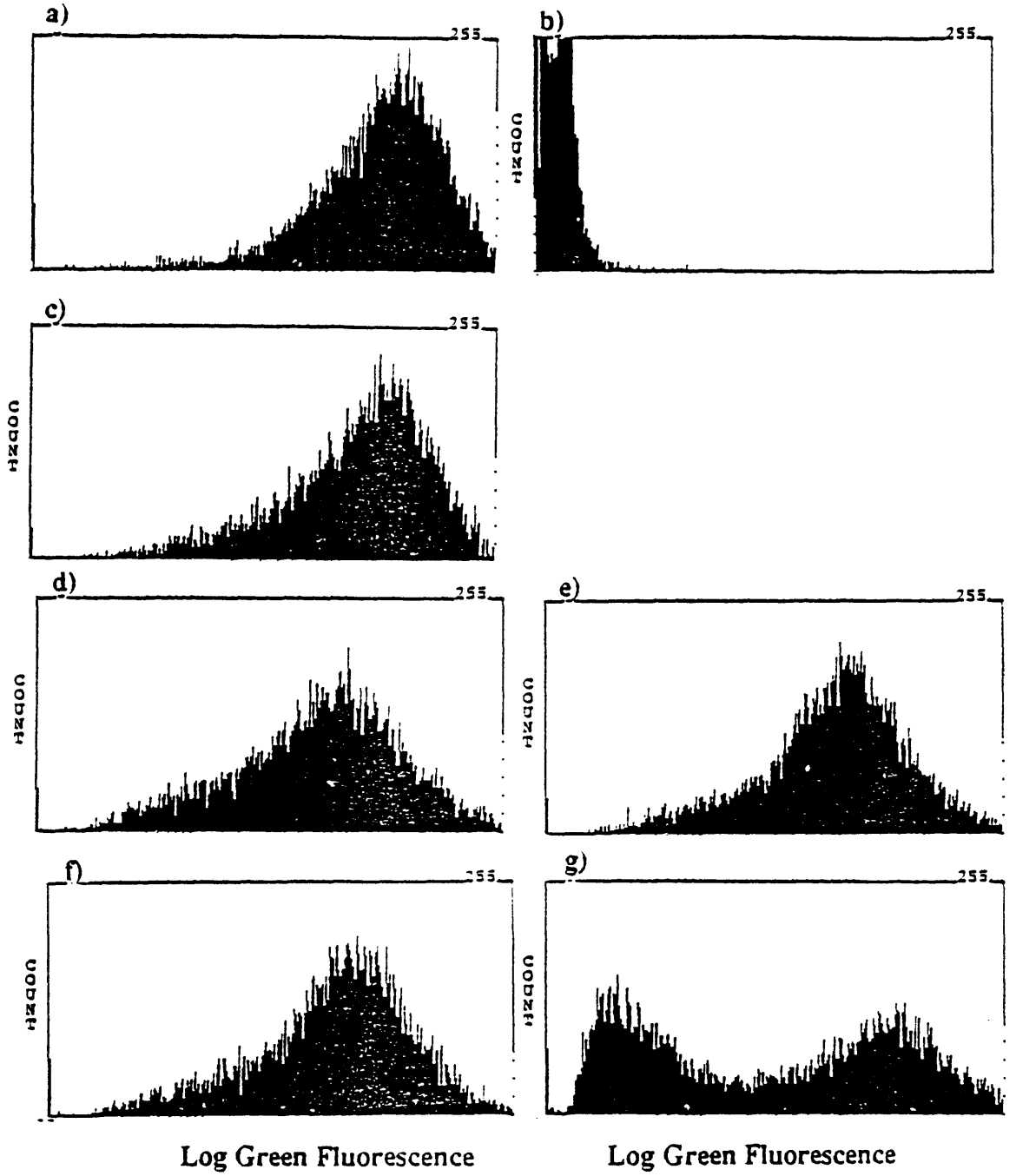
The goal of these experiments was to combine known changes to the medium and test for additional inhibition of antibody production at low density. Combinations of high lactate (25 mM sodium lactate), high ammonia (4 mM ammonia chloride) and low redox potential (-265 mVolts) were tested. The initial redox potential was set with low sodium pyruvate of 0.6 mM and high lactate of 25 mM. The effects of ammonia, lactate and pyruvate were compared to media removed from the outlet stream of the bioreactor at $18x10^7$ total cells/ml. High producing cells were inoculated at a low density (4×10^4 cells/ml) into spinner flasks, and intracellular antibody content was determined after two and four days by flow cytometry.

Histograms of intracellular antibody content after four days are presented in Figure 20. Additions of lactate and ammonia with redox of -248 mVolts (panel d), altered redox of -265 mVolts (panel e), or altered redox plus ammonia (panel f), all shift the distribution of antibody content compared to the positive control in fresh media (panel a). Addition of salt had a smaller effect (panel c). The negative control, the non-producing parental cell line stained as the other samples, is shown in (panel b). Unlike the other cases, cells placed in high density, low productivity (HDLP) media from $18x10^7$ cells/ml had a bimodal distribution, and therefore fewer positive staining cells. Note that the antibody content of the lowest producing peak is higher than the nonproducing controls because cells that have stopped secretion entirely still contain antibody containing vessicles according to EM studies (Al-Rubeai, *et. al.*, 1990). Results of growth, percent positive staining and intracellular antibody content are listed in the two following tables.

These histograms suggest two types of cell populations: (A) producing, (B) non-producing. As a comparison, samples removed from bioreactor #4, cells at day 3 were population type A, day 6 population A but with a lower mean antibody content, and day 10 and 13 mostly population B (data shown in Figure 21). Cells carried in the HDLP media for two and four days had 81% and then 69% percent positive staining cells (population A). Cells carried in combinations of lactate, ammonia and pyruvate had 94% or greater positive staining cells (population A). Variables other than lactate, ammonia and redox therefore shifted cells at low density to non-producing cells.

Figure 20. Histograms of Intracellular Antibody Content

after Four Days in Lactate, Ammonia and Redox Conditions



Cells were grown at various conditions for four days, passaged on day two. Panel (a) positive control in fresh media, (b) negative control of nonproducing cells in fresh media, producing cells in (c) fresh media with 25 mM sodium chloride, (d) 25 mM sodium lactate and 4 mM ammonia chloride, (e) fresh media with 25 mM sodium lactate and 0.6 mM sodium pyruvate, altered redox, (f) fresh media with 25 mM sodium lactate, 4 mM ammonia chloride and 0.6 mM sodium pyruvate, altered redox plus ammonia, (g) HDLP media from $18x10^7$ total cells/ml and 13×10^7 viable cells/ml.

Table 11: Two Days in Lactate, Ammonia and Redox Conditions

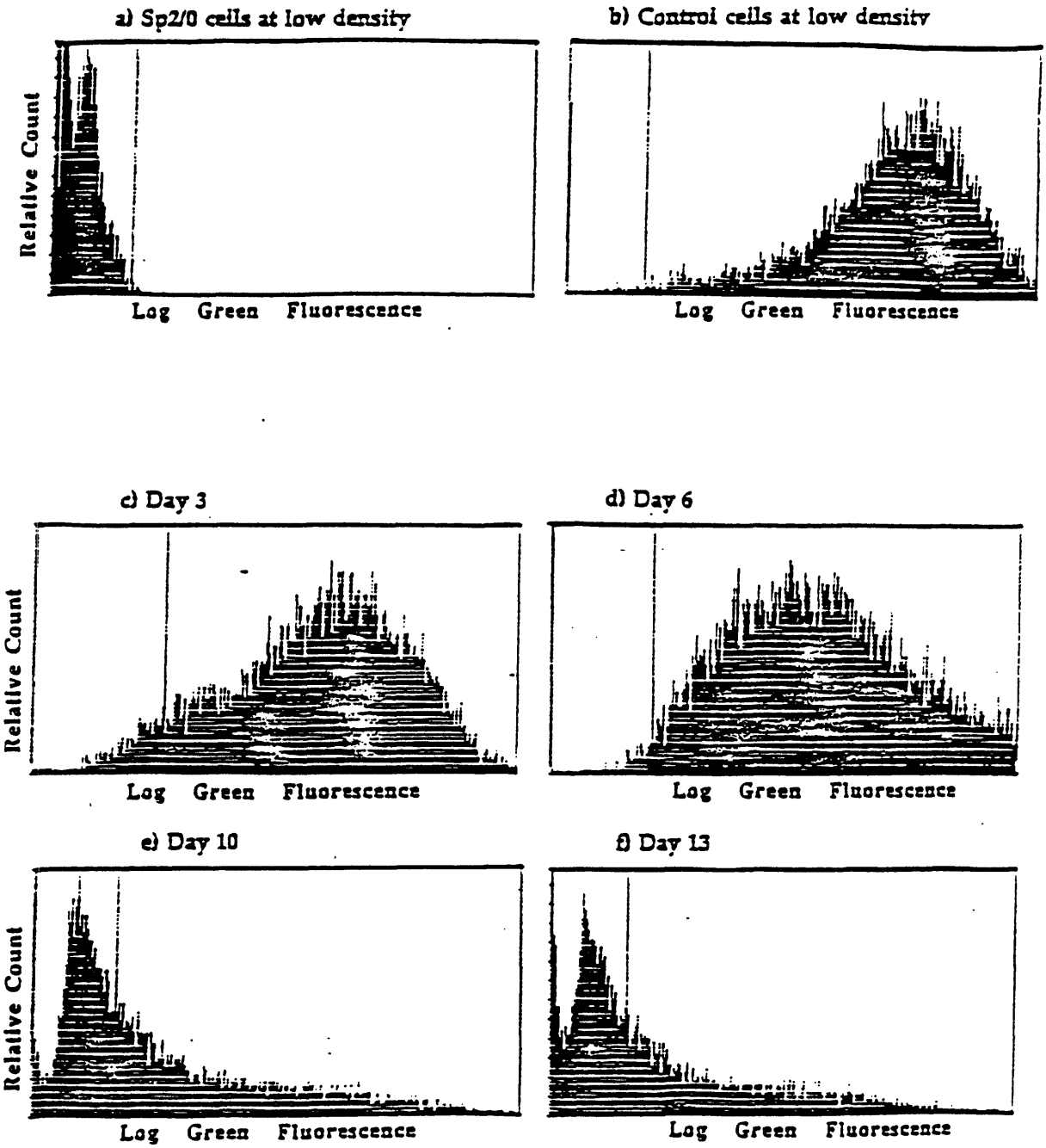
Medium	doubling time (h)	Percent positive	Relative IgG content
Fresh media with:			
a) Positive control	18	98	100
b) Negative control non producing cells	15	0	0
c) Salt (25 mM sodium chloride)	20	98	100
d) 25 mM lactate + 4 mM ammonia	21	95	66
e) Low Redox (-265 mV)	19	94	77
g) HDLP Media {18 x10^7 c/ml}	22	81	46

Table 12: Four Days in Lactate, Ammonia and Redox Conditions

Medium	doubling time (h)	Percent positive	Relative IgG content
Fresh media with:			
a) Positive control	16	99	100
b) Negative control non producing cells	15	0	0
c) Salt (25 mM sodium chloride)	17	98	74
d) 25 mM lactate + 4 mM ammonia	19	95	41
e) Low Redox (-265 mV)	18	96	42
f) Low Redox + ammonia	21	96	45
g) HDLP Media {18 x10^7 c/ml}	24	69	34

The doubling time for all cases was slower than the positive controls in fresh media. Cells were passaged on day two. At the second day in (d) high lactate and ammonia or in (e) low redox, the antibody section was reduced to 66 and 77 percent. Four days in either (d) high lactate and ammonia, (e) altered redox, or (f) altered reduce plus ammonia decreases antibody production to 41-45%. HDLP media at 18 x10^7 c/ml has lactate and ammonia of 22.9 mM and 4.2 mM and redox of -260 mVolts. Cells in this HDLP media gave even lower antibody secretion (of 46% and 34% at days 2 and 4) than any of the conditions tested. In conclusion, the lactate, ammonia and redox conditions account for most of the decrease in antibody production but not all. Also, the lower redox potential does not contribute more to the inhibition by lactate and ammonia.

Figure 21. Histograms of Intracellular Antibody Content of Cells Removed from Bioreactor #4



Cells were removed from bioreactor #4 stained and analyzed. Each day controls cells of producing CLC LT14 and non producing cells were analyzed, and one set is shown. Panel sample with {Relative Intracellular Antibody Content}: a) Sp2/0 cells (negative control), {1}; b) CLC LT14 cells maintained at low density (positive control) {100}; and cells removed from bioreactor #4 on c) day 3, {90}, d) day 6 {33}, e) day 10 {16}, and f) day 13 {15}. Figure taken from Figure III-45 of Buser, 1992.

5. A High Molecular Weight Inhibitor Was Not Found

Since the theories of nutrient limitation and cell to cell contact have been eliminated, the diffusible inhibitors were characterized by molecular weight. With the gel filtration media, it is possible to separate the higher molecular weight compounds of high density, low productivity media and replace the lower molecular compounds with fresh media. This experimental design has two notable advantages. Low molecular weight inhibitors such as lactate and ammonia are separated from any high molecular weight inhibitors. Also nutrient limitation can be prevented.

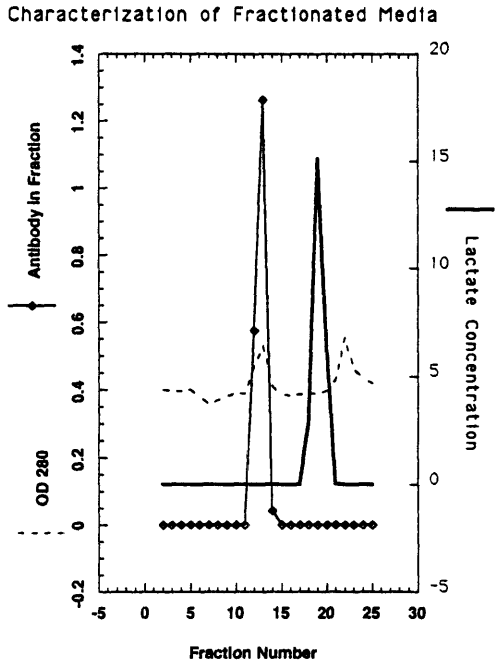
This strategy has been employed by Rønning *et. al.* (1991) to search for inhibitors secreted by 8 antibody producing cell lines. They separated conditioned media with the gel filtration media (sephacryl S-200 and sephacryl S-300, Pharmacia). They found two peaks of inhibitory activity. All 8 cell lines had low molecular weight inhibitory activity ("close to the salt fraction") and two cell lines also had high molecular weight inhibitory activity (coeluting with albumin). The authors assumed that substance(s) of about 5 kD is (are) growth limiting.

Using the same gel filtration media (sephacryl S-300, Pharmacia), I fractionated two samples of HDLP media eluted at 1.8×10^7 total cells/ml. The S-300 gel filtration material separates globular proteins of 1×10^4 to 1.5×10^6 MW. The 10 ml samples were loaded on a 200 ml column and eluted with DMEM*. The 10 ml fractions were sterile filtered. Samples of the fractions were characterized for OD 280 (for protein), antibody, lactate, ammonia and pyruvate concentrations. After adding 4% fetal bovine serum to the fraction, I tested for inhibition growth and of antibody content and secretion.

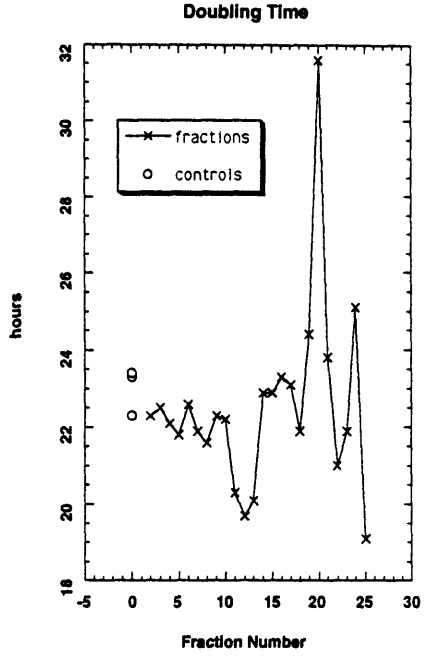
The elution characteristics of the first column run are shown in Figure 22a. The OD280 was measured to detect protein, and the first maximum was at peak 13. The maximum antibody concentration (MW150kd) was found by ELISA also at peak 13. As shown, the highest concentration of lactate, 15 mM, eluted at fraction 19. Not shown, the maximum ammonia concentration was 1.5 mM at peak 20, and the minimum pyruvate concentration was 0.6 mM in fraction 18. Therefore the elution of the salt fraction began by fraction 18. The calculated redox potentials for fractions 18, 19 and 20 are -236, -245, and -230 mVolts.

Figure 22: Fractionation of High Density, Low Productivity Media

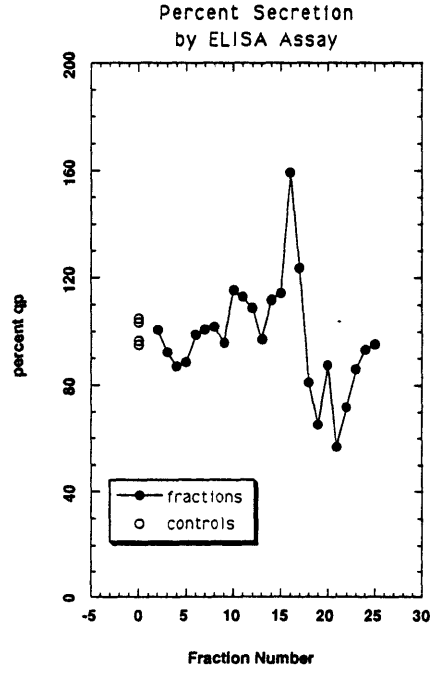
(a)



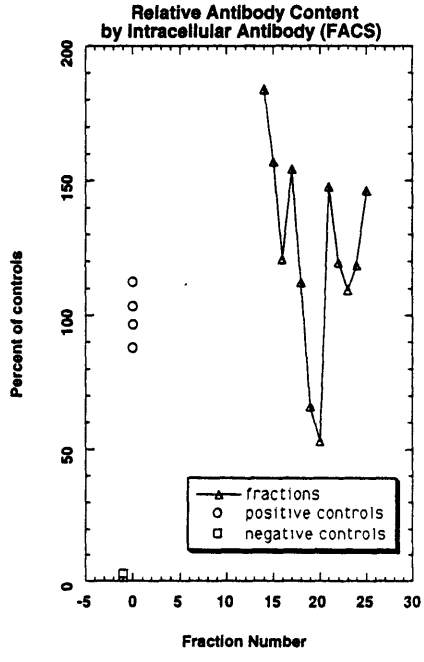
(b)



(c)



(d)



To test for growth and antibody inhibition, serum was added to 5 ml of each fraction, and cells were inoculated at $4x10^4$ c/ml, and grown for four days in 25 cm^2 t flasks. Total and viable cell density was measured. The calculated doubling times, plotted in Figure 22b, show one fraction, #20, that has a longer doubling time than controls grown in fresh media. The slightly faster doubling times at fractions 11-13 may be due to additional serum factors. Antibody secretion (Figure 22c) was at a maximum at fraction 16 (160%), and a minimum at fractions 18 and 20 (55 and 45%) compared to control cultures. The minimum intracellular antibody content of fractions 14 to 24 (Figure 22d) was at fraction 19 and 20, at ~50% of controls. None of the compounds measured account for the increase in antibody production characteristics of fractions 14 or 15.

Although the elution of lactate, ammonia and pyruvate coincide with the decrease in growth and production, do these factors, at the observed concentrations, account for all of the inhibition? To test this idea, reconstruction experiments were performed simultaneously with testing the second set of fractionated media. Media was supplemented to contain the amounts of lactate, ammonia and pyruvate observed in fractions 19 and 20, called R19 and R20. The higher amounts of 25 mM lactate and 4 mM ammonia present in the bioreactor runs was retested in samples "LN" (redox -248 mVolts), and "LNP" (redox -264 mVolts). The media "Mix" combines fractions excluding the inhibitory 19 and 20 (10 to 18 plus 21). Cells grew for four days from $4x10^4$ c/ml, and the results appears in the below.

Table 13: Comparison of Fractionated Test to Reconstruction Experiments

	Media: Fraction or Reconstruction Number							
	Fresh	19	R19	20	R20	LN	LNP	Mix
lactate (mM)	0	11.7	11.7	4.1	4.1	25	25	0
ammonia (mM)	0	1.1	1.1	1.8	1.8	4	4	0
pyruvate (mM)	2	1.2	1.2	2.1	2.1	2	2	2
redox (mVolts)	na	-245	-245	-224	-224	-248	-264	na
Doubling times (hours)		18	17	18	17	18	19	17
Intracellular Antibody Content		25	58	38	72	46	66	80

The factors of ammonia, and lactate, and pyruvate (and therefore redox potential) do not account for all of the inhibition in fractions 19 or 20. Note that the redox potentials of -230 to -245 mVolts give high production of 75 - 100% in the bioreactors (Figure 17), and ~80% secretion when tested at lower density (Figure 19). The "Mix" had 80% of the antibody content, and therefore no significant source of inhibitors. Inhibition in fraction 19 was 25% and 58% in the reconstruction. Inhibition in fraction 20, with the highest ammonia (1.8 mM), was 38% and 72% in the reconstructed media. The higher lactate and ammonia (LN), and the high lactate ammonia and low redox (LNP) had antibody content of 46% and 66%, respectively, which is more than fraction 19 or 20. Therefore the two fractions, 19 and 20, contain an additional inhibitory or limiting factor(s).

Although the design of the column filtration eliminated nutrient limitation in samples of high molecular weight, it is likely that the decreased concentrations of some nutrients separated into fractions 18 - 20. To check for nutrient limitation, amino acid content of the column fractions and samples after cell culture were analyzed (Biopolymers Lab, MIT). Since nutrient limitation is ruled out by these measurements, then any additional inhibitors are present in these fractions with a molecular weight excluded by the gel (less than 10,000 MW).

E. Shift in Amino Acid Utilization

The second major goal of this project is to investigate the metabolic shift in amino acid uptake and to close the mass balance on amino acid utilization. The metabolic shift is defined here as the decrease in the overall amino acid uptake rates. In this section, presented are the experiments to test the hypothesis that at low density the high rate of amino acid uptake is for lipid synthesis. In short term labeling experiments, the uptake rate of a ketogenic amino acid into lipid is shown to be density dependent. Also, the amino acid pools are density dependent. Samples from the bioreactor experiment were analyzed for protein and lipid content. Protein and lipid content declined with increasing cell density. A model to close the mass balance will be presented in section IV.C based on the experimental data present here.

1. Metabolic Shift in Amino Acid Uptake

The phenomenon of the down regulation is associated with a metabolic shift in amino acid uptake (Tyo, 1991). In the next two figures, the total amino acid uptake rates are plotted vs. time and vs. cell density. The total rate declines from about 250 to 50 fmole/cell-h over the first sixteen days in bioreactor #1. Since the change in amino acid uptake occurs at the same time as the cell density increases, another variable appears to correlate with density. Replotting the data vs. cell density gives a linear-log relationship. Cell density is a decreasing function of amino acid uptake (q_{aa}) of the form:

$$X_V = X_{VO} * \exp(-a_O\, q_{aa})$$

and for the data in Figure 24:

$$X_V = 15.6 * \exp(-0.013\, q_{aa}).$$

Experiments presented in section E.2 test the hypothesis that amino acid uptake is density dependent out of the reactor.

Figure 23: Total Amino Acid Uptake Rates vs. Time

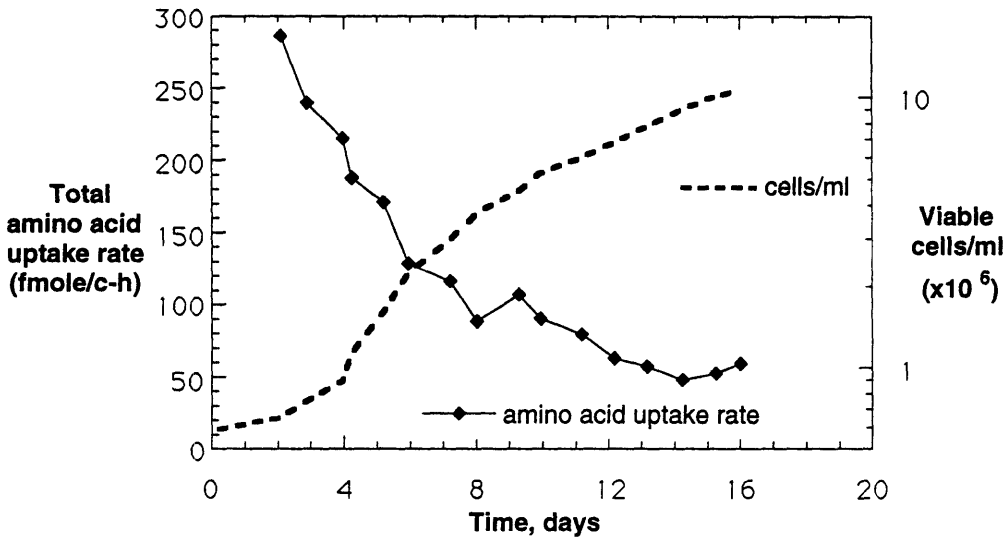
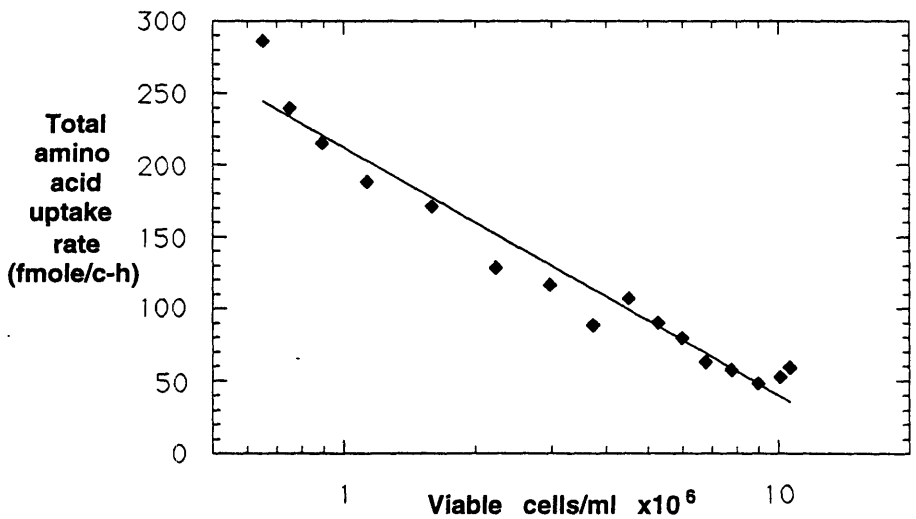
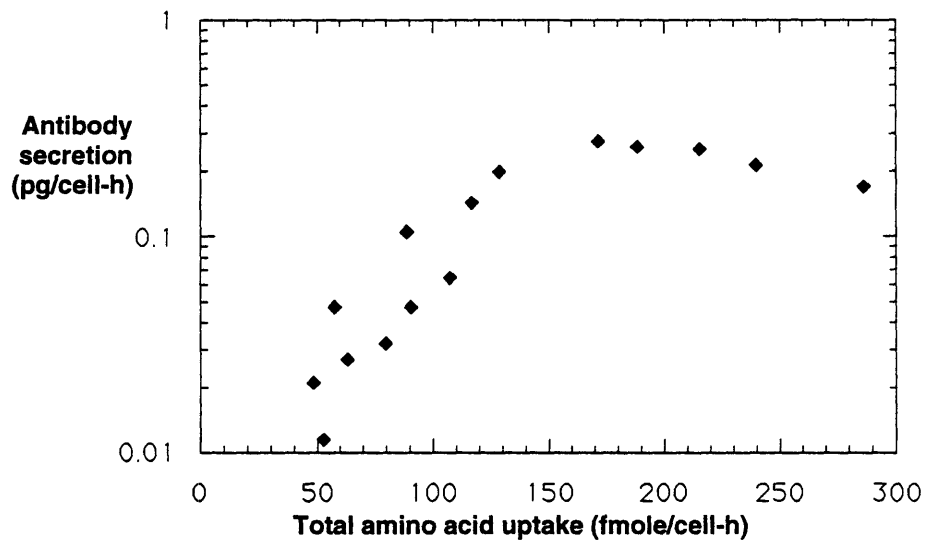


Figure 24: Total Amino Acid Uptake Rates vs. Cell Density



Plotted in the figure below are the antibody secretion rates vs. the amino acid uptake rates over the first sixteen days of bioreactor #1. From days 0 to 6, the cell density increases to about 1.6 million cells/ml, the total amino acid uptake declines but the antibody secretion does not decline, instead it increases slightly. From days 5 to 16 the cell density increases from 2 million to 10 million cells per ml, antibody production declines exponentially with declining amino acid transport. This data show an uncoupling of the variables from time. Antibody production may be high within a range of total amino acid uptake or alternately, the mechanism for the change in amino acid uptake may precede the change antibody production.

Figure 25: Antibody Production vs. Total Amino Acid Uptake



In the following table the rates of uptake of each amino acids are compared between day 5, when the viable cell density was 1.3 $x10^6$ cells/ml, and day 16, when the cell density was $1.1x10^7$ viable cells/ml. For example, the rates of uptake of the branched chain amino acids, isoleucine, leucine, lysine, threonine, valine, declined 5-9 fold.

The overall amino acid utilization at the high density condition declines to a level that is nearly accounted for by an estimate of DNA, RNA and protein synthesis (Tyo, 1991). To calculate the requirements for protein, RNA and DNA, the following assumptions were made by Tyo (1991):

1) protein content is 100 pg/cell,
2) RNA plus DNA content is 40 pg/cell, and
3) the doubling time is 22 hours.

At low density, the amino utilization exceeds these requirements by 3.5 fold, and this section shows that these "extra" amino acids are used for the production of lipids. I present a more detailed calculation of the amino acid requirements, and show the distribution among the cellular requirements.

Tyo (1991) found that the breakdown product of glutamine, pyrrolidone carboxylate (PCA), was taken up from the medium at a maximum rate of 62 fmole/cell-h and a minimum rate of 14 fmole/cell-h. He hypothesized that PCA was metabolized by the cells. Including PCA, the net uptake rate at low density was 184.6 fmole/cell-h and at high density 36.3 fmole/cell-h. When the utilization of PCA is included, the overall amino acid utilization at the high density condition declines to a level that is equal to the estimate for DNA, RNA and protein synthesis.

Table 14:
A Major Metabolic Shift Involves Amino Acid Utilization

Compound	High Productivity (fmole/c-h)	Low Productivity (fmole/c-h)	Ratio decrease [increase]
Utilized Amino Acids:			
GLN	40	10.4	4
ILE	17	1.9	9
LEU	19	2.6	7
LYS	14	2.8	5
THR	25	4.5	6
VAL	10	2.0	5
ARG	2.8	1.0	3
PHE	2.2	0.9	2
SER	6.5	2.3	3
TYR	18	0.6	30
CYS	5.4	1.4	4
HIS	2.6	-0.1	*
MET	1.5	0.2	7
TRP	1.2	0.9	1.3
Produced Amino Acids:			
ALA	-35	-8.9	[4]
ASP	-0.9	-0.1	[9]
ASN	-1.7	-0.3	[5]
PRO	-4.1	-0.2	[5]
GLU	-1.1	0.4	*
GLY	-3.8	-0.5	[7]
Total:	118.6	21.4	6
Rate Required: for protein and nucleotides	33.1	33.1	

Note: * rates not compared because of error due to measurement of concentration

With the CLC cells, large changes in amino acid uptake rates were observed. The uptake rates of all the consumed amino acids decline, and the production rates of the secreted amino acids also decline. For example, the uptake rate of glutamine and production rates of alanine declined 4 fold. The efficiency of conversion of glutamine to alanine was about 85% percent in each case, indicating a high rate for energy utilization. Glycine secretion, a by production of nucleotide synthesis decline 7 fold. The rates decreased for all the ketogenic amino acids (leucine, isoleucine, lysine, phenylalanine, tryptophan and tyrosine). Therefore, it is possible that the rates of lipid production change. Although the lipid production from amino acids may drop to zero at high cell density, the fraction of lipids in the cell cannot be zero. Cells can take up lipids from the media to compensate. However if the lipid profile changes, the ability of the cell to export antibody protein could be diminished. The final steps for antibody secretion are its transport through the golgi membranes and secretory vesicles.

2. Incorporation of Leucine into Lipid is a Function of Cell Density

Tyo (1991) showed that while the amount of amino acids incorporated at high cell density is sufficient for protein and nucleotide synthesis, much more amino acid is consumed at low cell density. The question remains, "what are the "excess" amino acids utilized for at low density?" Five of the amino acids that exhibit this behavior are known to be used for lipid synthesis. Therefore I tested the hypothesis that the high rate of uptake at low cell density is used for lipid synthesis.

To accomplish these goals, I incubated cells with the labeled ketogenic amino acid leucine, and measured the intracellular incorporation of leucine, specifically into:

a) the free amino acid pool (in an acid soluble fraction)
b) lipids only (by extraction of cells in an acid insoluble fraction), and
c) proteins (in the lipid free, acid insoluble fraction).

This was done under three cell culture conditions. The first condition was to vary the cell density in fresh medium. This experiment confirms that the decrease in amino acid uptake seen in the bioreactor also occurs under short incubation times in batch culture. The second condition was to transfer low density cells ($5x10^5$ cells/ml) into the high density, low productivity (HDLP) medium, incubate a few hours with the labeled leucine, and measure the mass balance. This experiment tests the hypothesis that the bioreactor medium immediately signals a change in metabolism of amino acid utilization. The third condition was to increase the period that cells are exposed to HDLP medium, since a lag time may be required to observed the down regulation. (The lag time for antibody production is between two and four days.) The labels chosen for these experiments were ^{3}H-leucine and ^{14}C-[U]-leucine.

^{3}H-Leucine Experiments

Preliminary experiments with ^{3}H-leucine are listed in the table below. Cells were labeled for four hours. Exponentially growing cells were resuspended in either fresh media or HDLP media {~10^7 cells/ml} at three different cell densities ($5x10^4$, $5x10^5$, and $5x10^6$ cells/ml). Parallel cells samples were washed with media without serum, precipitated with TCA for total protein and lipid or extracted with chloroform: methanol for total lipid. Rates were based on the specific activity of the ^{3}H-leucine added to the medium, then normalized to fresh media values at $5x10^4$ cells/ml. Directly switching cells into HDLP media did not have any effect on uptake rates. The uptake of leucine into lipid plus protein or total lipid alone was lowest at the highest density tested ($5x10^6$ cells/ml).

Table 15: Incorporation of ^{3}H-[3,4,5] -Leucine into Cellular Components

Media	Cell Density (cells/ml)	Total Lipid + Protein (relative rate)	Total Lipid (relative rate)
Fresh	50000	1.0	1.0
	500000	0.66	0.57
	5000000	0.12	0.04
HDLP	50000	0.91	1.07
	500000	0.37	0.23
	5000000	0.11	0.04

^{14}C-Leucine Experiments

The experimental methods with ^{14}-C-leucine were altered so that the samples could be processed in a series of steps, instead of in parallel. Cells were passaged in fresh or HDLP media four days before the experiment, and then were suspended in fresh or HDLP media. Cultures of 5 ml at various densities (ranging from $2x10^4$ to 3 x 10^6 cells/ml)were labeled for two hours. No protein or lipid secretion was detected in the media after the two hour incubation.

As shown in Figure 26, the incorporation of ^{14}C-leucine into lipid showed a trend of decreasing uptake (3 fold) with increasing cell density (about 100 fold). The incorporation of leucine into protein (Figure 27) did not show a trend. The incorporation

of leucine into the acid soluble, or free amino acid pool (Figure 28), shows a trend of decreasing uptake (40 fold) with increasing density in fresh media.

The different cell culture conditions can be compared with data from the total free leucine in the free amino acid pool. The use of high density, low productivity medium did not generally change counts into protein or lipid if the cells were preincubated or switched directly. For a given density in the experiment, the intracellular pools of leucine were not a function of media, fresh or HDLP. Further details are discussed in the next section.

Figure 26: ^{14}C-Leucine Uptake into Lipid

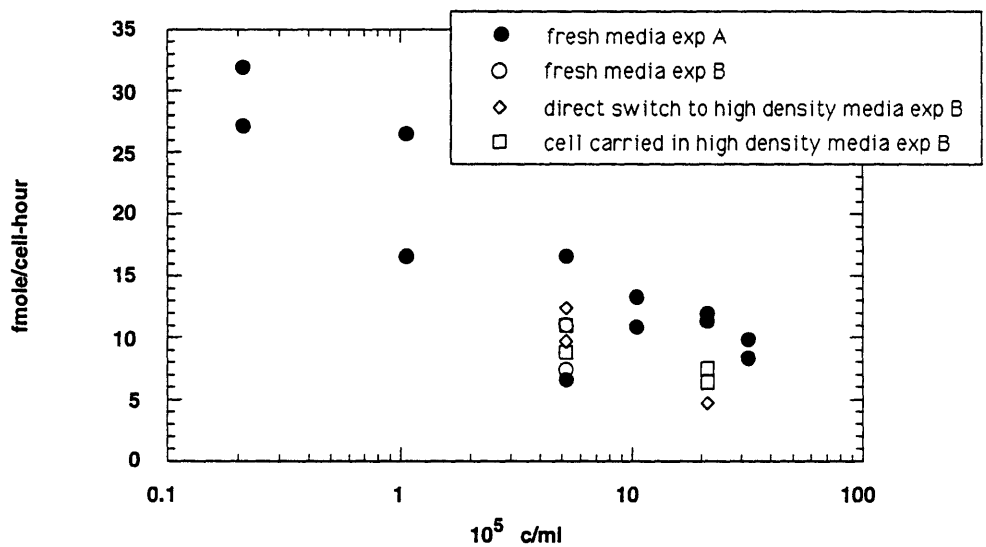


Figure 27: ^{14}C-Leucine Uptake into Protein

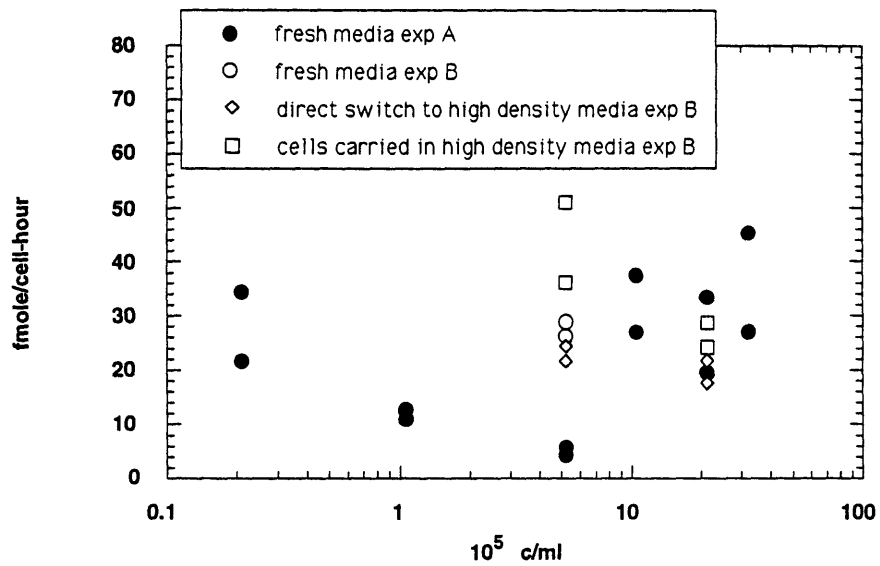
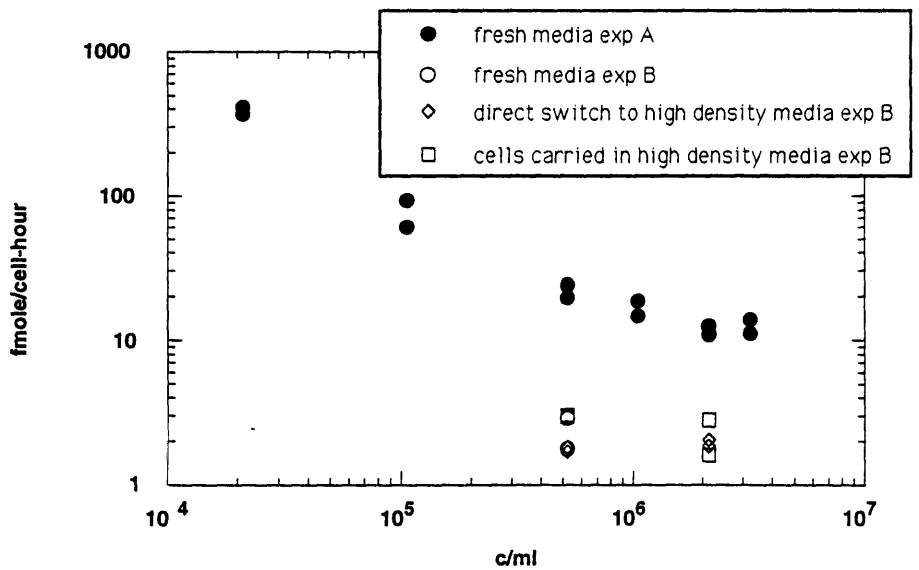


Figure 28: ^{14}C-Leucine Uptake into Free Amino Acids



3. The Free Amino Acid Pool is a Function of Cell Density

Investigation of the intracellular pools of amino acids showed that they decay exponentially with the log of increasing cell density. For the three secreted amino acids, the decay levels off at about 10^6 cells/ml.

Exponentially growing cells were resuspended at various densities in both fresh and in high density low productivity (HDLP) media. The intracellular amino acid concentrations were determined by HPLC analysis of TCA soluble extracts of cells labeled for two hours with ^{14}C-leucine (MIT Biopolymers Lab), and values were converted to fmole per cell.

The per cell values for leucine are plotted in Figure 29. The log of the pool content declines linearly with the log of the cell density. The pool of leucine declines from about 1000 to about 10 fmole per cell from $2x10^4$ cells/ml to $3x10^6$ cells/ml. The values for cells carried in fresh media and for four days in HDLP media are nearly identical. Extrapolation to 10^7 cells/ml gives a leucine content of 3 fmole/cell.

The amino acids that have a net uptake rate by the cells are plotted in Figure 30. For these amino acids, in fresh media, the free amino acid pool decreases with increasing cell density by a factor 40-120 over the cell density range of $2x10^4$ to $3x10^6$ cells/ml. The branched chain amino acids, isoleucine, leucine and valine, have the same slopes and intercepts, with the value at 10^7 cells/ml extrapolated to 3 fmole/cell. For cells carried in the HDLP media the intracellular pools are 0-30% higher than in fresh media. The extracellular concentrations of these consumed amino acids are supplied at 0.2 to 0.8 mM.

The amino acids that have a net secretion rate, alanine, proline and glycine, follow a different pattern vs. cell density, as shown in Figure 31. The log of the intracellular pool is linear with the log of cell density from $2x10^4$ cells/ml to about $4x10^5$ cells/ml. Then the intracellular values plateau to concentrations of 5.5, 2.5 and 20 fmole/cell for alanine, proline, and glycine, respectively. The intracellular and extracellular concentrations for glycine are similar for the fresh and for this sample of HDLP media. Comparing the HDLP cultures to those in fresh media at 10^6 cells/ml, the intracellular concentrations are 3.4 and 2.6 times higher for alanine and proline. The extracellular concentrations of alanine and proline are about 1.3 and 0.3 mM in the bioreactor (HDLP) media and are 0.3

and 0.01 mM in fresh media. The higher extracellular concentrations may make pumping these amino acids out of the cell less energetically favored.

Figure 29: Intracellular Concentration of Leucine vs. Cell Density

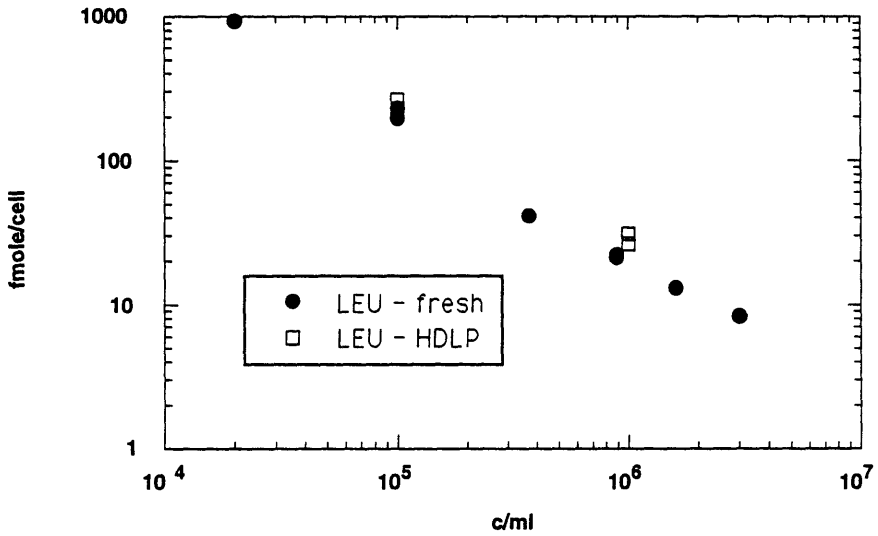


Figure 30: Intracellular Concentration of Imported Amino Acids vs. Cell Density

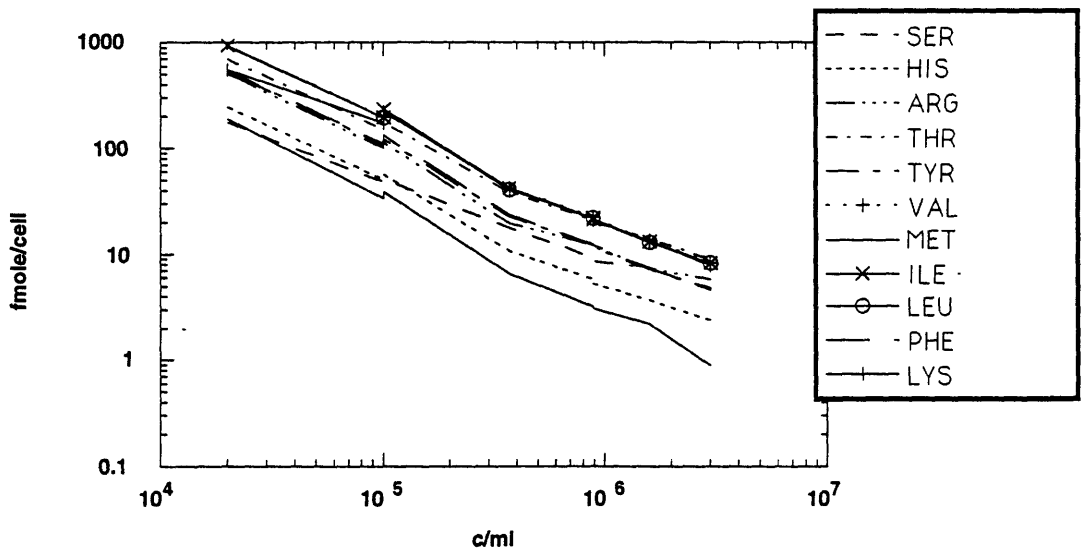
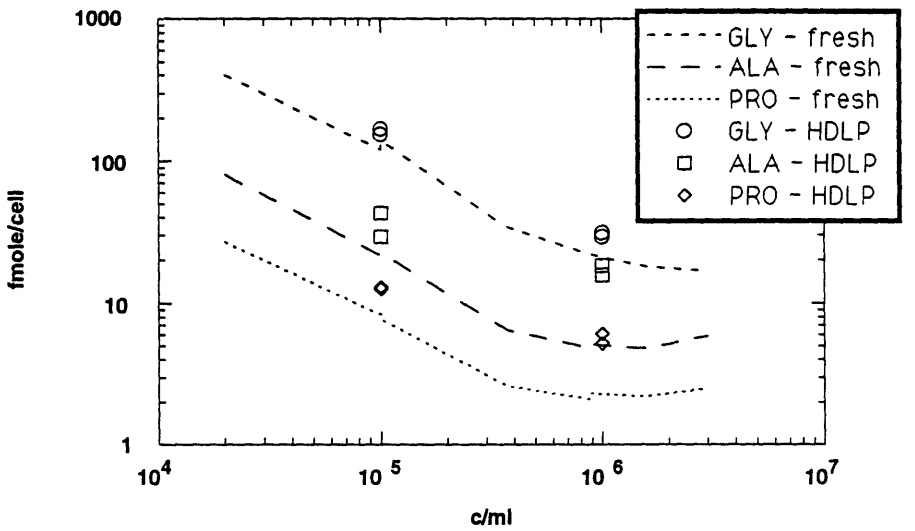


Figure 31: Intracellular Concentrations of Exported Amino Acids



The HDLP media did not suppress the incorporation of leucine into protein or lipid. The rate of uptake into protein and lipid was also determined in the same samples analyzed for intracellular amino acid pools. The specific activity of free leucine was calculated from the ratio in the acid soluble extract of dpm per cell to total leucine content per cell. Comparing fresh media to HDLP media, the pool of leucine and the counts per cell were the same. These results oppose those of Bhargava *et. al.* (1975). They found that media from higher density liver cells decreased the amino acid uptake of low density cells. Since the two hour labeling experiments with the HDLP media do not reproduce the decrease amino acid consumption, an unstable inhibitory factor or cell to cell contact mechanism is not supplied by the HDLP media.

4. Cellular Content of Protein and Lipid Declines with Cell Density

Protein Content as a Function of Cell Density

Protein content per cell decreased with increasing cell density. Protein content of cells removed from the reactor was determined by a modified Lowry assay. Plotted in Figure 32, the protein content per cell over 10^5 to $18x10^6$ cells/ml of samples removed from bioreactor #4 and of lower density controls. Curve fitting this data, give an equation of

Protein per cell = 56 * cell density ^(-0.25)..

From 10^5 to $2x10^6$ cells/ml, the protein content was clearly density dependent, declining from about 100 to 50 pg/cell. From the curve fit, from $2x10^6$ to 17 $x10^6$ cells/ml, the protein content declined 30% from about 45 to 30 pg/cell.

Lipid Content as a Function of Cell Density

Lipid content decreased with increasing cell density. Shown in Figure 33 is the lipid content per cell of the portions of the same samples analyzed for protein. Lipid content declined from 50 pg/cell at $1x10^5$ cells/ml to almost 15 pg/cell at $17x10^6$ cells/ml. Fitting the data to a exponential decline, results in the following equation:

Lipid per cell = 55.5 * exp (-0.077 *cell density)

From the curve fit, from $2x10^6$ to 17 $x10^6$ cells/ml, the lipid content declined 69% from about 48 to 15 pg/cell.

Lactate and ammonia apparently do not signal this change in lipid content. Cells were grown at lower density (< 2 million cells/ml) for four days in media supplemented with 25 mM lactate, 4 mM ammonia and either 2 to 6 mM pyruvate. Total lipid content was about 50 pg/cell, corresponding the values in the low density spinner controls of Figure 34.

Figure 32: Protein Content per Cell as a Function of Cell Density

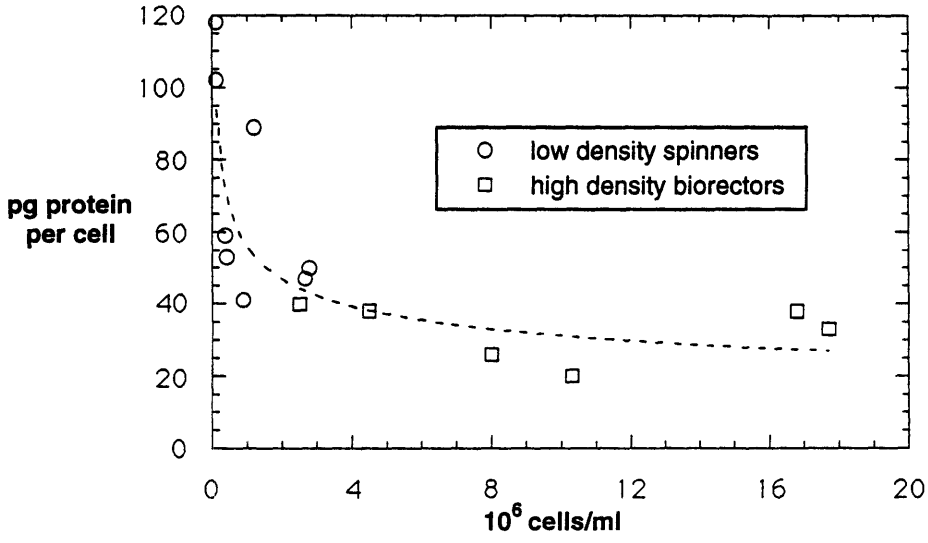
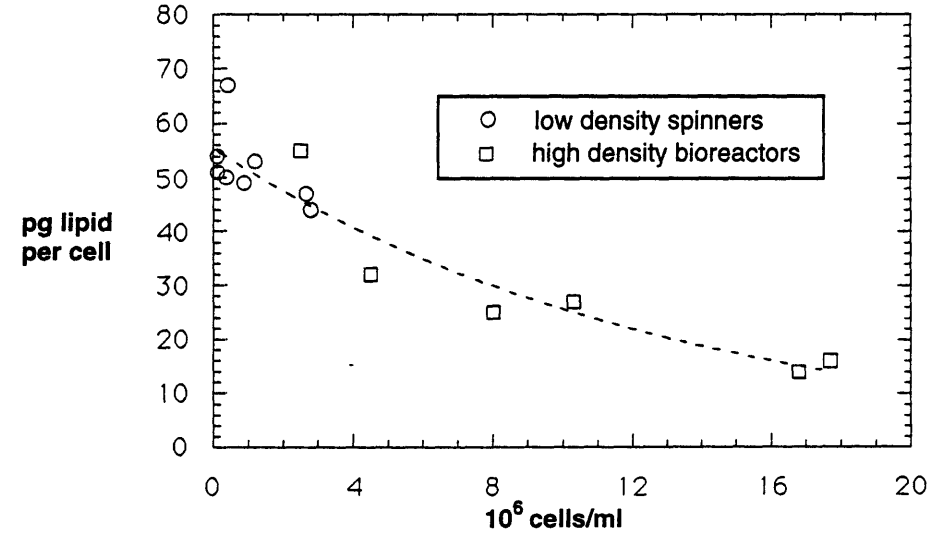
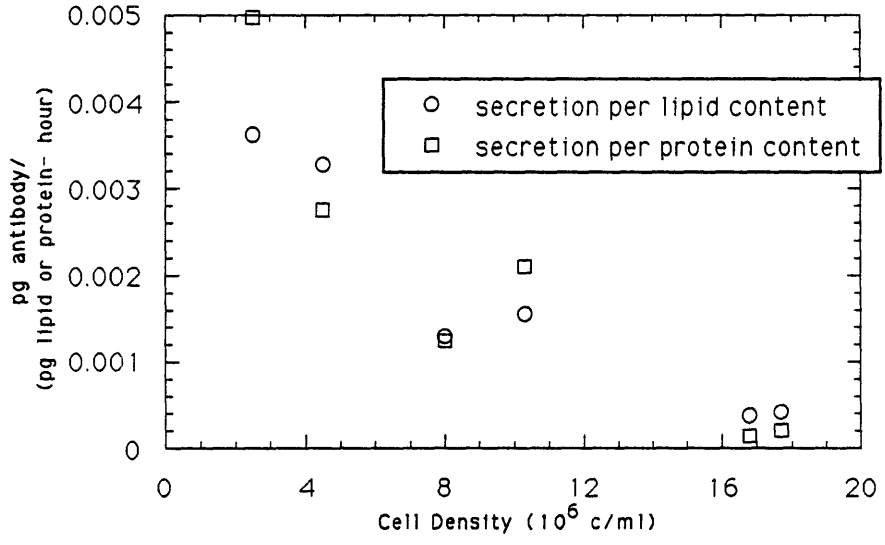


Figure 33: Lipid Content per Cell as a Function of Cell Density



Since antibody secretion declines with density (e.g. Figure 4) and protein and lipid per cell contents decline with cell density, it was suggested that the antibody secretion was a constant with respect to protein or lipid content. Dividing the secretion rate of antibody (pg/cell-hour) by either the protein content (pg/cell) or the lipid content (pg/cell) does not give a constant value for any cell density. Instead, as shown in Figure 34, as the cell density increases this relative secretion rate decreases. This trend results because the decrease in antibody secretion is much larger (down 80-90%) than the decrease in protein content (down by 30%) or lipid content (down by 68%).

Figure 34: Antibody Secretion Rate per Protein or Lipid Content



The decrease in total protein and lipid content is consistent with a decrease in cell volume. The combined lipid plus protein content declines two fold from 90 pg/cell at $2x10^6$ cells/ml and 45 pg/cell at $16x10^6$ cells/ml. If the cell volume decreases two fold, then the cell diameter would decrease by 20%. Comparing cell size at high density vs. low density by forward light scattering (Table 4), cell diameter decreased by 23 to 34%.

IV. Discussion

A. Cause of Decreased Antibody Production

One goal of this thesis was to find the signal for decreased antibody production as the cell density increased in the reactors. Experiments presented previously (Tyo, 1991; Buser 1992) and here show that the signal was not nutrient limitation or cell to cell contact. I conclude that diffusible inhibitors signaled the decrease in production, including lactate, ammonia, and an additional low molecular weight inhibitor(s).

1. Nutrient Limitation Did Not Occur

One goal of media optimization studies is to determine if one or more components are depleted and to increase the concentrations of these nutrients. In batch culture, cells cease growing when a nutrient concentration reaches zero. The final titers of antibody are a function of the limiting nutrient. The batch kinetics of the hybridoma OX-19 have been studied at various concentrations of serum and glutamine (Dalili and Ollis, 1989; Dalili *et. al.*, 1990). Duval *et. al.* (1991) improved final cell density from 1.5 to 3 $x10^6$ cells/ml by increasing all the amino acids. Final cell densities can be improved greatly in batch by fortifying media with glucose, amino acids, vitamins, etc. Fortification of medium for batch has been demonstrated by Jo *et. al.* (1990) and Franek and Dolníková (1991) as a way to improve final cell densities up to 10^7 cells per ml and subsequently increasing the final antibody concentrations.

In continuous culture, the medium is continuously supplied and cells are removed from the bioreactor. Robinson and Memmert (1991) found that for the transfected myeloma cell line (CRL 1581) antibody production declined with increasing doubling time. They measured amino acid concentrations and added back, one at a time, each depleted amino acid (histidine, isoleucine, methionine, phenylalanine, proline and tryptophan). Growth or production did not improve and they concluded that nutrient limitation by a single amino acid had not occurred.

In perfusion culture, the medium is continuously supplied and the cells are retained in the reactor. Designing media for high density requires studying nutrient consumption at high density. For example, Hosoi *et. al.*, (1991) examined the metabolism of a recombinant

Namalwa cell line, which secretes granulocyte-colony stimulating factor. To reach cell densities above 10^7 cells/ml, their bioreactor system used the single cone separator designed by Sato (1983) and a dialysis membrane to supply nutrients. They found that glucose, serine and cystine were nearly depleted. Increasing concentrations of glucose and amino acids by a factor of 2.5 improved the final titer of the protein product by 25%. Büntenmeyer *et. al.* (1991) measured amino acid concentrations and rates of a mouse hybridoma in a membrane type perfusion culture. They increased the concentrations of nine of the amino acids and improved antibody production by 60%.

In our perfusion culture system, cell densities of 1-2x10^7 cells/ml were achieved by continuously supplying nutrients and concentrating cells with the two layer, conical cell separator. The bioreactor experiments presented earlier were expected to be in a waste product limited condition, and not in a nutrient limited condition (Tyo, 1991), since none of the amino acids, glucose or pyruvate were depleted. Subsequent experiments were performed to eliminate the other nutrients as limiting.

Supplements to High Density, Low Productivity Medium (HDLP).

The goal of these experiments was to determine if the source of the limitation is a component from the defined medium or the undefined serum, and attempt to identify the limiting compound. The supplementation of either serum, vitamin mixture, or amino acid mixture to supernatants removed from the HDLP condition did not improve antibody production or growth rate of low density cells. However, in all cases the doubling time of healthy cells placed in bioreactor supernatants (16-18 hours) is faster than in fresh medium (25-60 hours).

Bioreactor Experiments to Test Several Nutrient Limitation Theories.

Nutrient limitation of a component of serum is not the external signal. Doubling the inlet serum concentration for one of three bioreactors during the down regulated phase did not increase in antibody production. Indeed, antibody production continued to decline after the serum was doubled. The serum is the only source of biotin and B_{12} in the medium of this study.

The sulfur-containing amino acids, cystine and methionine, and beta-mercaptoethanol are not limiting antibody production. In bioreactor #6, doubling the inlet concentrations of cystine and methionine and beta-mercaptoethanol during the down regulated phase did not increase the cell density or antibody production rate. Antibody production continued to decline after the media supplements were added.

2. Cell to Cell Contact Did Not Signal Decreased Antibody Production

High Density, Low Productivity Medium Decreases Antibody Production

The first approach to determine if high cell density was required for inhibition was to test the medium from the high density, low productivity (HDLP) state for ability to inhibit antibody production of low density, high producing cells. If cell to cell contact mediates inhibition, then the signal will be found in cell membranes and not in HDLP media.

In a series of experiments, cells were placed in HDLP medium at $5x10^4$ cells/ml and antibody production tested after four days. Since the cells decrease in growth in this medium after two days, cells were passaged on the second day. Medium from the high density, low productivity bioreactor was inhibitory to IgG production at low cell density. From this observation, the cell to cell contact hypothesis was eliminated as a primary down regulator.

Cell Membranes Do Not Inhibit Antibody Production

The second approach to determine if high cell density signals antibody production was to add membrane preparations to high producing cells in fresh media. Other researchers have tested the hypothesis that cell membranes act as a signal by this strategy. For example, membranes prepared from the contact dependent Swiss 3T3 cells inhibit cell growth and DNA synthesis (Whittenberger and Glaser; 1977; Whittenberger *et. al.*, 1978). Membranes prepared from the non contact dependent lymphoid cells inhibit growth and the inhibition is reversible as demonstrated by Stallcup *et. al.* (1984a, 1984b, 1986).

To determine if cell contact is the signal for antibody inhibition, cell to cell contact of live cells was mimicked by the addition of crude membrane preparations to cell cultures of low density, high producing cells. An advantage of this strategy was that the theory of cell to cell contact is separated from either nutrient limitation or diffusible inhibition theories. Membrane preparations from either the secreting or the non-secreting cell line gave the same results. Therefore the surface bound immunoglobulin is not a signal for the down regulation of antibody synthesis. Crude membrane fractions increased the doubling time, and within a range of doubling times observed in the reactor (20-40 hours)

they did not decrease antibody production. At the highest doses of crude membrane, antibody secretion was decreased 50%, but the doubling time was increased up to 60 hours. Therefore cell to cell contact does not signal a decrease in antibody production at doubling times observed in the bioreactor.

3. At Least Three Inhibitors Did Signal Decreased Antibody Production

In the section, the sources of diffusible inhibitors of antibody production will be discussed. Diffusible inhibitors present in the HDLP media decreased antibody production of low density cells. Since the bioreactor medium samples were stored at -20°C before testing, the possibility of a labile or volatile down regulator as the primary mechanism was eliminated. Experiments at low density, showed that the secreted amino acids were not inhibitors. Lactate and ammonia account for about 2/3 of the inhibition. The HDLP media was fractionated, and the remaining inhibitory compound(s) are less then 10,000 MW.

3.1 No Inhibition of Antibody Production by Secreted Amino Acids

One hypothesis for the inhibition of antibody production, was the inhibition of amino acid uptake by the secreted amino acids, alanine, proline and glycine. A summary of the amino acid transport systems that occur in lymphocytic cells is listed below. Systems A, ASC, L for neutral amino acids and system y^+ for basic amino acid are known to exist and a system for cationic amino acids is hypothesized. Information is taken from Christensen (1969), Christensen and Kilberg (1987), and Segel (1992).

In the bioreactors at high density, the uptake of branched chain amino acids declines 5-10 fold. Alanine, proline or glycine could potentially compete for uptake of other neutral amino acids in either the A or ACS sodium dependent transport systems or the L sodium independent system. In particular, the inhibition of the L system would decrease uptake of valine, isoleucine and leucine.

Pairwise tests of inhibition of amino acids have been in undertaken in the Ehlrich ascities cells (Johnstone and Scholefield, 1965). Alanine was found to inhibits the transport of many amino acids including arginine, glycine, histidine, methionine, β-phenylalanine, tryptophan and valine but not leucine. Glycine inhibited transport of alanine and α-aminoisobutyrate (among A system substrates) and also tryptophan and valine (among L system substrates). Proline inhibited histidine and tryptophan among L system substrates). These results suggest that the inhibition of transport by the secreted amino acids is possible.

Table 16: Amino Acid Transport Systems

Systems for dipolar amino acids		
	Na^+ dependent systems	
	A	all neutral amino acids
		glycine, proline, serine, methionine
		least reactive branched chain (valine, leucine, isoleucine)
	ASC	numerous dipolar amino acids
		3-4 carbon aliphatic and hydroxylaliphatic
		(valine, leucine, serine, threonine)
		plus proline and cysteine
	Na^+ independent system	
	L	most neutral amino acids
		prefers dipolar amino acids with bulky side chains
		valine, leucine and isoleucine
		and includes histidine, tryptophan
System for basic amino acids		
	Na^+ independent system	
	y^+	lysine, arginine
System for acidic amino acids		
	Na^+ independent system	
	x-	aspartate, glutamate

To test the hypothesis, high producing, low density cells were grown in fresh medium supplemented with additional 1.4 mM alanine, 0.5 mM proline, and/or 0.2 mM glycine in the maximum concentrations observed in the bioreactors. Addition of the secreted amino acids to fresh medium did not change antibody production or growth rate in low density culture.

The concept of amino acids as inhibitors of antibody production was also tested by Duval *et. al.* (1991). They also found that addition of 10 mM alanine had no effect on growth or antibody production of mouse hybridoma cells.

3.2 Ammonia and Lactate are Two of the Inhibitors

In experiments to test for inhibition of secreted antibody (Tables 10 and 13) or intracellular antibody content (Tables 11 and 12), I used lower density batch cultures and found that either ammonia or lactate will inhibit antibody production. Also the combination of lactate and ammonia are not more toxic than ammonia alone. Recreating the high density, low productivity media conditions with high lactate (25 mM), high ammonia (4 mM) and low redox (-265 mV) decreased production by ~55% in four days. HDLP medium inhibited production (intracellular content) by ~65-75% (Tables 8 and 12). In the bioreactor, intracellular antibody content was inhibited by 85% (e.g. Figure 21). After four day treatment, lactate and ammonia inhibit antibody production by 65% of the in- reactor conditions.

The inhibitor ammonium accumulates in cell culture as 1) glutamine is degraded to pyrrolidone carboxylic acid and ammonia and 2) glutamine is deaminated to glutamate by glutaminase and 3) glutamate is converted to α-ketoglutarate by glutamate dehydrogenase. Ammonium concentrations of about 4 mM have been found to be inhibitory to growth and antibody production of hybridoma cells (e.g. Glacken 1988a; Glacken *et. al.* 1988; Reuveny *et. al.* 1988; Ozturk *et. al.*, 1992). Glacken (1988b) theorized that ammonia alters ion gradients and increases the cells maintenance energy demands. Two of the proposed mechanisms for ammonia toxicity are 1) alkylination of the lysosomes by NH_3 and 2) acidification of the cytoplasm NH_4^+. Glacken (1988b) postulated that the first mechanism is more likely, and decreasing the pH of the culture over time decreases ammonia inhibition. Research by McQueen and Bailey (1991) has concluded that the first mechanism is a function of extracellular pH, and the second mechanism is not, and that the second mechanism is more important than the first for inhibition of hybridoma cells. Addition of ammonia to hybridoma cells alters some of the enzymatic steps, such as glutamate dehydrogenase and alanine aminotransferase (Miller *et. al.*, 1988). Martinelle and Hägström (1993) have suggested that NH_4^+ competes with K^+ for active transport into the cell and excess K^+ may decrease ammonia inhibition.

The metabolic inhibitor lactate is produced mainly via the glycolysis of glucose and partly by the glutaminolysis of glutamine. Lactate is an inhibitor of antibody production of some cell lines (Glacken 1988a; Glacken *et. al.*, 1988). The mechanism of inhibition by lactate may be to reduce the extracellular pH of the culture (Reuveny *et. al.*, 1986). Lactate may inhibit antibody secretion by chelating calcium and thereby inhibiting

protein excretion (Glacken *et. al.*, 1988). Lactate also alters the major redox pair, lactate/pyruvate and shifts the conditions towards a non-physiological state.

The combination of lactate and ammonia may not add to the inhibitory effects of one over the other. Doyle and Butler (1990) found the growth inhibition of ammonia is reduced at lower extracellular pH. Therefore lactate counteracts some of ammonia's effect by lowering the extracellular pH. The secretion of lactate may decrease some of the cytoplasmic acidification of ammonium by decreasing the intracellular acid content (Martinelle and Hägström, 1993).

Gaertner and Dhurjati (1993a, 199b) used fractional factorial analysis to study the separate effects of lactate and ammonia. They found that lactate and ammonia are separately inhibitory to antibody production. They also observed ammonia production to decrease with increasing glucose and lactate concentrations. Antibody production was modeled as function of medium, serum concentration and lactate and ammonia.

By definition, the redox potential is a function of the concentrations of lactate and pyruvate. Antibody production in the bioreactors declined by 85% as lactate concentrations reached 25 mM and redox values declined from -235 to -265 mV. To separate these variable I tested a variety of redox potentials (-225 to -265 mV) holding the total lactate plus pyruvate concentrations to 10 mM. No significant change in growth or antibody production was observed. In comparison in bioreactor #1, at a lactate concentration of almost 10 mM, redox was -250 mV and antibody production was 20% of initial value.

3.3 Additional Inhibitor of Less Than 10,000 MW

One third of the inhibition could not be accounted for by ammonia and lactate. To characterize the unknown inhibitors, media removed from the outlet of the bioreactors was separated by molecular weight in a gel filtration column. The gel media (Sephacryl S-300) separated proteins of $1x10^4$ to $1x10^6$ MW. Fresh media without serum was used as the elution buffer. Inhibition of growth and antibody production was tested in fractions supplemented with serum. No inhibition was observed at high molecular weight such as in samples coeluting with antibody protein or albumin. In the fractions containing lactate, ammonia, and pyruvate the doubling time was increased and antibody production

declined to 50%. Reconstruction experiments showed that adding the equivalent amounts of lactate, ammonia and pyruvate did not decrease production and growth to the same extent as the fractions. Fractions 19 and 20 decreased production to 25 and 28 percent, while reconstructions decreased production to 58 and 72 percent, respectively.

The results for inhibition of growth are consistent with those of Rønning *et. al.* (1991) described earlier. One procedural difference, instead of lactate or ammonia concentrations to mark the end of the column run, they used ^{3}H-thymidine. Although they did not measure antibody production of cells grown in fractions plus media, they showed that antibody production is improved with media that has been column filtered with Sephadex G-25 to remove small molecular weight compounds. My results and those of Rønning *et. al.* (1991) show that the inhibitory materials for growth eluted with the salt fractions. I found that antibody production was also inhibited in the column separated samples. I found that these inhibitors of growth and antibody production include lactate and ammonia. Additional diffusible factors are of a "low" molecular weight, that is less than the size of excluded compound, 10^{4} MW (or 10 kDa).

Other researches have concluded that multiple, diffusible inhibitors of growth and/or antibody production exist. Three inhibitors for growth have been reported by Holley *et. al.* (1978) for monkey epithelial cells, BSC-1. They conclude that lactate, ammonium and an unidentified unstable protein inhibitor exists. This protein inhibitor was degraded by shaking, gassing, heating (1 day at 37^{o}C), or freezing. Their research also suggested that an inhibitor is made by the cells at a constant rate. Three inhibitors of less than 10 kDa for growth of a hybridoma have been reported by (Büntemeyer *et. al.*, 1992). They also found that the lactate and ammonia concentrations observed in the reactor were not as inhibitory out of reactor in fresh media. They compared a perfusion culture of a 0.3 micron hollow fiber to separate cells and with one the 0.3 micron unit and an additional 10 kDa hollow fiber unit. The inhibition of antibody production was the same, and they therefore concluded that an inhibitor of less than 10 kDa exists in addition to lactate and ammonia. Consistent with the results here are the results of Takazawa *et. al.* (1988). These researchers used a 10,000 MW cutoff ultrafiltration unit for recycling cells and for concentration of antibody product and did not observe a decrease in antibody production. From their experiments and the experiments of this work, one concludes that the inhibitors of antibody production are less than 10,000 MW.

Some researchers find an inhibitor of size greater than 10,000 MW. Kidwell (1989) observed a decline in antibody production with two hybridoma cell lines entrapped in hollow fiber unit with molecular weight cutoffs of 4000 MW but not with 0.5 micron cutoff, and attributed the decline to the effects of the entrapped transforming growth factor β1 (TGF-β1). TGF-β1 has a molecular weight of 25 kDa. Rønning *et. al.* (1991) also found a second source of growth inhibition in the fraction coeluting with the albumin (67,000 MW). In contrast to these findings, the transfectoma used in these studies did not decrease production with either exogenously added TGF-β1 (data not shown) or high molecular weight fractions of the inhibitory media.

Merten *et. al.* (1985) found that conditioned media will inhibit antibody production while foreign antibody protein does not and suggested that the secreted antibody is a feedback inhibitor. Although the media was not analyzed for metabolic inhibitors, such as lactate and ammonia, this report is often referred to as an example of feedback inhibition of secreted antibody. The antibody protein itself is not a feedback inhibitor of the cell line in this work. We have found that the antibody concentration in the perfusion culture is less than that observed in a batch culture. Furthermore, the antibody concentration and the specific production rate show no correlation (Buser, 1992). The fractionation of HDLP media separated the antibody protein from the other metabolic inhibitors. I found inhibition in samples coeluting with lactate, ammonia and pyruvate and not in samples coeluting with the antibody protein. Other perfusion systems trap the antibody protein with the cells, increasing the concentrations 10 to 100 times over batch culture. For example, in the perfusion method of (Takazawa *et. al.*, 1988), antibody concentrations reached 2 mg/ml and no feedback inhibition by the antibody protein occurred. I conclude that the secreted antibody protein is not a feedback inhibitor of its own production.

3.4 Identity of Unknown Inhibitor

The identity of the low molecular weight inhibitors other than ammonia and lactate (and redox) is not yet determined. Other researchers have planned to search but results are not as yet published (e.g. Büntemeyer *et. al.*, 1992).

The effectiveness of the reducing agents in the medium may be a factor in the inhibition of antibody production. Glacken (1989) found that thiol compounds are growth limiting at low serum or low inoculation densities. He hypothesizes that the oxidation of thiols in

culture medium degrades the growth promotion of serum at low density. In this work with the transfectoma C46, β-mercaptoethanol was added at 50 μM since it is required for growth. Separate experiments were done to test for limitation of serum or reducing compounds. In bioreactor #6, doubling the concentration of β-mercaptoethanol along with cystine and methionine did not improve antibody production. In bioreactor #4, doubling the serum concentration did not improve antibody production. The effects of other compounds such as DTT, glutathione, and cysteine have not been tested with this cell line.

Other possible inhibitory mechanisms may have been an increase in osmolarity or change in the ion balance. The defined media DMEM is supplied at about 320 mOsm. In low density experiments, the addition of 25 mM NaCl did not decrease growth or antibody production. Effects of larger changes in osmolarity with our cell line have not been studied. With the mouse hybridoma 167.4G5.3, Ozturk and Palsson (1991) found the growth rate to decline with increasing osmolarity. Antibody production was increased above 400 mOsm/kg. With the mouse hybridoma 6H11, Øyaas *et. al.* (1994) also found that growth decreases as a function of osmolarity, depending on the compound added and growth will stop with the addition of 100 mM NaCl (510 mOsmol/kg), or 60 mM KCl (435 mOsmol/kg) or 175 mM sucrose (510 mOsmol/kg). With a cell line that produces better at slow growth rates (growth associated production) Oh *et. al.*, (1993) used a combination increasing the osmotic pressure to 400 mOsm/kg and the addition of sodium butyrate to slow growth and improve antibody production. The ion balance may be altered by changes in pH or increase in ammonia concentration.

An overall amino acid imbalance may inhibit antibody production. In the extreme case of starvation conditions, the transport of amino acids can signal changes in amino acid transport and other cellular functions. For example, the transport of amino acids by the A or N systems is a function of the extracellular concentrations of amino acids. A model for repression/derepression of the A system was proposed by Gazzola *et. al.* (1981). Starvation via low concentrations of amino acids "de-represses" the transport mechanisms. Carrier protein is synthesized and inhibitors are not synthesized. Conversely, high concentrations of amino acids "represses" the synthesis of the carrier protein and stimulates synthesis of inhibitors. The concentrations of amino acids can signal more than changes in amino acid transport. For example, amino acid starvation signals changes gene expression that alter the development of M. Xanthus. Amino acid

starvation signals the release of proteases, grouping together of single cells and cell to cell signaling factors leading to the formation of fruiting bodies (Kim and Kaiser, 1990).

Some researchers have had success in improving product production by altering the amino acids concentrations of nutrients to prevent low level starvation conditions. In general, increasing the concentrations of many of the amino acids has been most successful. In batch culture, Duval *et. al.* (1991) did not improve hybridoma cell density when increasing the concentrations of the four most consumed amino acids (leucine, isoleucine, valine and tryptophan) but did improve final cell densities when the concentrations of all the amino acids were increased. In continuous culture, Büntemeyer *et. al.* (1991) found no improvement by increasing isoleucine concentrations, but found a 20% increase in growth by increasing methionine and tryptophan. In perfusion culture, they increased concentrations of nine of the amino acids, and maintained cell density at $7x10^6$ cells/ml and improved antibody production from 0.2 to 0.33 pg/cell-hour.

With the CLC cells, the amino acid uptake rates decreased at high density. Many of the concentrations of amino acid in the bioreactor were close to the concentrations in the inlet media. Other concentrations of amino acid were decreased, but not depleted. The cells did not increase overall amino acid uptake as if they were in a starvation condition. Methionine and cystine were at low concentrations in the bioreactor, but doubling these concentrations did not improve growth or antibody production. The effects up-regulation or down regulation of all the amino acid concentrations on the transport systems of these cells is not known.

3.5 Growth Associated Behavior

Growth associated behavior is defined as a positive correlation between cell growth and product production; in other words, the faster the cells grow, the more product is produced. Cells may behave differently in batch than in continuous culture, so this definition is generally applied to continuous culture conditions.

Production of antibody protein by hybridoma cells is most often "non-growth associated" (reviewed by Al-Rubeai *et. al.*, 1992). Such cell types produce best at slow growth rates (e.g. Al-Rubeai and Emery, 1990; Frame and Hu, 1991; Miller *et. al.*, 1988b; Reuveny

et. al., 1986). For example, Miller *et. al.* (1988b) found that antibody production increased by a factor of two as doubling time increased from about 15 to 28 hours.

Antibody production via non-growth associated kinetics is theorized to be related to cell cycle kinetics. Cells with slow growth rates spend more of their cell cycle time in G_1. Production of immunoglobulin is highest in late G_1 and early S phase as shown by lymphoid cells (Buell and Fahey, 1969; Takahashi *et. al.*, 1969), myeloma cells (Byars and Kidson, 1970), and hybridoma line (Al-Rubeai and Emery, 1990). By flow cytometry analysis of a hybridoma cell line, Ramirez and Mutharasan (1990) found that the smaller cells secreted more antibody and postulated that more of the cells were in G_1. Therefore, the theory is that production is improved by slowing the growth rate by any means including suboptimal pH, high lactate concentrations, etc.

In contrast, the production kinetics of the Sp2/0 transfected cells line in this work may be called "growth associated" because cells in batch culture only produce antibody while they are exponentially growing and stop when the cell density plateaus. Also, in any condition where the growth rate is slower, the antibody production is decreased.

The growth dependence has been tested for one other transfected cell line. For the Sp2/0 transfected cell line (ATCC CRL 1581) Robinson and Memmert (1991) found a positive correlation of antibody production and growth ("growth associated"). Antibody production decreased linearly from 3.2 to 5.8 pg/cell-hour as the growth rate decreased from 1 to 0.4 day $^{-1}$. This growth rate range corresponds to a doubling time range of 16 to 43 hours. From doubling times of 16 to 24 hours antibody production declined from 3.2 to about 1.0 pg/cell. Cell cycle kinetics for either transfectoma are not known, but highest production is expected to be in G_2.

The difference in kinetic behavior of production of the transfected cell lines versus the myeloma or hyridoma cell lines may be due to effects of the promoters on mRNA expression. The transfected cell lines were created with two plasmids each containing their own promoter. The integration site of the plasmids is unknown, and the control of the genes may be under other promoters.

In the reactor I showed that the fraction of cells in G_1 increased and calculated an increase in doubling time from ~17 to ~20 hours. Such an increase in doubling time was observed in tests for inhibition of antibody production by the HDLP media, or lactate or

ammonia. Growth rate and inhibition of a diffusible inhibitor are variables that have not been uncoupled.

B. Further Characterization of Density Effects

Cells in culture alter many metabolic functions as the cell density changes. The uptake of almost every nutrient has been found to be a function of cell density in one cell type or another. Nutrients that have been shown to be taken up in a density dependent manner include glucose, phosphate, vitamins, oxygen, and amino acids. The density dependence of metabolism has been studied in detail for cells which are "contact dependent". Contact dependent cells grown only when attached to surfaces and stop growing when they cover the surface. Factors in their membranes or diffusible factors signal changes in metabolism. Suspension cells do not adhere to surfaces but may also stop growing do to signals secreted into the media.

1. Density Dependence of Nutrient Uptake

The following discussion presents examples of density dependence of uptake other compounds other than amino acids.

The growth and uptake of ^{32}P- phosphate of 3T3 cells is a function of cell density (Bladé *et. al.*, 1966). This inhibitor is a diffusible substance (Harel *et. al,* 1978). By fractionating conditioned media, they found the inhibitor to have a molecular weight of 40,000 and it is thermostable. This partially purified, diffusible inhibitor also inhibited DNA synthesis and protein content (Harel *et. al.*, 1983).

The effects of vitamins on culture sometimes depends on the cell density. Ability to take up a vitamin acting as either a nutrient or as an inhibitor can change with cell density. For example, Matsuya and Yamane (1986) found the uptake of vitamin B12, folate and folinic acid to be required for growth of mammary ascities tumor cells, Ehlrich cells, and L cells a very low densities (10^3 -10^4 cells/ml inoculum). Conversely, vitamin A (retinoic acid) inhibits growth of L cells from multilayers to monolayers and this inhibition is increased at higher cell densities (Dion *et. al.*, 1977).

The per cell uptake of oxygen may decline with increasing cell density. This phenomenon was noted with lymphocytes cells by Sand *et. al.* (1977). They resuspended exponentially growing cells at varying densities (10^3 to 10^8 cells/ml) and observed a 100 fold decrease in the specific uptake rate of oxygen. During the one hour experiments, they concluded that nutrient limitation had not occurred and inhibition by lactate or cell

handling was not the cause. They hypothesized that "humoral factors" regulated the respiration rate. The phenomena was tested with hybridoma cells by Wohlpart *et. al.* (1990). They resuspended exponentially growing cells at varying densities (10^5 to 10^7 cells/ml) and observed a four fold decrease in the specific uptake rate of oxygen. Fresh media or conditioned medium from 8 x 10^5 cells/ml did not change the uptake rate, and they concluded that the cell density itself was a signal for the decrease in oxygen uptake.

Resistance to a metabolic inhibitor can be density dependent. The uptake declines with increasing cell density. Protein synthesis and amino acid uptake decline with increasing cell density in ascities tumor cells (Bladé *et. al.* 1968b). When protein synthesis was inhibited with puromycin, amino acid uptake was still a function of cell density (Bladé *et. al.* 1968a). The inhibition of protein synthesis by the antibiotic puromycin for pig kidney cells is also density dependent (Cass, 1972).

2. Density Dependence of Amino Acid Pools

Generally, amino acid transport and incorporation are interrelated with the amino acid pool. In experimental circumstances, amino acid transport and amino acid incorporation into protein can be uncoupled by addition of separate inhibitors (Riggs and Walker, 1963).

I found the intracellular amino acid pool is a function of cell density. Exponentially growing cells were resuspended at densities ranging from $2x10^4$ to $3x10^6$ cells/ml. The change in amino acid pools occurred within the two hours of the experiment. For the consumed amino acids, the intracellular concentration decreased with increasing density in a log-log relationship. For the secreted amino acids, glycine, alanine and proline, the relationship was log-log to about $4x10^5$ cells/ml and then leveled off.

Intracellular amino acid concentrations of hybridoma cells have been estimated by one other research group. The intracellular amino acid pools were measured in continuous culture under three glutamine feed concentrations (Schmid and Keller, 1992). At a 2.7 mM glutamine steady state, the viable cell density was about 1.7 $x10^6$ cells/ml and the doubling time was about 23 hours. Their extracellular steady state values for alanine glycine and proline were 1000, 230 and 190 mM. The corresponding intracellular values for alanine, glycine, proline were 23, 12, and 6 fmole/cell. These values are consistent

with the values I observed in the two hour experiments at the same cell density. I found 5, 18, and 2 fmole/cell in fresh media and 17, 6 and 30 fmole/cell in HDLP media. The extracellular amino acid concentrations of most of the other amino acids were 5-10 fold lower than in my fresh or HDLP media, and their intracellular values were about 5-10 fold lower than my values. In general, the higher the extracellular concentrations the higher the intracellular pool concentration. The amino acid pools were not compared at different cell densities by Schmid and Keller (1992).

The dependence of uptake of amino acid into the intracellular amino acid pool on cell density has been demonstrated in other cell lines. For example, uptake of histidine and methionine into the intracellular amino acid pools decreased 10 fold and 2 fold with increasing cell density (from 0.25 to ~$2x10^6$ cells/ml) in rat liver parenchymal cells (Bhargava *et. al.*, 1975). I observed at least a two fold decline in leucine uptake over this cell density range (Figure 28).

3. Density Dependence of Amino Acid Uptake

At the lower density when antibody production was highest, the amino acid uptake was greater than expected for nucleotide and protein synthesis. Other researchers have noted this phenomenon with exponentially growing cells (reviewed by Johnstone and Scholefield, 1965). For example Bhargava *et. al.* (1976) observed that dividing tumor cells take up a large excess of amino acids as compared to resting liver cells.

Over the course of the bioreactor runs with the CLC cells, an overall decrease in amino acid uptake with increasing cell density occurred (Tyo, 1991). Research with other cells lines has shown that some amino acid transport systems are affected by cell density or growth rate. As reviewed by Guidotti and Gazzola (1992) these systems include the A, ASC, and L systems for neutral amino acids and the X^-C and X^-AG systems for cationic amino acids. In general, the maximum rate of transport (V_{max}) is altered but not the K_m value.

A number of researchers have tested the effects of cell density and growth rate on amino acid uptake for attachment dependent cells. The following presents some examples of results. Bhargava and Bhargava (1962) increased the cell density of suspension hepatic cells from 0.5 to 2.7 $x10^6$ cell/ml and observed a 4 fold decline in overall uptake rates of

a mixture of amino acids. Foster and Pardee (1969) observed lower uptake rates for non-growing (confluent) vs. growing 3T3 cells for both the A and L systems with nonmetabolizable amino acid analogs. In contrast, Robinson (1976) concluded with the Shionogi 115 carcinoma cells that the sodium dependent A system for amino acid transport is cell density-dependent, but the sodium independent L system is not. Otsuka and Moskowitz (1974) found that metabolizable leucine uptake decreases with 3T3 cell density. Borghetti *et. al.* (1980) showed that the A and ASC systems are density dependent with contact dependent 3T3, SV3T3 and SV3T3 reverent cells. The noncontact dependent SV40 3T3 cells showed density dependence of the L system (Petronini *et. al.,* 1982) and the ASC system (Piedimonte *et. al.,* 1982). In one report, one transport system was increased with density. Dall'Asta *et. al.* (1983) tested glutamine uptake by diploid human fibroblasts and found uptake via the A, ASC or x^-_C (for glutamate and cystine) systems decreased with density but increased with density via the X^-_{AG} (for glutamate and aspartic acid) systems.

Griffiths (1972) compared contact inhibited cells (MRC-5) and non contact inhibited (L cells). For MRC-5 but not for L cells, he observed as cells become confluent, doubling in density, their ability to transport amino acids (arginine, leucine, valine, methionine, and aminobutyric acid) decreases three fold. He found that confluent cells require higher concentrations of amino acids for maximal protein synthesis.

The suppression of amino acid utilization also occurs in lymphocyte derived cells, which do not attach to surfaces. Piedimonte *et. al.* (1989) varied growth and density of Namalwa cell line, derived from Burkitt's lymphoma , and found a correlation of cell density. Uptake into the A or ASC system decreased with cell density below $4x10^5$ cells/ml. Recently, Hiller *et. al.* (1991) controlled growth of a murine hybridoma cell line by varying the dilution rate in chemostat culture. The steady state cell densities ranged from .2 to 1.8 x 10^6 cells/ml. They observed a decrease in amino acid uptake with increasing doubling time and decreasing cell density. The dependence on growth rate is partly because rapidly growing cells take up more amino acids into protein than resting cells.

The signal for the decrease in amino acid transport is not known for any cell line. Once again, a phenomenon is somehow causally related to cell density, and may be mediated by cell to cell contact or a diffusible inhibitor.

Bhargava *et. al.* (1975) proposed that the cause for the decrease in amino acid uptake with increasing cell density was not mediated by cell to cell contact but was caused by a factor in the conditioned medium. They showed that the rate of uptake of low density cells in high density medium is equivalent to the rate of uptake of high density cells in fresh medium. This inhibitor had properties of a protein since its activity was degraded by proteases and heat treatment. This research group partially purified the "protein inhibitor of transport" from the supernatant of perfusion liver by ultrafiltration and gel chromatography (Bhargava *et. al.*, 1979). They inhibited the uptake rates of amino acids of liver cell types that display a density dependence. The active fractions also inhibited growth.

The possibility of an unstable inhibitor for the density dependence of amino acid uptake and protein synthesis in ascities tumor cells was considered by Bladé and Harel (1968b). They conditioned media at high density (10^7 cells/ml) for 15 minutes, spun down the cells, and resuspended different cells in this medium at 10^6 cells/ml. No inhibition was observed.

I found that the HDLP media frozen down during the bioreactor experiments did not inhibit amino acid uptake. I compared the incorporation of leucine in either fresh media or the HDLP media from the bioreactors and I did not observed an inhibition of incorporation of leucine. Also, most of the amino acid pools were not altered by the HDLP media. The exceptions were the pools of the secreted amino acids, alanine, proline and glycine, which were higher by 3 times in the HDLP media. The diffusible inhibitors of antibody production in the HDLP media did not alter the leucine incorporation. The inhibitor proposed by Bhargava's group may have been degraded by the handling the bioreactor media. Alternately, the mechanism of the inhibition of amino acid transport of the transfectoma is cell to cell contact.

4. Density Dependence of Cell Size

In this section I will present the evidence that supports the conclusion that the cell size decreases with increasing cell density. Previously, Tyo (1991) showed that the cells in the reactor change cell size distribution at different flow rates. Using flow cytometry, I comparing cells at high ($2x10^7$ cells/ml) versus low ($1x10^6$ cells/ml) density and found

that cell size decreased by about 25%. The decline in total protein and lipid content also supports a 25% decrease in cell size.

Tyo (1991) measured cell size distributions at various flow rates with a coulter counter The cell size in the reactor at three volumes per day was smaller than at 9 volumes per day. At relatively fast flow rate of 9 volumes per day, (conditions where excess nutrients are supplied and waste metabolites are reduced), the distributions of cells in the reactor were gaussian with a maximum value at 12.1 microns, respectively. At the three volumes per day, (the waste metabolite limiting condition studied here), the distribution was not gaussian. Instead the distribution ranged from 7 to 16 microns and was maximum at 7.8 microns.

I compared the cell size at high vs. low density at three volumes per day by flow cytometry. The mean forward light scattering values give a relative estimation of size (diameter). From 10^6 to 10^7 cells/ml, the cell size (diameter) decreases on average by 26% (cell volume decreases by 59%).

The decline with protein and lipid content is consistent with decline in cell diameter. From low to high cell density, the protein content per cell declines from 47 to 27 pg/cell. Similarly, lipid content declines from 47 to 14 pg/cell. Total lipid plus total protein content declines from 94 to 41 pg/cell (or 56%). If one assumes that the 56% decrease in total protein plus lipid is caused by a decrease in cell volume, then this implies the cell diameter decreases by about 24%.

Using Tyo's estimate of 8 microns at high cell density, then at low cell density the mean cell size is 10 microns.

It is possible that the change in cell cycle accounts for part or all or part of the change in cell size. Cell volume increases as cells pass through the cell cycle phases. Newly divided cells begin in the first gap (G1) phase, increase DNA in the synthesis (S) phase, then reach double the DNA content in the second gap (G_2) phase and divide in mitosis (M) phase. By staining cells for DNA content and analysis by flow cytometry, percentages of cells either G_1, S or G_2+M phases were measured. Since the time spent in S plus G2 + M phase is approximately constant, cell volume and cell cycle phase have been experimentally correlated.

Cell size by forward light scattering with flow cytometry and percentage of cells in S plus G2+M phases have been linearly correlated with the TB/C3 mouse hybridoma (Al-Rubeai *et. al.*, 1991). I observed a decrease in the percentage of cells in S plus G2 + M phases from 70 to 56% (Table 4). From their data, a 22% decrease in cell size occurs. In other words, the diameter would decrease from 10 to 7.8 microns.

Cell volume by coulter counter measurement of the HB-32 mouse hybridoma cells has been linearly correlated to the percentage of cells in S phase (Ramírez and Mutharasan, 1990). I observed that between high and low cell density, the percent of cells in S phase decreased from 58% to 41% (Table 4). From the data of Ramírez and Mutharasan (1990), at 58% S phase, the volume of cells was 1200 μ^3 (diameter of 13.2 μ) and at 41% S phase volume was 850 μ^3 (diameter of 12 μ). The cell volume decreases by 29% and the diameter decreases by 9% between these two cases.

C. Mass Balance of Amino Acids

In general for this and other cell lines, cells in culture at low density or fast growth take up more amino acids than high density or slow growth. Cells take up amino acids from media and incorporate them into the free amino acid pool, cellular components of protein, DNA, RNA, and lipid and sometimes secrete them as protein. For the cells studied in this thesis, which of these uses for amino acids changed between low cell density (10^6 cells/ml) and high cell density (10^7 cells/ml)?

- **DNA.** The incorporation in DNA is constant per cell and therefore depends solely on growth rate.
- **Secretion.** At low density .2 pg/c-h of antibody is secreted. At high density .02 is excreted.
- **Free Amino Acid Pool.** The free amino acid pool is a function of cell density. At $1x10^6$ cells/ml the total free amino acid pool is 351 fmole/cell. Extrapolating to $1x10^7$ cells/ml gives 56 fmole/cell.
- **Protein.** Cellular content of protein was found to decrease from 45 to 30 pg/cell.
- **RNA.** Since Buser (1992) found the number of ribosomes associated with the house keeping gene α-tubulin decreased 2.6 fold, the change in RNA content can be estimated to follow the same trend.
- **Lipid.** Cellular content of lipid was found to decrease from 45 to 15 pg/cell.

Requirements of Amino Acids

Now with quantitative data for the cells' behavior, the mass balance on amino acids can be closed. This calculation is summarized in the following table

This mass balance compares the two conditions of low density (1-2 $x10^6$ cells/ml), high productivity to high density (1-2$x10^7$ cells/ml), low productivity. This calculation assumes a change in doubling time between the two states. The growth rate of 17 hours for low density and 21 hours for high density was estimated by flow cytometry (Table 4). The change in amino acid requirement per cell per hour is based on the growth rate, the cellular content and the relative rates of incorporation.

Cellular content values are taken from experimental estimates. Experimental data for the cellular content of protein (Figure 32) and lipid (Figure 33) are used. Between these two densities protein content declines by 1/3 and lipid content declines by 2/3. Tyo (1991) showed the sum of the RNA and DNA content at low density is about 40 pg/cell. Glazer and Webber (1971) showed that the RNA content is triple the DNA content for rat cells, and this ratio is applied here. Buser (1992) showed RNA content declines by a factor of 2.6 between low and high density based on polysome data on a house keeping gene. The content of the free amino acid pool was determined from data used to plot Figures 29-31. The value at low density, 351 fmole/cell, was the sum of all free amino acids observed at $1x10^6$ cells/ml. The value at high density, 56 fmole/cell, was obtained by extrapolating the curves to $1x10^7$ cells/ml.

The information about the relative rates of incorporation is based on the rate of leucine incorporation at different densities. In the labeling experiments, the cellular content of protein or lipid is not expected to change significantly over the two hour incubation. From 10^6 cells/ml to 10^7 cells/ml, I did not observe a change in the rate of uptake of leucine into protein or free amino acids, but I did observe a decrease into lipid synthesis. I estimate a 3.6 fold decrease into lipid synthesis between these two densities by extrapolating the data in Figure 26.

The calculations for amino acid requirements assume exponential growth at different doubling times (td). Let X be the number of cells. Since the cell density increases exponentially:

$$X = X_o 2^{t/td}$$

Now let P be the per cell protein content (e.g. pg/cell). Then:

$$P = P_0 2^{t/td}$$

To calculate the change in content per cell hour, $P_f - P_o$,

$$P_f - P_o = P_o 2^{1/td} - P_o$$

To convert a content in pg/cell to a rate of fmole/cell-hour, a molecular weight conversion factor is needed. Average molecular weights of 110 and 325 are used for amino acids and nucleotides. The amino acid requirements for DNA, RNA, and the free amino acid pool are calculated by the same method.

The requirements of amino acids for the secretion of antibody (Mab) are directly calculated from the secretion rates (pg per cell-hour).

The calculation for amino acid requirements for lipid production are not so straightforward because a molecular weight conversion factor (e.g. fmole amino acids per pg lipid) is not available. However, the following is known: Let L be the amount of lipid (pg/cell). Taking into account the changes in doubling time and lipid content gives:

$$(L_f - L_o)_{low} / (L_f - L_o)_{high} = 3.7$$

In other words the change in lipid content over one hour is 3.7 times larger at low density than at high density. Second, based on the labeling experiments, the rate of incorporation of leucine into lipid is a function of density. The change in rate of leucine uptake into lipid was 3.6 between the two densities. This factor of 3.6 was observed in conditions where no change in the lipid content is expected. This may or may not indicate a change in the stoichiometry. Multiplying the change in rate (3.6) and the changes doubling time and content (3.7) yields a relative change in amino acid incorporation of a factor of 13.3, though its absolute value is determined by the following calculation.

To solve the mass balance for the factor x, the calculated rates of the amino acid required are set equal to the observed net rate amino acid taken up. For the low density case,

$$124.6 = 38.5 + 13.3x$$

and x = 6.5. Similarly, for high density one obtains approximately the same number, x=6.8. Therefore the balance closes if the amino acid requirement for lipid at low density is 86.5 fmole/cell-hour and at high density is 6.8 fmole/cell-hour.

Table 17: Mass Balance on Amino Acid Uptake

	Low Density High Productivity	High Density Low Productivity
secretion of Mab (pg/cell-h)	0.2	0.02
density (cells/ml)	1-2 x 10^6	1-2x10^7
doubling time (hours)	17	21
Cellular content:		
protein (pg/cell)	45	30
lipid (pg/cell)	45	15
DNA (pg/cell)	10	10
RNA (pg/cell)	30	21.5
free amino acids (fmole/cell)	351	56
information on relative rates:		
ratio leucine into protein	1	1
ratio leucine into lipid	3.6	1
ratio leucine into free amino acid	1	1
calculated required rates of amino acids (fmole/c-h) :		
protein	17.0	9.2
lipid	13.3*x	x
DNA	1.3	1.0
RNA	3.8	2.2
free amino acids	14.6	1.9
secreted Mab	1.8	0.2
total	**38.5+13.3x**	**14.5+x**
Observed net amino acid uptake rates (fmole/cell-h)	118.6	21.4
Solving for x (fmole/cell-h):	6.5	6.9
then lipid requirement is(fmole/cell-h):	79.8	6.9

To confirm that the values for the requirements of amino acids for lipid are realistic, one can examine the uptake rates of the ketogenic amino acids (isoleucine, leucine, lysine, phenylalanine, tryptophan and tyrosine). The sum of the uptake of these amino acids are 77.4 and 9.7 fmole/cell-h at low and high density, respectively. Based on the occurrence of amino acids in various proteins (Creighton, 1984), 27% the ketogenic amino acids are used for protein synthesis. Given that 18.8 fmole/cell-h of amino acids are used at low density for both cellular protein and secreted antibody, the contribution of the ketogenic amino acids is predicted to be 5.1 fmole/cell-h. Similarly, at high density 2.5 of the 9.4 fmole/cell-h are contributed by ketogenic amino acids. Subtracting, 72.3 and 7.2 fmole/cell-h of the ketogenic amino acids remain for lipid synthesis at low and high density, respectively. These values are in good agreement with the modeled values of the requirements for lipids of 79.8 and 6.9 fmole/cell-h.

This investigation into the amino acid utilization began with the observation that as cells reached the high density, low productivity state, the net uptake into amino acids dropped to 1/5 of the low density rates. From the mass balance, the high density uptake rates are the following proportions of the low density rates:

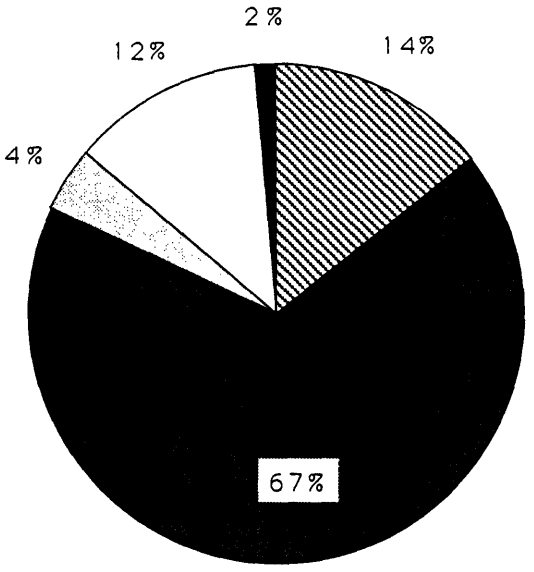
1/2 for cellular protein,
1/12 for lipid,
3/4 for DNA,
3/5 for RNA,
1/9 for free amino acids, and
1/10 for secreted antibody.

The smallest changes in requirements were for DNA, RNA and then protein. The largest changes are for the requirements for free amino acids and lipid.

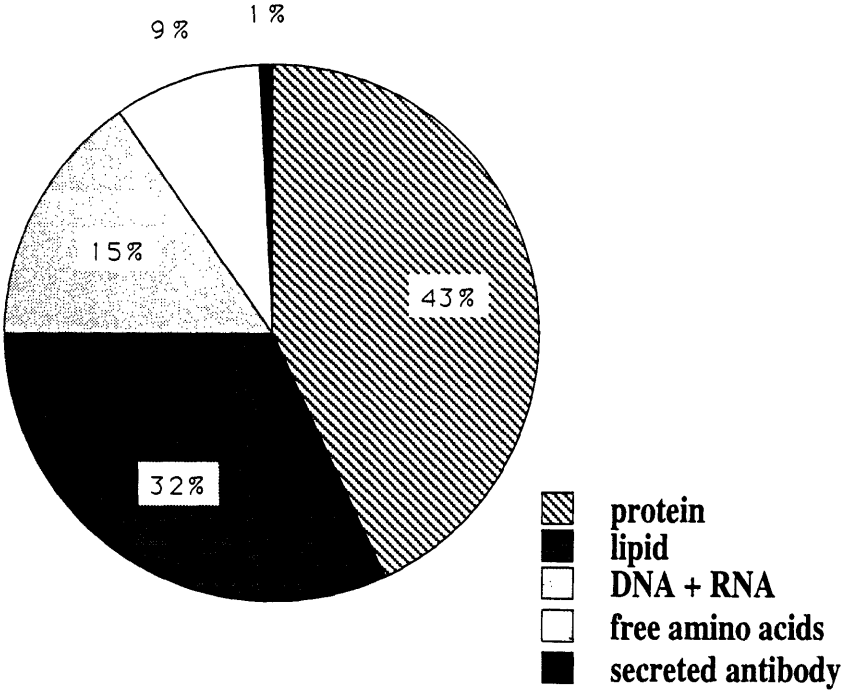
At high cell density, not only are overall uptake rates of amino acids for each component decreased, but the distribution of amino acids into cellular needs changes. In the following figure, the rates required are recalculated as a percentage of the total for the two cases.

Figure 35: Distribution of Amino Acids for Cellular Needs

Net low density rate = 118.6 fmole/c-h



Net high density rate = 21.3 fmole/c-h



The mass balance confirms that at low density the observed uptake rates are in excess of the requirements for protein and nucleotides. This mass balance shows the excess (2/3 of the total) is consumed by lipid. At high density, the distribution changes. At high density, more than half of the uptake of amino acids is for protein and nucleotides. The lipid requirement only consumes 1/3 of the total. The percentage for the free amino acid pool is about constant. The percent of total amino acids used for antibody is also constant.

The amino acid uptake of the transfectoma studied here declined with increasing cell density in perfusion culture and in short term batch culture. Resuspending exponentially growing cells at different densities decreased amino acid transport and thereby decreasing free amino acid pools. The smaller intracellular pools would decrease the availability to synthesize new cellular components of proteins, nucleotides and lipids. This would cause cells to grow slower and to be smaller. The decrease in lipid synthesis would decrease membrane production and thus decrease growth and protein secretion.

The high density, low productivity medium from the bioreactors inhibited antibody production of low density cells. The lactate and ammonia in the HDLP media caused 2/3 of this inhibition. However, the HDLP media, and therefore lactate and ammonia, did not inhibit the uptake of leucine in the labeling experiments. Therefore the inhibitor of amino acid transport is either an unstable, diffusible inhibitor, or is cell to cell contact.

The common link between the antibody secretion and the metabolic shift is the growth rate. The antibody secretion kinetics of this cell line appears to be growth associated. Any mechanism that slows growth also decreases antibody production. The transient increase in cell density is linked to the decrease in growth and the decrease in amino acid uptake. Other cells lines at high cell density decrease growth but secrete the same or more antibody. From the mass balance model, the transfectoma studied in this thesis uses a constant percentage of the available amino acids for antibody secretion.

V. Conclusions

The overall goal of this thesis was to characterize the phenomenon associated with the decrease in antibody secretion as the cell density increases in perfusion bioreactors. The table below summarizes the results of this characterization.

Table 18: Summary of Sp2/0 Transfectoma Behavior

Characteristic	Low Density ($1\text{-}2\times10^6$ c/ml)	High Density ($1\text{-}2\times10^7$ c/ml)
Antibody Secretion (pg/c-h)	0.2	0.02
Lactate (mM)	5	25
Ammonia (mM)	2	4
Redox (mVolts)	-245	-265
Cell Cycle (% G1)	30	46
Doubling Time (hours)	17	21
Cell Diameter (microns)	10	8
Lipid Content (pg/cell)	45	15
Protein Content (pg/cell)	45	30
Free Amino Acid Pool (fmole/cell)	351	56
Total Amino Acid Uptake (fmole/c-h)	118	21
Distribution of Amino Acid Uptake		
Secreted Antibody (%)	2	1
Nucleotides (%)	4	15
Amino Acid Pool (%)	12	9
Protein (%)	43	14
Lipid (%)	67	32

The first goal of this thesis was to find the signals that shut down antibody production. The conclusions the first goal of this thesis were reached with the following experimental information:

The nutrient limitation theory has been eliminated by a number of experimental techniques. Although a flow rate of 1 volume per day is sufficient for the cell densities of interest, 3 volumes per day were used. Tyo (1991) measured concentrations of nutrients by HPLC in the reactor and did not find any nutrients that were depleted. Due to the metabolic shift, at high cell density the cells were consuming some nutrients at such a low rate that the concentration of these nutrient was unaffected (for example leucine). Subsequent experiments showed that serum, β-mercaptoethanol, cystine, methionine, and vitamins were not limiting nutrients.

The cell to cell contact theory has been eliminated. Since effluent of the bioreactors minus the cells is capable of decreasing antibody production, this implies that the signal is conveyed by soluble factors and not present on the cell surface. Cell membrane fractions prepared from either the antibody secreting or the nonsecreting parental cell line do not decrease antibody production of lower density cells. These experiments also show that membrane bound antibody is not a feed back signal for antibody production.

Diffusible inhibitors cause the decrease in antibody production. Inhibition of antibody production of various compounds were tested with high producing cells at low density. Lactate and ammonia were identified as inhibitors. The molecular weight of the remaining unknown inhibitors was characterized by column fractionation. Further details follow.

The secreted amino acids did not inhibit antibody production. Grew high producing low density cells in fresh medium supplemented with one or all of the following: 1.4 mM alanine, 0.5 mM proline, or 0.2 mM glycine. Addition of the secreted amino acids to fresh medium did not change antibody production or growth rates.

The redox potential does inhibit antibody production. The redox potential, a function of the lactate and pyruvate concentrations, declined from -235 to -265 mV as the cell density increased in the bioreactors. The redox potential effects were tested at low

density by varying the redox potential and maintaining the lactate concentration below 10 mM. No change in growth or production was observed.

Lactate and ammonia are two of the diffusible inhibitors that cause of the decrease in antibody production. The theory that lactate and ammonium inhibit antibody production was tested at low density. These two compounds inhibit antibody production as measured by flow cytometry by 65% in four days. Additional inhibitors are present in the bioreactor media, since inhibition was about 75%. Also, combining lactate, ammonia with redox did not increase the inhibition.

Additional diffusible inhibitors of antibody production are of low weight (<10,000 MW). I searched for other diffusible inhibitors by fractionating the conditioned medium. The effects of a possible high molecular inhibitor were tested without the effects of lactate or ammonia during the experiment. No inhibition of antibody secretion was observed. Large proteins, such as transforming growth factor-β or the antibody protein itself, are not inhibitors. The inhibitory fractions coeluted with the low molecular weight compounds. Comparing antibody production in reconstruction experiments, the inhibition in the fractionated media was higher than accounted for by the lactate, ammonia and pyruvate concentrations. The molecular weight of the other inhibitors are the size of the excluded salt fraction, which is 10,000 MW or less.

The second goal of this thesis was to further characterize the concurrent metabolic shift in amino acid uptake, and to close the mass balance on amino acid utilization. The conclusions the second goal are as follows:

The doubling time increases from about 17 to 21 hours between low and high cell density. From kinetic modeling analysis, I estimated that the doubling time increases from 17-18 hours to 19-23 hours. Experimentally, the cell cycle distribution was analyzed by staining for DNA content and flow cytometry. The percentage of cells in the G1 phase increased from 30 to 46%. The doubling time increased from about 16-17 to 18-21 hours.

The overall cellular content of protein and lipid change by 2/3 and 1/3, respectively, between low and high cell density. Reactor samples were directly analyzed for protein and lipid content. Protein content declines from by 1/3 from 45 to 30 pg/cell, and lipid content declines from 2/3 from 45 to 15 pg/cell between the cell densities of $2x10^6$ to $2x10^7$ cells/ml.

The cell size decreased by 25% from low to high cell density. The decrease in protein in lipid content indicated a decrease in cell volume of about 50% and a decrease in cell size (diameter) by 25%. Cell size as determined by forward light scattering decreased by 25%. The change in cell cycle also decreased the mean cell size of the population. I estimate the mean cell size shifted from 10 microns to 8 microns from the cell densities of $2x10^6$ to $2x10^7$ cells/ml.

The uptake of leucine into lipid is a function of cell density. Exponentially growing cells were resuspended at densities of $2x10^4$ to $3x10^6$ cells/ml for two hour labeling experiments. The uptake rates of leucine into lipid declines with the log of cell density. From $2x10^6$ to $2x10^7$ total cells/ml, the change in rate declined by 1/3.

The free amino acid pools decrease in size as function of cell density. Cells used for labeling experiments were assayed for the free amino acid pools by HPLC. The amount of each amino acid declined from 10 to 100 times from $2x10^4$ to 3×10^6 cells/ml. The total free amino acid pool at 10^6 cells/ml was 351 fmole/cells, and by extrapolation to 10^7 cells/ml the total pool size is 56 fmole/cell.

The inhibitors of antibody production did not inhibit amino acid uptake. Medium removed from the high density, low productivity conditions in the bioreactors inhibits antibody production of lower density cells. The inhibitors are lactate, ammonia and other low molecular compound or compounds. Comparing cells incubated in either fresh media or the high density, low productivity media, the free amino acid pools of the consumed amino acids were not changed. The high density, low productivity media did not inhibit the incorporation of leucine.

The mass balance for the utilization of amino acids for lipid synthesis was closed. Calculations on the requirements of amino acid utilization at high and low density closes the mass balance using the experimental information of the increase in doubling time, and decreases in protein and lipid content, amino acid pool size, and rates of incorporation of leucine into lipid. The requirement for the secreted antibody is about 1/100 of the net amino acid uptake at either high or low cell density. The requirements for protein and nucleotides increases from 1/5 at low density to about 1/2 at high density. The requirement for lipid declines from 2/3 at low density to 1/3 at high density.

VI. Suggestions for Future Research

A. Suggestions to Improve Antibody Production at High Density

Antibody production of this cell line can be improved by changing the operation of the perfusion system by controlling maximum cell density. The media could be altered chemically to reduce lactate, ammonia and the unknown soluble inhibitor. The cell line could be re-engineered to avoid the problems of secreted inhibitors.

Optimize Density

The simplest method to have high specific production is to prevent high cell density by maintaining a medium density of a few million cells per ml in continuous culture, or a perfusion culture with a bleed stream. This short term solution does not use other information known about the cell line.

Alter media formulation to eliminate sources of lactate and ammonia.

Some researchers (Glacken *et. al.*, 1986; Reuveny *et. al.*, 1986) have reduced lactate levels by maintaining a very low concentration of glucose in the bioreactor. They have found that the yield of lactate per glucose is lower at low glucose concentrations than at high glucose concentrations. This strategy requires monitoring the glucose levels and control of the glucose feed stream. This method could reduce the lactate concentration and partially reduce the decrease in antibody production in the bioreactor.

To reduce both lactate and ammonia, Kurokawa *et. al.* (1993) reduced the inlet concentrations of both glucose and glutamine. In continuous cultures of a mouse hybridoma, they found that residual concentrations of less than 2.5 mM glucose and 1 mM glutamine resulted in a 3 fold increase in specific antibody production rates. Uptake rates of both glucose and glutamine were reduced. The yield of lactate from glucose was decreased, but the yield of ammonia from glutamine was not.

To reduce ammonia, replacing glutamine with a similar compound or a dipeptide has been accomplished with other cell lines. Possible media changes include replacing glutamine with glutamate, 2-oxoglutarate, glutamine-glutamine dipeptide, etc. (Griffiths

and Pirt 1967; Hassell and Butler 1990; Minamoto *et. al.*, 1991). For example, Hassell and Butler (1990) replaced glutamine in medium with either glutamate or 2-oxoglutarate. They adapted three cell lines, McCoy, Vero and BHK-21, and observed lower yields of ammonia per compound. They also observed higher cell yield per glucose, and lower lactate per hexose. For this cell line media with glutamate or pyrrolidone carboxylate instead of glutamine does not support growth. Dipeptides of glutamine have the advantage of being broken down slowly by the cells, keeping the glutamine concentration low, and also of being stable enough for autoclaving. Dipeptides have the disadvantage of being expensive. The compounds 2-oxoglutarate and glutamine-glutamine dipeptides have not been tested for this cell line.

To reduce lactate, replacing the sugar source has been successful with some cell lines. For example, Imamura *et. al.* (1982) replaced glucose in DMEM with fructose or maltose for MDCK microcarrier culture. The reduced lactate concentration kept the ratio of lactate to pyruvate in physiological range of 6 to 15, and therefore the redox potential remained in the physiological range. Replacing glucose with galactose also reduces the lactate concentration as observed with L-15 media (Leibovitz, 1963). After several experiments, I concluded that this cell line would not grow in the DMEM based media with glucose replaced with either fructose, maltose or galactose.

Adaptation of Cell Line

Adapt the cell line to lactate and ammonia. For example, select a clone of cells that produces well in high lactate and ammonia concentrations.

Remove the chemical ammonia from the media.

Iio *et. al.* (1985) reduced ammonia concentrations of batch cultures of myeloma and hybridoma cells with the silica alumina adsorbent ZCP-50 placed in dialysis tubing. Operation of a long term culture with this absorbent has not been tested.

Reduce effects of ammonia with potassium

Martinelle and Hägström (1993) have suggested that potassium ions may decrease inhibition of ammonium. Altering the ion composition of this media has not been tested

Exchange All Low Molecular Weight Components

Rønning *et. al.* (1991) demonstrated use of G25 column to remove low molecular weight compounds and recycle high molecular weight compounds with supplements. The operation of a column in conjunction with a bioreactor has not been tested.

Genetic Engineering

Instead of changing the environmental conditions of cell culture for this cell line, change the cell line. One approach would be to construct a mammalian cell line with an inducible promoter or multiple copies of the gene. For example, dihydrofolate gene amplification has been used to express an IgM antibody in Chinese hamster ovary cells (Wood *et. al.*, 1990). Alternately, with glutamine synthetase as the amplifiable gene, a recombinant antibody was expressed in a myeloma cell (Bebbington *et. al.*, 1992), and glutamine in the media was replaced by glutamate and ammonia (Omasa *et. al.*, 1992). Another approach would be construct a bacterial *E. Coli.* cell line to express part of the antibody protein. For some applications, such as immunotherapeutics, an antibody fragment is desirable since is not cleared from the body as fast as the whole molecule. Expression of antigen binding regions of the antibody linked together in a single chain (called scFv) produces a monovalent antibody (Bird *et. al.*, 1988). Recently, bivalent systems of two linked scFv fragments have been developed and are successful in producing correctly folded proteins (Pack *et. al.*, 1993). The production of antibody in bioreactors of bacterial cells is expected to be cheaper than the production by mammalian cells.

B. Further Characterization of Unknown Inhibitors

Further research is suggested to identify the unknown inhibitor of antibody production. Inhibition of antibody production by lactate and ammonia accounted for only part of the inhibition signaled by the diffusible inhibitors.

The unknown factor can be further characterized by molecular weight. In the media fractionation experiments, an additional factor (or factors) appears to have coeluted with lactate and ammonia. The molecular weight of the factor must be below the molecular weight cuttoff of the column of 10,000. Rønning et. al. (1991) found a growth inhibitory

fraction below 5,000 with a similar column material. The unknown factor(s) is likely to be of similar molecular weight to lactate and ammonia. To indirectly confirm that the molecular weight is below 1000, the bioreactor media could be dialyzed vs fresh media or ultrafiltered. If the dialyzed media does not inhibit production then the inhibitors are below 1000. Combining the low molecular weight fraction of the inhibitory media and the high molecular weight fraction of fresh media, one would expect inhibition of antibody production. Alternately, the fractionated samples could be run on polyacrylamide gels to exclude a substance in the 500 to 10,000 molecular weight range.

The unknown factor could be characterized as an acid or base. The inhibitory fractions could be further fractionated by ion exchange chromatography with cationic and anionic columns. The fractions from these columns could then be tested for inhibition of antibody production. Lactate or ammonia may interact with the third compound. Identification of the third compound would then require adding lactate or ammonia to the fractionated samples in the bioassay.

In his measurements of the bioreactor samples by analytical HPLC Tyo (1991) he did not note the existence of an unidentified peak increasing in size over time. He used three different column types to separate samples: an organic acid column (Bio-Rad HPX-87H), and organic base column (Bio-Rad HPX-870), and a reverse phase C18 column (Beckman Ultrasphere) for amino acids separation. Due to the presence of serum, not all of the peaks were identified. It is possible that the unknown inhibitor of antibody production was not separated by the elution techniques and remained bound to a column or coeluted with a known factor. For example, the organic acids were eluted at a low pH, and amino acids and nucleotides were bound to the column and were not separated. In theory, short chain fatty acids can also be separated by this method.

The fractionation strategy may not identify the additional inhibition of antibody production. One such possible inhibitor would be increased osmolarity. Separate experiments to test the sensitivity of this cell line to increased osmolarity (e.g. increasing sodium chloride) would be performed. Alternately, lactate or ammonia may reduce uptake of a nutrient not measured in these studies or interact with a cellular component.

Identification of the inhibitor (or inhibitors) would then lead to strategies to remove or block the inhibitor and therefore increase antibody production.

C. Further Analysis of Amino Acid Uptake

The density dependence of the amino acid transport systems for this cell line could be determined with amino acid analogs. Cycloleucine and 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid (BCH) are a non-metabolizable analogs of leucine that are used to measure uptake by the L system. Alpha-aminoisobutyric acid (AIB) and n-methyl AIB are non-metabolizable analogs that are taken up by the A system.

The changes in amino acid utilization between high and low density could be characterized further by types of lipids. For example, Lazo (1981) found the incorporation of [U-^{14}C] leucine in fast growing tumor ascities cells is partitioned to 78% sterols, 13% free fatty acids, 5% saponified lipids and 4% glycerol.

The cellular components could be assessed by electron microscopy. Al-Rubeai *et. al.* (1990) compared non-producers to producers cells and found that the non-producers had less endoplasmic reticulum than the producers. Such a study has not been done comparing cells at high vs. low cell density.

Another line of research would be to find the mechanism for the decrease in amino acid uptake at high density and block the mechanism. The mechanism may require cell to cell contact or be mediated by a diffusible inhibitor. Bhargava (1975) proposed a complex model to account for the inhibition of amino acid uptake into protein and the inhibition of DNA synthesis at high cell density. The protein inhibitor central to this model was not identified.

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