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OPTIMIZING THE GROWTH ENVIRONMENT FOR A RECOMBINANT MYELOMA CELL LINE USED IN THE PRODUCTION OF NEW CANCER VACCINES

by

ANTHONY O. S. STEINICKE

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The undersigned certify that they have read, and recommend to the Faculty of Graduate studies for acceptance, a thesis entitled, "Optimizing the Growth Environment for a Recombinant Myeloma Cell Line used in the Production of New Cancer Vaccines", submitted by Anthony Steinicke in partial fulfillment of the requirements for the degree of Master of Science.

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Date: August 31, 1994.

ABSTRACT

A serum free medium (SFM) has been developed for both the recombinant myeloma (C174) and hybridoma (M174) cell lines. The major components of this SFM are BSA (bovine serum albumin), insulin, transferrin, ethanolamine, and selenium.

Adaptation of both cell lines to serum free conditions was attempted using three different adaptation protocols. For each protocol three different media were tested. Experiments to evaluate the effect of various supplements on cell growth and antibody production were also performed. Surprisingly, none of the supplements tested were found to enhance the antibody production of either cell line. In terms of growth, the myeloma cell line responded favorably to all the supplements tested. The optimization of the more expensive components in the formulation was the final step before carrying out carefully controlled batch runs in a 1.5 L Celligen bioreactor. For the myeloma cell line the optimum concentrations of BSA, insulin, transferrin, and ICN LDL were 130 mg/L, 5 mg/L, 10 mg/L, and 150 mg/L respectively. For the hybridoma cell line the optimum concentrations for BSA, insulin, and transferrin were 430 mg/L, 5 mg/L, and 5 mg/L respectively.

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Firstly, I would like to thank my supervisor Dr. Leo A. Behie for all his guidance and support during my research work. I would also like to thank Patrick Farrell for all his help and good suggestions, without his help this thesis would not have been completed as quickly as it has been. I am also grateful to Dr. Nicolas Kalogerakis for all the helpful discussions we had concerning the experimental setup. I would also like to extend my gratitude to Minxieu Chen and Andrea Behie for all the help they gave with my thesis project. Finally I would like to thank the rest of the group at the PPRF laboratory who made coming into work every morning a little easier.

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Chapter 1: Introduction

1.1 Biotechnology

Biotechnology is an area in which chemical engineering principles are applied to biological processes in order to operate them efficiently at an industrial scale. The applications for biochemical engineering in bio-processes are numerous ranging from the production of rare pharmaceuticals such as platelet regulatory factor thrombopoietin (TPO) to the production of monoclonal antibodies (MAb's). TPO is believed to be able to increase platelet levels in the blood, and is essential for cancer patients undergoing chemotherapy who often develop thrombocytopenia (a disease in which the blood becomes deficient in platelets). MAb's have many uses including cancer treatment, and diagnosis of many different diseases including the HIV virus. By applying chemical engineering principles the production of these pharmaceuticals can be done in large quantities and at significantly lower costs than previous methods would allow. Other applications of biotechnology are listed in Table 1.1.

1.2 The Immune Response of Vertebrates

Acquired immunity for a human being or animal refers to an organism having an immune system which specifically recognizes and eliminates foreign microorganisms and molecules. Acquired immunity displays specificity, diversity, memory, and self/non-self recognition (Kuby, 1992).

The humoral immune system of a body recognizes components within the body as self and non-self. Non-self material found within human bodies includes pollen, insect venom, viruses, parasites, fungi, and bacteria. When the immune cells come in contact with the invaders, a part of the invader located on its surface known as the "antigen" triggers the immune response. Typical antigens include cell walls, virus coats, flagella, and toxin fragments. The humoral branch and cell-mediated branches of the immune system are shown in Figure 1.1.

Table 1.1- Applications of Biotechnology (Lee, 1992)

PHARMACEUTICALS:
Antibiotics, antigens (stimulate antibody response), diagnostics, gamma globulin (prevent infections), human growth hormone (treat children with dwarfism), human serum albumin (treat physical trauma), immune regulators, insulin, interferon, interleukins, lymphokines, monoclonal antibodies, neuroactive peptides, tissue plasminogen activator, vaccines, etc.
ANIMAL AGRICULTURE:
Products similar to those being developed in the pharmaceutical industry; development of disease free seed stocks and healthier, higher yielding food animals.
PLANT AGRICULTURE:
Transfer of stress-, herbicide-, and pest-resistant traits to important crop species; development of plants with the increased abilities of photosynthesis or nitrogen fixation; development of biological insecticides and nonice nucleating bacteria.
SPECIALTY CHEMICALS
Amino acids, enzymes, vitamins, lipids, hydroxylated aromatics, and biopolymers.
AGRICULTURAL CHEMICALS
Pesticides, fungicides, and herbicides.
ENVIRONMENTAL APPLICATIONS
Mineral leaching, metal concentration, pollution control, toxic waste degradation, and enhanced oil recovery.
FOODS AND BEVERAGES
Alcoholic beverages, sweeteners, single-cell protein.
COMMODITY CHEMICALS
Acetic acid, acetone, butanol, ethanol, and many other products from biomass conversion processes.
BIOELECTRONICS
Biosensors, biochips.

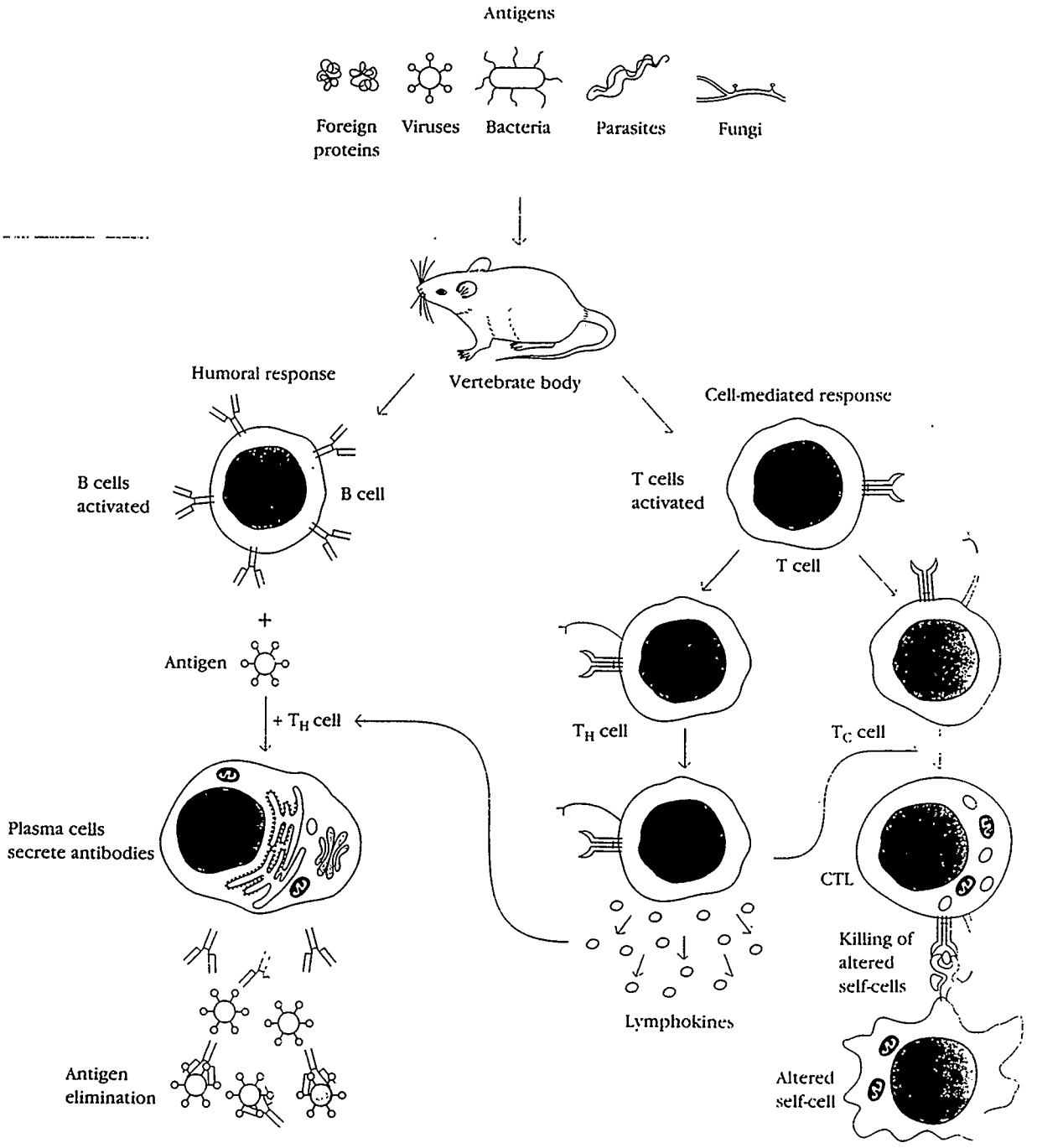


Figure 1.1- The humoral branch and cell mediated branches of the immune system for vertebrates (Kuby, 1992).

The immune response can be divided into humoral and cell mediated responses. The humoral response involves interaction of B lymphocytes (B cells), which arise in the bone marrow of humans, with the antigen and their differentiation into antibody secreting plasma cells. The secreted antibody binds to the antigen and facilitates its removal from the body in a number of different ways. One way is that the antibodies can bind a large number of antigens together into clumps, which enables phagocyte cells to ingest them more readily. Also the antibodies can bind to and immobilize viruses and toxins which prevents them from harming the vertebrates cells.

The cell-mediated response involves T lymphocytes (T cells), which stem from the thymus, and recognize the antigen present on self-cells. T_H (helper) cells respond to the antigen with the production of lymphokines (responsible for activation of B cells, T_c cells and phagocytes in the immune response) and cytoxic T lymphocytes (CTL) which kill the cells that have become infected with the antigen (e.g. virus-infected cells;Kuby, 1992).

After being infected with a particular antigen, the immune system protects itself from a second attack by creating lymphocyte memory cells which can recognize the antigen much more quickly. The immune system can then respond almost immediately significantly reducing the danger of the antigen.

1.3 Monoclonal Antibodies

Immunoglobulins are protein molecules that function as receptors for antigen on the B-lymphocyte cells and as secreted products from the plasma cells. These secreted antibodies circulate throughout the body and attach themselves to antigens, eliminating them from harming the body. The human immune system is able to produce more than 10^{12} different antibodies each binding specifically to one antigen or protein. As explained in the clonal selection theory the specificity of each T and B lymphocyte is determined by the random rearrangements of the membrane receptor genes. An antigen will bind to and stimulate a particular cell to undergo mitosis, producing cells with the same

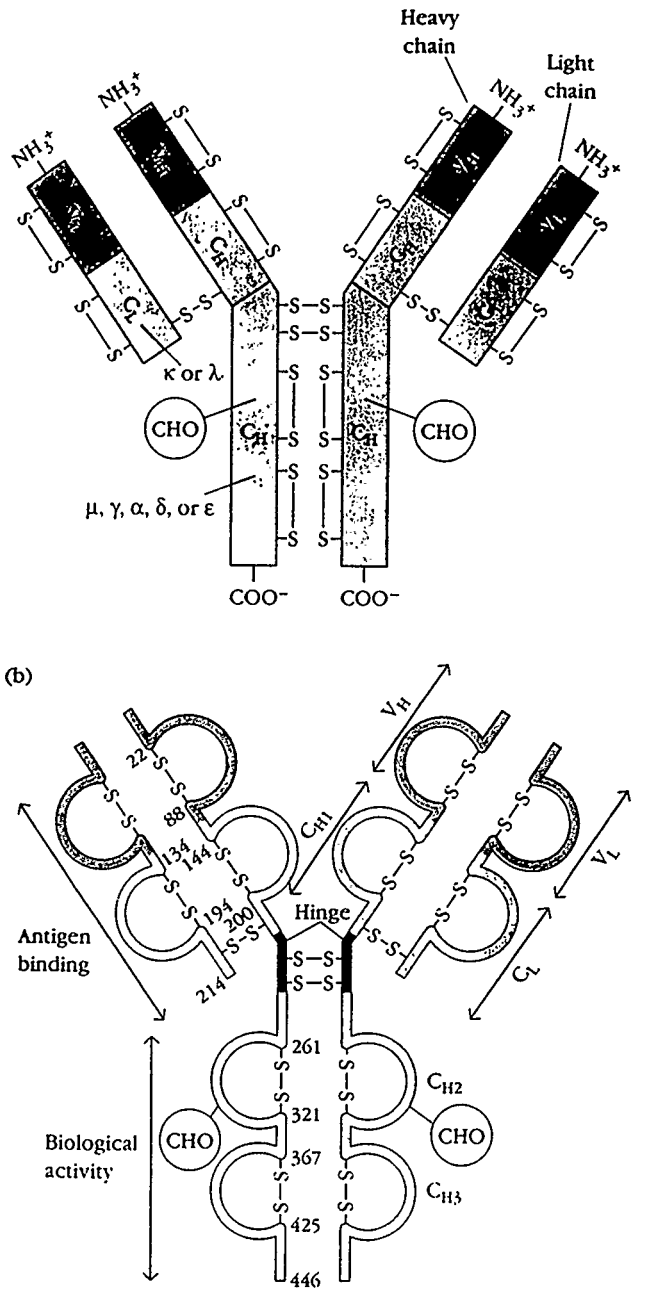


Figure 1.2: A typical antibody structure (Kuby,1992)

antigenic specificity as the parent cell (Kuby, 1992). A typical IgG antibody structure (Y shaped) is shown in Figure 1.2. The antigen binding capability of the molecule resides in the hypervariable or complimentarity-determining regions and are labelled in the figure as V_L and V_H. The constant regions give the antibody its biological activity and are labelled with C's in the figure. The 150,000 MW IgG molecule is composed of two polypeptide chains of 50,000 MW which are called the heavy chains, and two 25,000 MW polypeptide chains or light chains. The heavy chains are joined by two disulphide bonds in the "hinge region" whereas the light chains are joined by a single disulphide bond to the heavy chain also in the hinge region. A single antibody has two identical heavy chains and two identical light chains. The heavy chains of a given antibody determine the class: IgM, IgG, IgA, etc. Biotechnology has found many uses for these antibodies including diagnostics, in-vivo therapeutics, purifications, and catalysts.

1.3.1 Diagnostic Reagents

There are over 100 different monoclonal antibodies which are used for detecting pregnancy, diagnosing infectious protozoan, detecting diabetes, detecting tumor cells, and many other applications. Monoclonal antibodies labelled with low level radioisotopes can also be injected into patients to detect a variety of cancers, including ovarian, breast, and lung cancer. As an example iodine 131 attached to a monoclonal antibody specific to breast cancer has been injected into the patient to determine if the cancer has spread to the lymph nodes. The major benefit of using antibodies in cancer detection is that the cancer can be detected far earlier than conventional techniques allow, thus increasing the chances for survival of the patient. As monoclonal antibodies have become more and more popular in the detection and treatment of diseases, their use has reached in the kilogram quantities per year production levels.

1.3.2 Purification

Monoclonal antibodies can be used to purify specific protein products by affinity chromatography. Antibodies specific for a desired protein are contained within a gel packing in a separation column. The protein mixture is passed through the column and

the desired protein becomes absorbed into the gel and binds to the monoclonal antibody. The bound protein molecules in the gel are eluted out by using suitable elution buffer. In this manner antibodies can be used to purify almost any protein. The purification of interferon using an immunosorbent column is shown in Figure 1.3. In the figure the interferon binds to the monoclonal antibody and is eluted out using a salt solution.

1.3.3 *In vivo* Therapeutics

Tumor specific monoclonal antibodies conjugated to bacterial toxins or radioisotopes are injected into cancer patients where they bind to cancer cells. The toxins kill the cancer cell without harming any other cells in the body. Examples of some bacterial toxins include Diphtheria toxin, Shigella toxin and Ricin. This technique is similar to chemotherapy in that a toxin is used to kill the cancerous cells, however it has the advantage of having no real side effects as does radiation treatment.

1.3.4 Catalysts

R. A. Lerner created antibodies which catalyzed the hydrolysis of an ester (Lerner and Benkovic, 1988). The antibodies where created against a molecule which resembled the transition state of ester undergoing hydrolysis. The theory is that the antibody binds to and stabilizes the transition state thereby catalyzing the reaction.

1.3.5 Large Scale Production of Monoclonal Antibodies

In 1975 Kohler and Milstein won a Nobel prize for fusing mouse myeloma cells with mouse lymphocytes from the spleen of mice immunized with a particular antigen (Kohler and Milstein, 1975). The resulting cell was called a mouse-mouse hybridoma cell which could produce antibodies like B-lymphocytes and was immortal like cancer cells. This opened the door to the production of antibodies on a large scale since the hybridoma cell could be cultured indefinitely. The fusion agent most commonly used in polyethylene glycol (PEG). The PEG facilitates attachment of the cell membranes of adjacent cells, and ultimately the two cells fuse into one. A schematic representation of the fusion technique as well as the technique used for clonal selection (In this case clonal selection

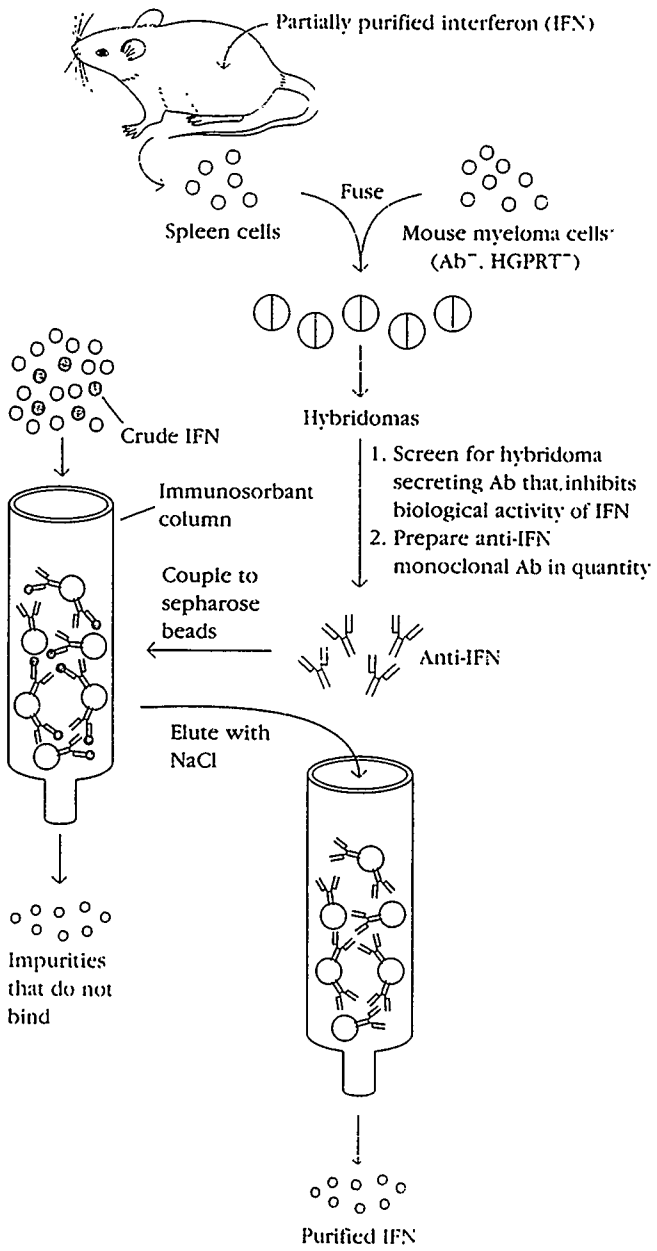


Figure 1.3: The purification of interferon using an immunosorbent column (Kuby, 1992).

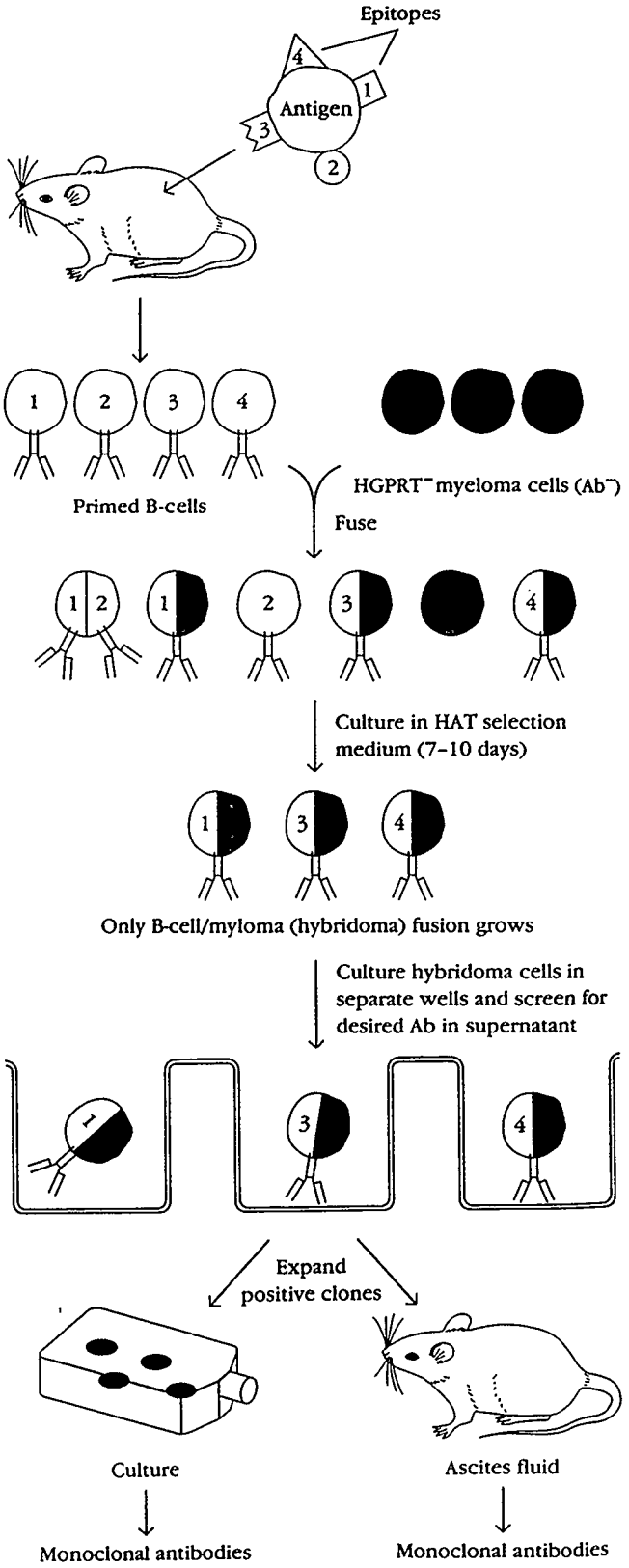


Figure 1.4- A schematic representation of the process of cell fusion as well as clonal selection (Kuby, 1992).

refers to the selection of a producing hybridoma clone) is shown in Figure 1.4.

1.4 The Role of Bioreactors

The large scale production of monoclonal antibodies is carried out in bioreactors in which the temperature, dissolved oxygen, pH, and nutrients may be carefully controlled. The four most common modes of operation for bioreactors are batch, fed batch, continuous, and perfusion and are depicted in Figure 1.5.

The batch mode of operation allows the most flexibility of operation, since once the batch run is completed a different cell line can be grown using the same reactor. Also, this reactor avoids the problems associated with long periods of operation, such as equipment malfunction and higher risk of contamination. The cells are first inoculated into the reactor at a cell density ranging from $1.0x10^5$ to $2.0x10^5$ cells/mL. After a short lag period (at most a few hours for hybridoma cells) the cells begin to grow exponentially until they reach their maximum cell density generally $1.0x10^6$ to $2.5x10^6$ cells/mL after which the depletion of nutrients and build up of metabolic wastes causes the cells to die. The one major drawback of batch operation is that the product concentrations are generally much lower than for other reactor types. For example the antibody concentration for a hybridoma culture grown in batch mode ranges from 40 to 100 mg/L, whereas for perfusion culture the antibody concentration can be 20 to 50 times higher because of the higher cell densities achieved. By using fed batch systems the nutrient environment of the cells can be controlled avoiding problems such as overflow metabolism and leading to higher product yields.

The biggest advantage in using continuous operation is that the antibody concentration is generally much higher (due to higher cell densities) for these bioreactors. In perfusion reactors the cells are retained within the reactor and very high cell densities and high antibody production rates can be achieved. This however is balanced by the fact that cost of operation as well as the initial investment required is generally much higher than for batch systems. Another important use of continuous culture is the determination of

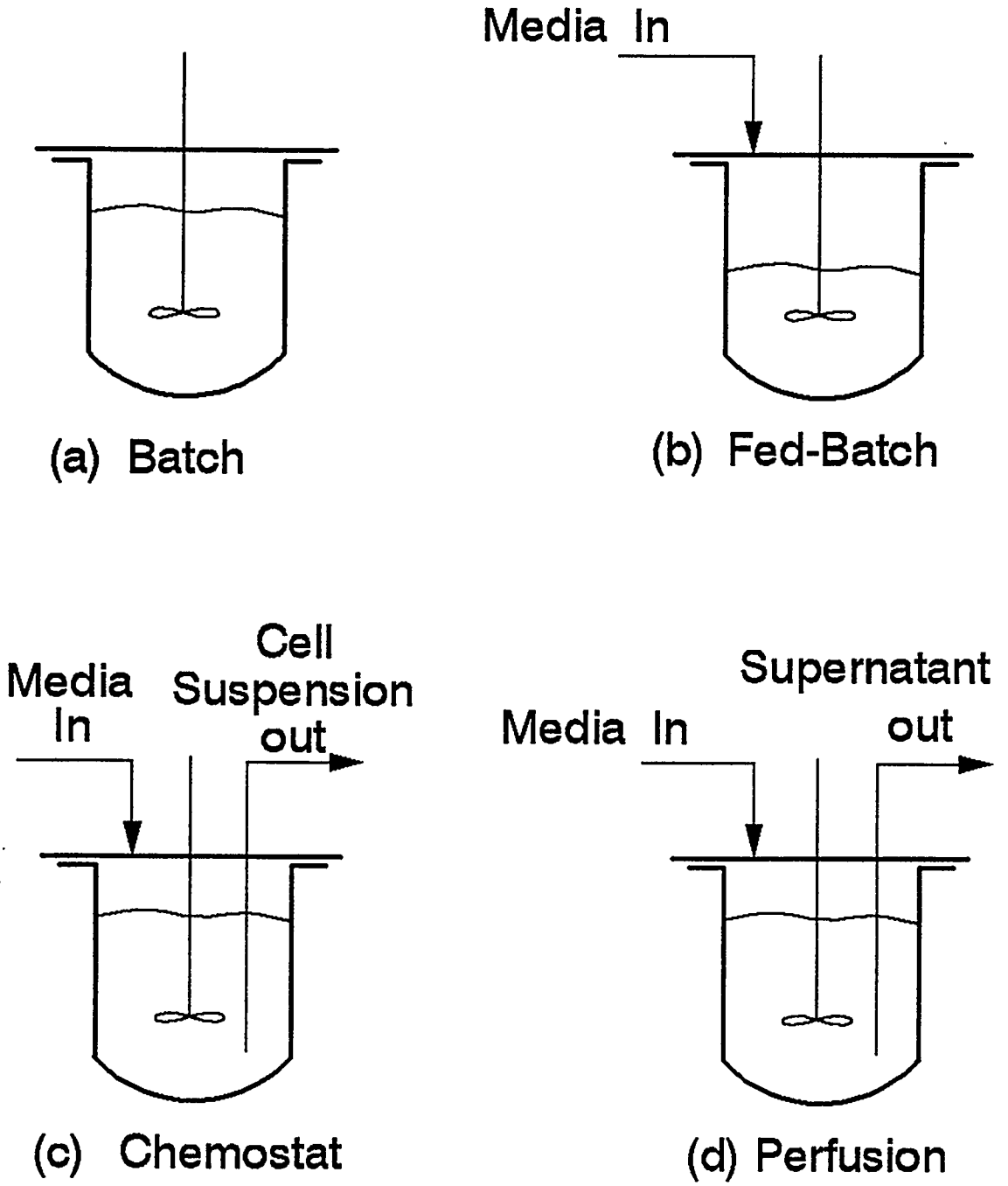


Figure 1.5: Four common types of bioreactors used in the animal cell biotechnology industry; (a) batch, (b) fed batch, (c) continuous, (d) perfusion.

cell growth and product formation kinetics, something which is difficult in batch culture. By using continuous culture the system can reach a steady state, in terms of nutrient, metabolic waste, and product concentrations. By studying a variety of steady states the kinetics can be readily determined.

1.5 Monoclonal Antibodies from Recombinant Cells

In order to bind sufficient amounts of monoclonal antibodies to the tumor cells when treating a cancer patient, the antibodies must usually be given in high concentrations. However, because these antibodies are generally mouse monoclonal antibodies the human body recognizes them as non-self material and an immune response to these proteins is set in motion. This results in complexes of mouse and human antibodies which can build up in the organs of the patient and cause serious allergic reactions. This makes the use of mouse monoclonal antibodies for cancer detection and treatment in humans highly undesirable. The obvious way to overcome this problem is to use human monoclonal antibodies. However, the production of human monoclonal antibodies has been hindered by several obstacles. The biggest problem is obtaining B cells from humans which have been exposed to the specific antigen. It is of course morally wrong to immunize a human with a deadly antigen in order to harvest the persons cells after they die. Therefore immunization of the human system is usually done *in vitro*, which has proved much less effective than *in vivo*.

The other major difficulty in the production of human monoclonal antibodies is finding a suitable fusion partner for the human B cell. The fusion partner must be immortal, be susceptible to HAT selection, and be incapable of antibody production. Only a few human hybrids have been created to date which can be cultured indefinitely. Another possibility is to fuse human B cells with mouse myeloma cells. These hybrids are very unstable exhibiting loss of chromosomes and hence loss of antibody production.

Another method which has proven successful is to transform healthy B lymphocytes with Epstein Barr Virus (EBV) into cancer cells. When the B cells are cultured with the

antigen and EBV, some of the cells become immortal (myeloma), while continuing to produce antibody. This however is not always successful. Because of the problems associated with using mouse monoclonal antibodies in humans, and the problems associated with the production of human monoclonal antibodies, recombinant DNA technology is now being used to create cells which will produce the desired antibody. The engineered monoclonal antibodies consist of human constant regions and mouse variable regions. The variable regions are responsible for attachment to the antigen and must be obtained by harvesting the spleen cells from a mouse injected with the antigen. Obviously human cells cannot be used because injecting human beings with various antigens including cancers is not morally or ethically correct. These chimeric antibodies have a far less chance of invoking an immune response when used for *in vivo* purposes. A schematic representation of various engineered monoclonal antibody molecules are shown in figure 1.6. The figure shows a chimeric monoclonal antibody containing the variable regions of a mouse antibody and the constant regions of a human antibody.

1.6 Research Objective

The ultimate objective of this experimental project is to develop a serum free low protein medium for the cultivation of myeloma and hybridoma cells. A target protein content of 150 mg/L up to 450 mg/L is established in order to increase the chances for success and to develop a medium which will significantly reduce the costs associated with purification of the antibody (estimated at 70 % of the total production cost). To achieve this objective several obstacles must be overcome. Firstly an appropriate basal medium must be selected in which the cells grow well in serum free conditions. This requires the use of commercially available formulation in order to be able to perform the experiments in serum free medium. Nutridoma NS was chosen since it is considered the best serum free medium on the market today. Secondly the adaptation of the cells to a base formulation (consisting of BSA, insulin, transferrin, ethanolamine, and selenium) constructed from a literature review must be performed. A number of successful adaptation protocols have been reported and several of these will be attempted. The choice of the protocols are not only based on the likelihood of success but also on the

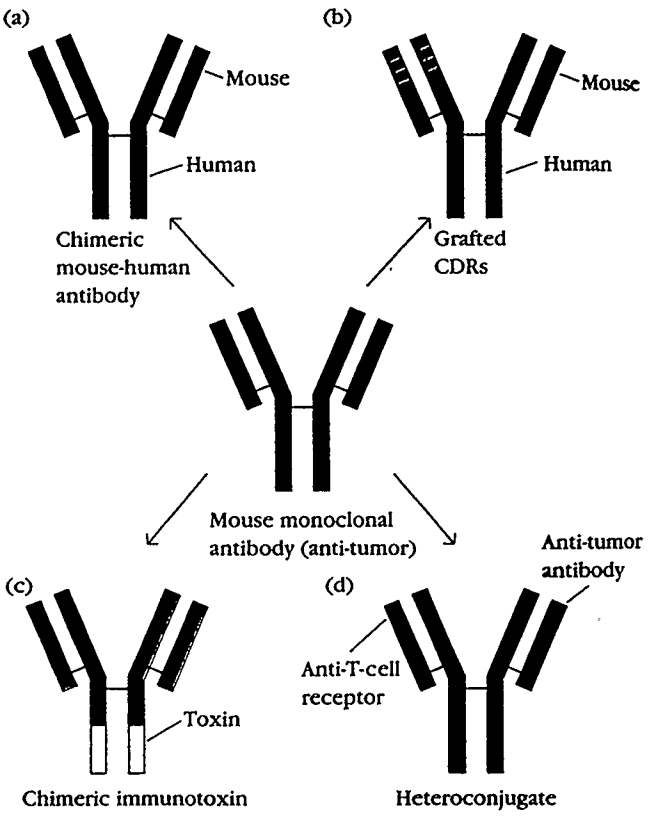


Figure 1.6: A schematic representation of an engineered mouse-human monoclonal antibody (Kuby, 1992)

fact that there is a time restriction posed on the completion of the project. The result is that only short and medium term adaptations will be attempted. Finally the testing of other supplements to determine their growth supporting effect will also be examined. This will hopefully culminate into the development of a economically viable serum free medium for use with hybridoma and myeloma cells. The cost of using 10 % serum in medium is approximately 49.00 $/L and the cost of Nutridoma NS is 54.40 $/L (for the recommended concentration). Generally a higher concentration of Nutridoma NS must be used resulting in a cost in excess of 100 $/L.

Chapter 2: Introduction To Serum Free Medium

2.1 Overview

The first studies on the substances required for cell growth of mammalian cells began in the 1950's, in order to satisfy the large demand for vaccination programs. In 1959, Eagle introduced a minimal essential medium (MEM) consisting of amino acids, vitamins, and glucose in a balanced salt solution used specifically for the culturing of mammalian cells. This medium had to be further supplemented with 5 to 10 % blood serum in order for the cells to grow in cultures. This chapter briefly reviews the approaches to serum free medium (SFM) development in the literature including a summary of the SFM formulations (and concentrations when disclosed), possible functions of the key components, and the approaches to adapting cells to the formulations.

2.2 The Role of Serum

Hybridoma cells (artificially created by fusing a cancer cell with a lymphocyte) and recombinant myeloma cells (genetically engineered cancer cells) are used to produce monoclonal antibodies (MAb) for diagnostic purposes as well as cancer treatment. These cells are grown in a medium which contains sugars, amino acids, and vitamins, and is usually supplemented with foetal bovine serum (FBS). This serum provides hormones, growth factors, binding and transport proteins and other nutrients which are necessary for cell growth. Serum also binds to and neutralises toxins, provides protease inhibitors (which inactivate trypsin), and provides protection from shear in agitated suspension cultures.

Although the serum is essential for cell growth there are problems associated with its use. These include high cost, variable composition, interference in product purification, and is a possible source of contamination (mycoplasmas, viruses, etc.). The factor affecting the final cost of the produced antibody is mainly the cost of downstream

purification of the produced monoclonal antibody (which can be as high as at 70 to 80 % of the total production cost). This is partially due to serum proteins which are similar in size to the antibody and therefore are difficult to separate out. Therefore the use of a low protein serum free medium can greatly improve the economics of antibody production. The major functions of serum as well as the disadvantages in using serum are listed in Tables 2.1a and 2.1b. The advantages and disadvantages of using serum-free media for hybridoma and myeloma cells are listed in Table 2.2.

2.3 Serum Free Medium Formulations

A summary of fourteen published serum free medium (SFM) formulations is shown in Table 2.3. As seen from the table, most of the formulations contain three proteins namely albumin, insulin, and transferrin. The trace element selenium, as well as supplements of ethanolamine, 2-mercaptoethanol and fatty acids are also frequently present. In addition, to these already mentioned, some formulations include steroids, hormones, fatty acids, lipid complexes, vitamins, and trace elements. Total protein concentrations vary from 1 to 2000 mg/L in this list of SFM formulations.

Bovine Serum Albumin (BSA)

BSA is the major protein in serum and typically present at levels of 3000 to 5000 mg/L in a basal medium containing 10% fetal bovine serum (FBS). Albumin's primary function is to bind and present fatty acids (insoluble in water) and lipids to the cells (Kovar, 1987). In addition, it may have a detoxifying effect (Barnes and Sato, 1980), as well as protecting against shear damage from bioreactor agitation.

Transferrin

Transferrin is an iron binding glycoprotein that may have multiple functions. Besides transporting iron to the cell, and being essential for some intracellular activities, it may also act independently through cell surface receptors to stimulate proliferation as a growth factor (Kovar and Franek, 1985) at levels of 1.5 to 45 mg/L in SFM. Transferrin may also act as a chelating agent for toxic trace elements in vitro (Lambert

Table 2.1a - Functions of fetal bovine serum (FBS) in cell culture

1. Carrier/buffer/chelator for labile or water-insoluble nutrients.
2. Binds and neutralises toxins.
3. Provides protease inhibitors which inactivate trypsin.
4. Provides essential low-molecular-weight nutrients.
5. Provides hormones and peptide growth factors.
6. Protective effect (e.g. in agitated suspension culture).

Table 2.1b - Problems associated with the use of FBS in suspension culture

1.	Variability in composition.
2.	High cost and product scarcity.
3.	Source of contamination (mycoplasmas, bacteriophages, viruses).
4.	Source of toxins.
5.	Prevents definition of nutritional environment.
6.	Interference in product purification (e.g. by immunoglobulins in immunological products).

Table 2.2 - Advantages and Disadvantages of using serum-free medium (Glassy *et al.*, 1988).

Advantages
1. Consistent and chemically defined composition.
2. Improves reproducibility of cell culture growth and product yield.
3. Decreases potential of contamination.
4. Reduces difficulty and cost of product purification.
5. May provide cost savings with a low protein serum-free formulation.
Disadvantages
1. Requires optimization for each cell line.
2. Applicable serum free media have not been developed for all cell lines.
3. May require expensive hormones and growth factors which are difficult to purify
4. Frequently results in longer lag periods.
5. Reduction in cell growth rate, maximum cell density, and viability usually occur.
6. Protease inhibitors present in serum are not present in SFM formulations.

Table 2.3 -Published Serum Free Formulations For Hybridoma Cell Cultures

AUTHORS (see references)	Brown	Cho	Heath	Hill	Jäger	Glassy	Kawa	Darfler	Faze	Hagi	Mura	Kovar	Ozturk	Sato
Basal Medium	I	IF	RD	DF	IF	R	RDF	DF	D	RDF	RDF	R	DIF	RDF
Albumin (μg/mL)		100		100	1000	500	500		1100	1000	1000	1000	5000	2000
Insulin (μg/mL)		10	5	5	10	10	10	15		10	5	10	2.5	5
Transferrin (μg/mL)	5	10	5	30	10	10	10	1.5	31	10	35	45	2.5	35
Ethanolamine (μM)		10	10	20	20	10	10			1	20	20	2.5	20
2-Mercaptoethanol (μM)		50				10	10		4.4	1			2.5	
Selenium (nM)		30	10	20			1		1.6	1	2.5	5	2.5	2.5
Trace Elements		yes												
Sodium Pyruvate (μg/mL)	110		110		22									
GHL peptide (ng/mL)					10									
Linoleic acid (μg/mL)								1				5		
Oleic Acid (μg/mL)					8		4							
Egg Yolk Lipoprotein (μg/mL)											5			10
Soy Bean Lipoprotein (μg/mL)									22.6					
Bovine Lipoproteins (μg/mL)					10									
Human Lipoproteins (μg/mL)							2							
Casein (μg/mL)								500						
Cholesterol (μg/mL)									1.6					
Progesterone (ng/mL)													2	
Ascorbic Acid (nM)													3	
Polyethylene glycol (%)		0.05												
TOTAL PROTEIN (mg/L)	5	120	10	135	1020	520	520	16.5	1131	1020	1040	1055	5040	2040

Note: I - IMDM medium ; IF - IMDM+F12 (1:1) medium ; RD - RPMI+DMEM (1:1) medium ; DF - DMEM+F12 medium ; R - RPMI medium
RDF - RPMI+DMEM+F12 (1:1:1) medium ; D - DMEM medium ; DIF - DMEM+IMDM+F12 (1:1:1) medium

and Birch, 1985)

Insulin

Insulin is a peptide hormone that stimulates metabolic activities such as glucose uptake, and RNA, protein and lipid synthesis (Schubert, 1979). It acts via cell surface receptors at levels of 2.5 to 15 mg/L in SFM.

Selenium

Selenium is an essential trace element enhancing cell growth that probably acts as an antioxidant, protecting the cell from damaging and toxic effect of peroxides (Barnes and Sato, 1980). It is supplemented at levels of 1 to 30 nM in SFM, although it becomes toxic at higher levels.

Ethanolamine

Ethanolamine stimulates the growth of hybridomas, and is associated with lipid synthesis. Mammalian cells use ethanolamine to synthesize phosphatidylethanolamine, a major constituent of membrane phospholipids (Lambert and Birch, 1985). It is usually present at 1 to 20 μM in published SFM formulations.

2-Mercaptoethanol (2ME)

This compound acts to reduce intracellular toxicity by indirectly maintaining glutathione in a form that prevents peroxide damage and facilitates the transition from the G_1 to S phase in the cell cycle (Kristensen *et al.*, 1981). It is generally present at 1 to 50 μM.

Fatty Acids

Fatty acids, such as oleic and linoleic acid, are naturally coupled to crude serum albumin, and at least play a role in the cell membrane integrity. They may have a direct function in controlling cell growth (Kovar, 1987). By themselves, fatty acids are generally insoluble in culture medium, unless complexed to carrier protein - usually albumin at levels of 1 to 10 mg/L are used in SFM.

2.4 Protein Free Medium Formulations

Table 2.4 summarises selected published protein free medium formulations. Most reports claim to have replaced iron transporting protein transferrin with ferric citrate or iron (II) sulphate. Results on cell growth with these media surprisingly do not include viability, an indicator of the success of adaptation to these media.

2.5 Difficult Animal Cell Lines

In general, problems have been reported in the literature with the growth in serum free medium of some hybridoma cell lines, such as those derived from the NS-1 myeloma cell line. This is likely due to a defective cholesterol synthesis pathway, as exogenous cholesterol has been demonstrated to be absolutely essential for the survival and growth of NS-1, P3-X63-Ag8, and X63-Ag8.653 myeloma cells (Sato *et al.*, 1984 and 1987).

Other reports have shown that raw cholesterol cannot support the growth of NS-1 cells unless presented in low density lipoproteins (LDLs), either from human, chicken yolk, bovine, or soybean sources (Shinohara *et al.*, 1993). LDLs are highly complex molecules comprising of apoprotein B, phospholipid, and cholesterol (free or esterified to fatty acids), the cholesterol constitutes approximately 50% of their mass. In mammalian cells, after endocytosis the LDL is transported to lysosomes where the apoprotein B is degraded to amino acids, and the cholesterol esters are hydrolysed to cholesterol and fatty acids. The cholesterol is directly incorporated into cell membranes. Approximately 40% of the published articles have used lipoproteins in their SFM formulations.

Isolated components of LDLs, such as phospholipids, were unable to support the growth of LDL dependant cell lines by themselves (Murakami *et al.*, 1988). It appears that cholesterol is a key ingredient to a successful serum free formulation for some hybridoma and myeloma cell lines, although the form in which the cholesterol must be presented to the cells may vary from cell line to cell line. In fact, it is likely that most of the useful cholesterol in serum is contained in lipoproteins.

Table 2.4 - Summary of Published Protein Free Media Formulations used for Hybridoma Cell Culture

AUTHORS *(see references)*	Schneider	Shinmoto	Kovar	Cleveland
Basal Medium	DNF	RDF	R	IF
Ferric Citrate (μM)	500		500	
Iron (II) Sulphate (μM)		80		
Ethanolamine (μM)		20	20	
2-Mercaptoethanol (μM)	50			
Selenium (nM)		25		
Ascorbic Acid (μM)			20	
Estradiol (nM)	10			
Trace Elements			yes	yes
Hydrocortisone (nM)			5	
Progesterone (ng/mL)				10
Thioglycerol (ng/mL)				
yes				
Pluronic F68 (w/v %)	0.1			

Note: RDF - RPMI + DMEM + F12 (1:1:1) medium ; R - RPMI medium ; IF - IMDM + F12 (1:1) medium ; DNF - DMEM + N + F12 (1:1:1) medium

2.6 Adaptation Protocols for Serum Free Media

There are three approaches to the adaptation of cells from serum to serum free media.

i) Long term adaptation to SFM over a period longer than 6 months relies on clonal selection where only the cells with the appropriate mutations can survive. This procedure is not considered suitable for hybridoma or genetically engineered cells since these cells are inherently unstable and should not be propagated for long periods for fear of losing MAb productivity (Ozturk and Palsson, 1991). That is, over a long period of time a clone which does not have the burden of MAb production may take over the culture.

ii) Medium term adaptation assumes that the cells make major physical changes to their metabolic pathways over a period of less than 6 weeks to adjust to the absence of serum.

iii) Short term adaptation over a period of 1 or 2 weeks assumes that the cells make minor physical changes over several subcultures to adjust to the new culture conditions.

The procedures for the medium and short term adaptation of cells to the serum free environment vary considerably, particularly with the cell line used and the total decrease in total protein concentration, however most adaptation protocols fall into three categories:

a) The initial addition of serum free supplements at final levels, followed by the gradual reductions in serum concentration (Radford *et al.*, 1991)

b) Stepwise increase in serum free formulation with simultaneous decrease in serum levels (Shacter, 1989)

c) Direct transfer to serum free medium with perhaps a trace of serum for several subcultures (Shinmoto and Dosako, 1993)

2.7 Summary

Most hybridoma and myeloma cell lines require trace elements, albumin, insulin, transferrin, and antioxidants to survive in a serum free environment. The cultivation of cells in protein free formulations is also possible but more difficult to achieve. In

addition, a cholesterol source is often essential for growth in serum free conditions. The transition from serum to serum free culture requires an adaptation anywhere from 2 weeks up to several months, depending on the severity of the change and the cell line in question. Only a short term adaptation (1-2 weeks) and a medium term adaptation (8 weeks) are considered in this study due to the instability of the two cell lines examined.

Chapter 3: Materials and Methods

3.1 Introduction

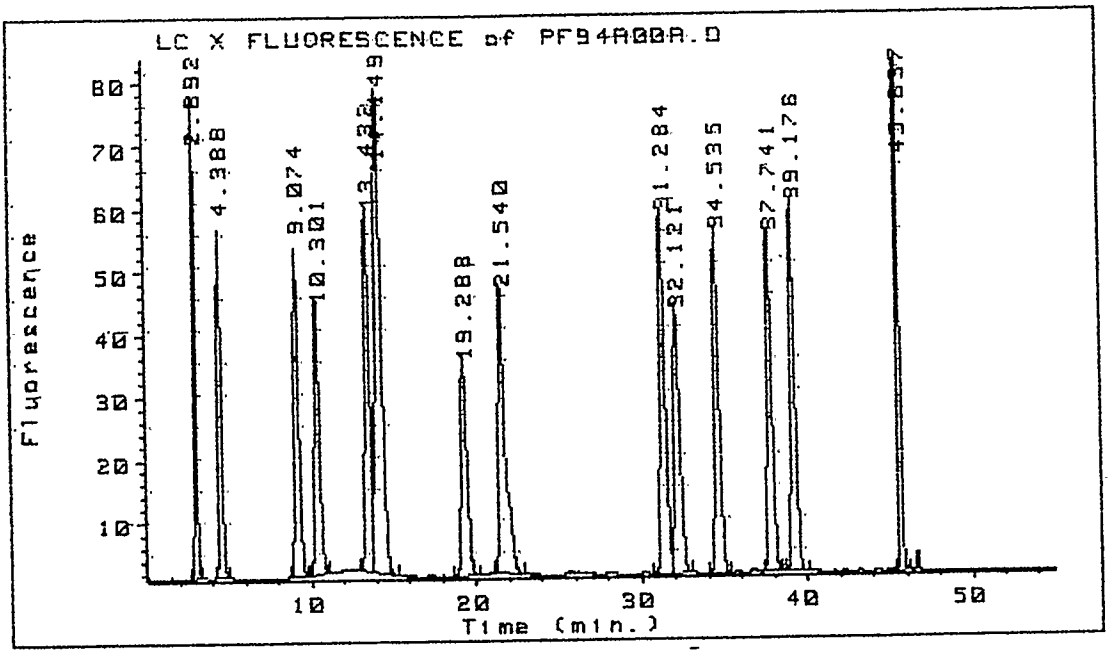
The following sections describe the analytical techniques used at our PPRF laboratory at the University of Calgary to determine the cell density, amino acid concentrations, cell cycle distribution, antibody production, glucose and lactate concentrations, ammonia production and LDH activity of a cell culture. Measurements were made in duplicate when possible.

3.2 Cell Density

The cell density and viability was measured by the trypan blue exclusion method. The trypan blue stain (cat no. T-8154) obtained from Sigma was mixed in a 1:1 ratio with a cell culture sample, and the cells were then counted using a haemocytometer. Fresh suspension samples were centrifuged at 3000 rpm for 5 min and then stored at -20 ° C for future analysis. Cell counts were done before freezing and immediately after the sample was taken from the tissue culture flask.

3.3 Amino Acid Concentrations

Amino acid analysis was performed by measuring the absorbance at 340 nm of orthophthaldialdehyde (OPA) derivatives after separation on a reverse phase HPLC column (Forestell, 1991) obtained from Supelco Inc. The amino acids proline, cysteine, and hydroxyproline do not form fluorescent derivatives with OPA and were not included in our analysis. A Hewlett-Packard 1090 liquid chromatograph connected to a computer work station was used to detect and record the fluorescent intensity of each amino acid. Standard curves were generated using amino acid standard solutions (Sigma) at various dilutions. The standard curves were then used to determine the concentrations of the amino acids. A typical HPLC output is shown on the following page, along with the residence times for various amino acids. The residence time refers to the time it takes for a particular amino acid to make its way through the column. The residence time distribution of each separate amino acid is fairly ideal.



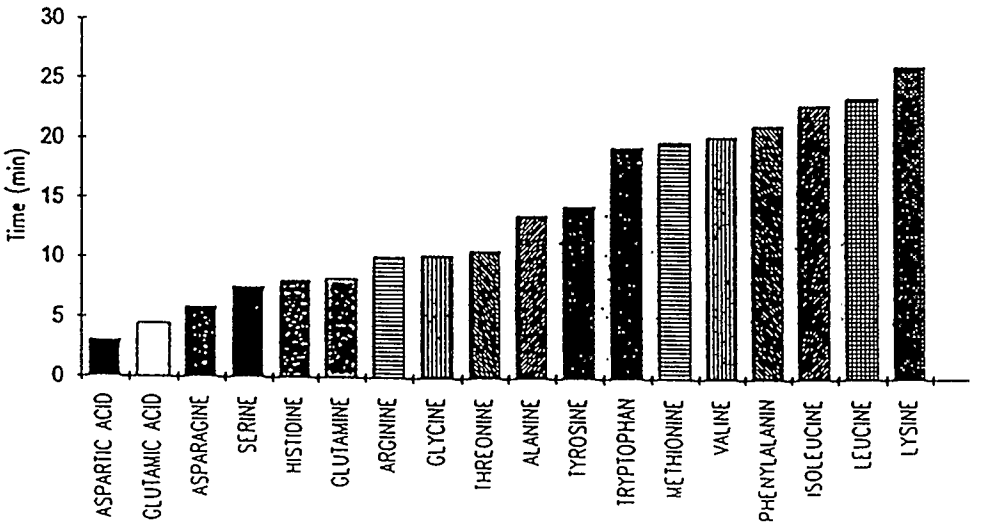


Figure 3.1: A typical HPLC peak analysis for amino acid determination at PPRF. Residence times for various amino acids, are shown in the bar graph (Farrell, 1993).

3.4 Cell Cycle Distributions

Our observations on cell cycles were achieved using a Becton-Dickinson Facscan flow cytometer equipped with a 488 nm argon laser. A cell culture sample was placed in the flow cytometer and the instrument measured the forward and right angle scatter. The forward angle scatter is a measure of the cell's size, whereas the right angle scatter relates to the internal complexity of the cell (granularity). Acridine orange is used to stain RNA and DNA of the cell. RNA and DNA emit red and green light respectively when excited by the laser beam. By using the flow cytometer and measuring the cell size, internal complexity, RNA content, and DNA content the different phases of the cell cycle can be determined.

3.5 Antibody Production

Antibody concentrations were determined using an enzyme linked immuno-sorbent assay (ELISA). The same assay procedure was used for each cell line, the only differences were that different coating agents, antibodies, and conjugated antibodies were used for each cell line.

For the murine hybridoma (M174) cell line, Anti-idiotype 174 (an antibody specific to the IgG antibody produced by the hybridoma cell line obtained from Biomira Inc, Edmonton.) was diluted to 3 μg/mL in cold carbonate buffer and then used to coat a 96 well plate (50 μL per well). Idiotype refers to the sum of all the antigenic determinants on a immunoglobulin molecule. The plate is then incubated overnight at 4°C. Samples diluted at a 1:7000 ratio were loaded on the plate, and incubated for one hour at 37°C in a water saturated (humidity = 100 %) incubator. Finally, the conjugated antibody (alkaline phosphatase anti-mouse IgG obtained from Jackson Immuno Research Laboratories Inc.) was added to detect the murine antibody. The substrate PNPP (para nitro phenyl phosphate) was added in the last step and the plate was incubated at 37°C for 30 minutes. The absorbance was then measured using a spectrophotometer at a wavelength of 405nm.

The myeloma cell line protocol used Anti-human IgG_3 as a coating agent and alkaline phosphatase anti-human IgG_3 to detect the chimeric antibody. More complete details on the ELISA for both cell lines can be found in the standard operating procedure obtained from Biomira Inc.

3.6 Measurement of Glucose, Lactate and Ammonia

An enzymatic YSI Model 2000 Analyzer (Yellow Springs Instruments) was used to determine the D-glucose and L-lactate concentrations. A probe (Orion Research) was used to measure the ammonia concentration using a Fischer Scientific Ion Selective Meter.

3.7 LDH Activity

The LDH (Lactate Dehydrogenase) activity was measured using an LDH Optimized Lactate-Dehydrogenase Kit obtained from Sigma Diagnostics. The enzyme activity was measured based on the "optimized standard method" recommended by the German Society for Clinical Chemistry. LDH activity is commonly used as a measure of cell lysis occurring in a cell culture. The lysis mainly occurs due to the shear in stirred bioreactors caused by the impeller. Polymers such as Pluronic F68 can be used to reduce the shear damage to cells by increasing the medium viscosity.

3.8 Calculation of Specific Rates

The equations used in calculating the specific uptake and productions rates are as follows

(i) Maximum Specific Growth Rate (μ_{max}, h^{-1}) and Doubling Time (t_d, d) during the exponential phase $[t_1, t_2] \Rightarrow \therefore \mu(s) = \mu_{max}$ where,

$$\frac{dX_v}{dt} = \mu_{max} X_v \quad \Longrightarrow \quad t_d = \frac{\ln(2)}{\mu_{max}}$$

μ_{max} can be determined using the following equation using linear regression,

$$\ln(X_v) - \ln(X_{vo}) = \mu_{max} t$$

where, X_v = viable cell concentration at end of exponential phase (cells/mL).

X_{vo}= initial viable cell density at start of exponential phase (cells/mL).

(ii) Average Specific MAb productivity q_{MAb}, μg/(cell·h) during the exponential phase $[t_1, t_2]$

$$\frac{d[MAb]}{dt} = q_{MAb} X_v \quad \Longrightarrow \quad \Delta[MAb] = \overline{q}_{MAb} \int_{t_1}^{t_2} X_v dt$$

where, [MAb] = monoclonal antibody concentration (mg/L)

Note: q_{MAb} can be obtained as the slope in a linear plot of $[MAb]_t$ vs $\int X_v dt$ with limits t_1 to t_2.

(iii) Average Specific Substrate Uptake Rates [q_s , μg/(cell·hr)]

$$\frac{dS}{dt} = q_S X_v \quad \Longrightarrow \quad \Delta[S] = \overline{q}_S \int_{t_1}^{t_2} X_v dt$$

where, [S] = concentration of substrate (μg/mL)

Note: q_s can be obtained as the slope in a linear plot of $[MAb]_t$ vs $\int X_v dt$ with limits t_1 to t_2.

3.9 Preparation of the proposed Serum Free Formulation (BITES)

The word **BITES** refers to **B** (bovine serum albumin - oleic acid complex), **I** (insulin), **T** (transferrin), **E** (ethanolamine), and **S** (selenium) which comprise the base components in all serum free formulations currently used for mammalian cells. The preparation of

the serum free formulation as stock solutions is as follows. All substances listed below were purchased from Sigma Chemical Co. unless otherwise noted.

1. BSA-Oleic acid complex (B) - Slowly add 1.0 μL freshly diluted oleic acid (20.0 mg/mL in ethanol) obtained from Sigma Chemicals (catalogue # L1012) per mL of fatty acid free BSA (50 mg/mL in PBS; catalogue # A8551). The mixture is then complexed overnight at 4°C. The following day sterile filter and store solution at 4°C.

2. Insulin (I) - Add 10.0 mL of distilled water followed by a 100.0 μL of glacial acetic acid to 100.0 mg of insulin powder (catalogue # I6634). Sterile filter and store at 4°C.

3. Transferrin (T) - Add 20.0 mL of sterile culture medium to 60.0 mg of transferrin (catalogue # T1147), rotating gently to dissolve. Sterile filter and store at 4°C.

4. Ethanolamine (E) - Add 40.0 mL of sterile culture medium to 24.0 μL of ethanolamine (catalogue # E0135) to make a concentration of 10 mmol/L . Sterile filter and store at 4°C.

5. Selenium (S) - Dissolve 6.92 mg of sodium selenite powder (catalogue # S5261) in 40 mL of sterile culture medium to make 1.0 mmol/L solution. The stock concentration of 1.0 μmol/L is made by diluting this solution 1000 times. Sterile filter and store at 4°C.

3.10 Other Chemicals Required

Other chemicals which were used during the course of the experimental program include,

1. 2-Mercaptoethanol - Add to sterile culture medium.

2. Cholesterol - Add 10.0 mg of cholesterol powder (catalogue # C3045) per mL of 100 % ethanol then sterile filter and store at 4°C.

3. Trace Elements - dissolve contents of a purchased bottle trace elements (Sigma Chemical Co.) in 10.0 mL of sterile culture medium to make a 100X concentration solution. Sterile filter and store at 4°C.

4. ICN Low Density Lipoprotein - dilute 1:1 in PBS to make a 25.0 mg/mL solution. Sterile filter and store at 4°C (catalogue # 152429). Dilution in PBS facilitates the filtering of the lipoprotein emulsion. Excite lipoprotein (catalogue # 81-129-1) can also be used as the lipoprotein source for the myeloma cell line. No dilution prior to filtering is required for the Excite lipoprotein.

5. Chitosan/Locust Bean Gum/Citric Pectin - dissolve 10.0 mg of the polysaccharide in 10 mL of 0.1 N HCl solution to prepare a 1mg/mL stock solution. Dilute the stock solution in PBS to make the desired concentration. Sterile filter and store at 4°C. The respective catalogue numbers are C0792, G0753, P9135 for the chemicals as listed above.

6. Dexamethasone - add 1.0 mL absolute ethanol to 1.0 mg of dexamethasone (catalogue # D2915). Swirl gently to dissolve. Add 49.0 mL of sterile culture medium to prepare a 20.0 μg/mL stock solution. Sterile filter and store at 4°C.

7. Prostanglandin E_1 - add 1.0 mL absolute ethanol to 1mg of prostaglandin E_1 (catalogue # P8908). Swirl gently to dissolve. Add 19.0 mL of sterile culture medium to prepare a 50.0 μg/mL stock solution. Sterile filter and store at 4°C.

8. Interleukins - dilute the concentrated interleukin (10000 U/mL) at 1-10 % (v/v) in PBS. The catalogue numbers are 1271-164, 1468-138, 1444-581 for interleukin-2, interleukin-4, and interleukin-6 respectively.

Chapter 4: Basal Media Selection

4.1 Media Comparison

One of the most important decisions to be made for this project was to choose a basal medium for each of the cell lines being studied. The medium should provide enough nutrients for the cells to grow well in serum free conditions. Since each cell line is unique and behaves differently experiments must be performed to find a suitable basal media. All basal media used in subsequent experiments were purchased from Gibco BRL Ltd. in powder form. Table 4.1 shows a comparison of RPMI-1640, DMEM, IMDM, and Ham's F12 on a component by component basis. The table shows that there are some ingredients in F12 that are not found in the other formulations. These include fatty acids (linoleic acid, and lipoic acid), some inorganic salts, and some amino acids (l-cysteine). The table also shows that DMEM and RPMI-1640 are very similar in composition, this being the case RPMI-1640 was eliminated from the basal media selection tests.

4.2 Media Tested

The following basal media and combinations thereof were considered in the development of a serum free medium. These media were chosen since they all have been used in previous serum free media developments that have been published.

1. DMEM - typical media used in animal cell culture.
2. Ham's F12 - lipid rich media recommended for serum free media development
3. IMDM - contains HEPES for greater buffering capacity and has been recommended for use in serum free medium development.
4. RPMI-1640 - Similar to DMEM but with less buffering capacity and thus no testing was done with this medium (not considered)

Experiments were conducted comparing the growth and antibody production of the two

Table 4.1 - Comparison of Selected Commercially Available Basal Media Formulations

COMPONENTS	RPMI-1640	DMEM	IMDM	F-12
Inorganic Salts (mg/L)				
$CaCl_2$ (anhydrous)	0.0	200.0	165.00	33.22
$Ca(NO_3)_2.4H_2O$	100.00	0.0	0.0	0.0
$CuSO_4.5H_2O$	0.0	0.0	0.0	.0025
$Fe(NO_3)_3.9H_2O$	0.0	0.1	0.0	0.0
$FeSO_4.7H_2O$	0.0	0.0	0.0	0.834
KCl	400.0	400.0	330.0	223.6
$MgCl_2$ (anhydrous)	0.0	0.0	0.0	57.22
$MgSO_4$ (anhydrous)	48.84	97.67	97.67	0.0
$MgSO_4.7H_2O$	0.0	0.0	0.0	0.0
NaCl	6000.0	6400.0	4505.0	7599.0
$NaHCO_3$	2500.0	3700.0	3024.0	1176.0
Na_2HPO_4	800.0	0.0	0.0	142.04
NaH_2PO_4 (anhydrous)	0.0	109.00	0.0	0.0
$NaH_2PO_4.H_2O$	0.0	0.0	125.0	0.0
$Na_2SeO_3.5H_2O$	0.0	0.0	0.0173	0.0
$ZnSO_4.7H_2O$	0.0	0.0	0.0	0.863
Vitamins (mg/L)				
p-Aminobenzoic Acid	1.00	0.0	0.0	0.0
d-Biotin	0.2	0.0	0.013	0.0073
d-Ca Pantothenate	0.25	4.0	4.0	0.48
Choline Chloride	3.0	4.0	4.0	13.96
Folic Acid	1.0	4.0	4.0	1.3
i-Inositol	35.0	7.20	7.20	18.00

Table 4.1 (continued)

COMPONENTS	RPMI-1640	DMEM	IMDM	F-12
Niacinamide	1.0	4.0	4.0	0.037
Pyridoxal.HCl	0.0	4.0	4.0	0.0
Pyridoxine.HCl	1.0	0.0	0.0	0.062
Riboflavin	0.20	0.40	0.40	0.038
Thiamine.HCl	1.0	4.0	4.0	0.34
Vitamin B_{12}	0.005	0.0	0.013	1.36
Other Components (mg/L)				
Glutathione	1.0	0.0	0.0	0.0
d-Glucose	2000.00	1000.0	4500.0	1802.0
Hypoxanthine (Sodium Salt)	0.0	0.0	0.0	4.77
Linoleic Acid	0.0	0.0	0.0	0.084
Lipoic Acid	0.0	0.0	0.0	0.21
Phenol Red	5.0	16.0	15.0	1.20
Putrescine.HCl	0.0	0.0	0.0	0.161
Sodium Pyruvate	0.0	110.0	110.0	110.0
HEPES	0.0	0.0	5958.0	0.0
Thymidine	0.0	0.0	0.0	0.73
Amino Acids (mg/L)				
l-Alanine	0.0	0.0	25.0	8.90
l-Arginine (Free Base) l- Arginine.HCl	200.0 0.0	0 .0 84.0	0.0 84.0	0.0 211.0
l-Asparagine l-Asparagine.H_2O	50.0 0.0	0.0 0.0	25.0 0.0	0.0 15.01
l-Aspartic Acid	20.0	0.0	30.0	13.30
l-Cysteine l-Cysteine.HCl	0.0 0.0	0.0 0.0	0.0 0.0	0.0 35.12
l-Cystein.2HCl	65.0	62.57	91.24	0.0
l-Glutamic Acid	20.0	0.0	75.0	14.70
l-Glutamine	300.0	584.0	584.0	146.0
Glycine	10.0	30.0	30.0	7.50

Table 4.1 (continued)

COMPONENTS	RPMI-1640	DMEM	IMDM	F-12
l-Histidine (Free Base) l-Histidine.HCl.H_2O	15.0 0.0	0.0 42.0	0.0 42.0	0.0 20.96
l-Isoleucine	50.0	105.0	105.0	3.94
l-Leucine	50.0	105.0	105.0	13.10
l-Lysine.HCl	40.0	146.0	146.0	36.50
l-Methionine	15.0	30.0	30.0	4.48
l-Phenylalanine	15.0	66.0	66.0	4.96
l-Proline	20.0	0.0	40.0	34.50
l-Hydroxyproline	20.0	0.0	0.0	0.0
l-Serine	30.0	42.0	42.0	10.50
l-Threonine	20.0	95.0	95.0	11.90
l-Tryptophan	5.0	16.0	16.0	2.04
l-Tyrosine (disodium salt)	28.83	103.79	103.79	7.78
l-Valine	20.0	94.0	94.0	11.70

Experiments were conducted comparing the growth and antibody production of the two cell lines using the basal media combinations shown in Table 4.2. By using basal media combinations it is hoped to achieve a better nutrient balance which will result in improved cell growth and antibody production. The basal media were supplemented with Nutridoma NS, a commercially available serum free medium manufactured by Boehringer Mannheim for use with murine myelomas, hybridomas or lymphocytes. This supplement (currently considered the best SFM formulation on the market) was used in order to choose the basal medium under serum-free conditions.

4.3 Experimental Setup

The following protocol was used in the experiment:

1. The cells were subcultured for 2 weeks on different basal media supplemented with 2 % and 3 % Nutridoma NS and no serum for the myeloma and hybridoma cells respectively. The recommended Nutridoma NS concentration is 1 % (protein content < 150 mg/L), however due to poor growth of the cells we adjusted the concentration. It should be noted that Nutridoma NS was being used at Biomira at levels of 1.75 % for these cell lines, which is also above recommended levels.
2. The cells were transferred into 150 mL Corning spinner flasks in an incubator with a 5 % CO_2 atmosphere for 3 days to generate an inoculum for the experiment.
3. Cells were inoculated into spinner flasks at 4 x 10^5 cells/mL.
4. Samples were taken every 12 hours and frozen down for ELISA assay when cell counts were performed.

4.4 Results

The growth rate, viability, and antibody production for the hybridoma (M174) cell line is shown in Figure 4.1. The graphs show that IF produced the highest cell density (approximately 2.5x10^6 cells/mL) and MAb levels (approximately 40 mg/L) of the media tested. However the viability of the hybridoma cells in the IF medium was quite low

Table 4.2 - Basal Media Combinations

Media Combinations	Symbol	Relative Costs
DMEM	D	1.66 $/L
IMDM	I	4.70 $/L
F12	F	1.93 $/L
IMDM:F12 (1:1)	IF	3.32 $/L
DMEM:F12 (1:1)	DF	1.80 $/L
DMEM:IMDM:F12 (1:1:1)	DIF	2.76 $/L

Note: The glucose and glutamine levels of all the media combinations above were equalized to 4.5 g/L and 4 mM to match concentrations in DMEM.

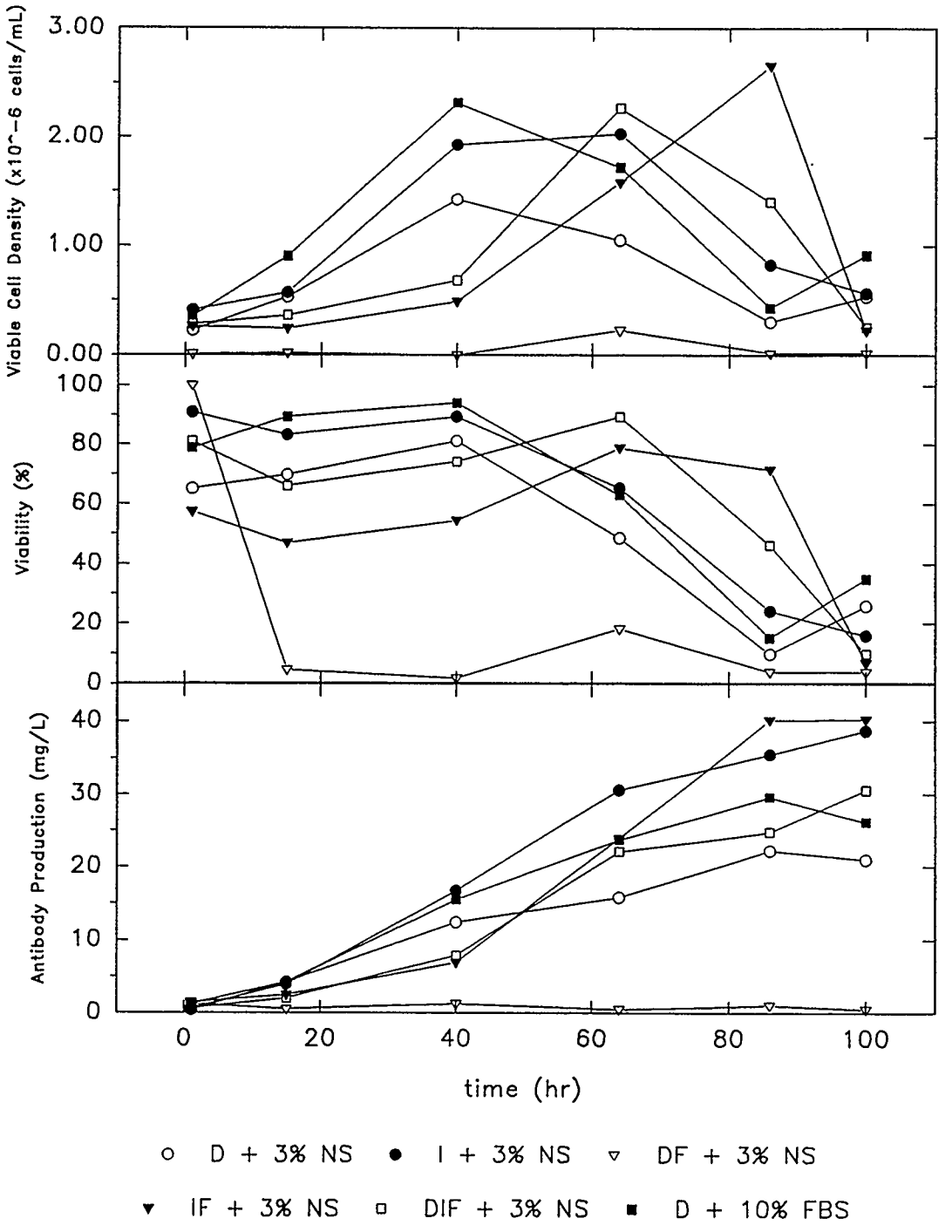


Figure 4.1: Viable Cell Density, Viability, and Mab production for the hybridoma cell line in the basal media selection experiment. The experiment was performed in 150 mL Corning spinner flasks in an incubator with a water saturated atmosphere and 5 % CO_2.

starting at about 60 % and reaching a maximum of 80 %. The IMDM and DIF media (having viabilities in the range of 80 %) performed better in terms of viability than the IF medium and almost as well in terms of antibody production and viable cell density. The DF and DMEM media performed the poorest in this experiment. No curves are shown for the Ham's F12 medium because the cells could not survive in the medium when the inoculum was generated. It is also important to note that during subculturing to generate inoculum the DIF medium performed the best in terms of growth and viability (i.e. the cells recovered better when subcultured on DIF medium). Based on this information DIF was chosen as the basal medium to be used in the serum free medium development.

The growth rate, viability and antibody production for the myeloma cell line are shown in Figure 4.2. As before, we observed that subculturing and response to stress was much better in the DIF basal medium, and moreover the cells could not be passaged at all on F12 alone. The graphs show that there was good growth and viability in all media, with the DIF and IMDM basal media providing the highest MAb levels (approximately 60 mg/L). DIF was again chosen as the best basal medium because it gave good growth, viability, high antibody production, and facilitated cell subculturing. Another advantage in using the same basal medium for both cell lines is reduced preparation time.

4.5 Conclusions

The major conclusions arising from the basal media experiments are:

- The growth, antibody production, and viability for the hybridoma cells (M174) in the DIF medium were almost identical to the IF medium which performed the best.
- Good growth and viability was observed in all medium for the myeloma cell line (C174), with DIF giving the highest antibody titers along with IMDM.
- Subculturing the hybridoma and myeloma cells was easiest using the DIF basal medium.

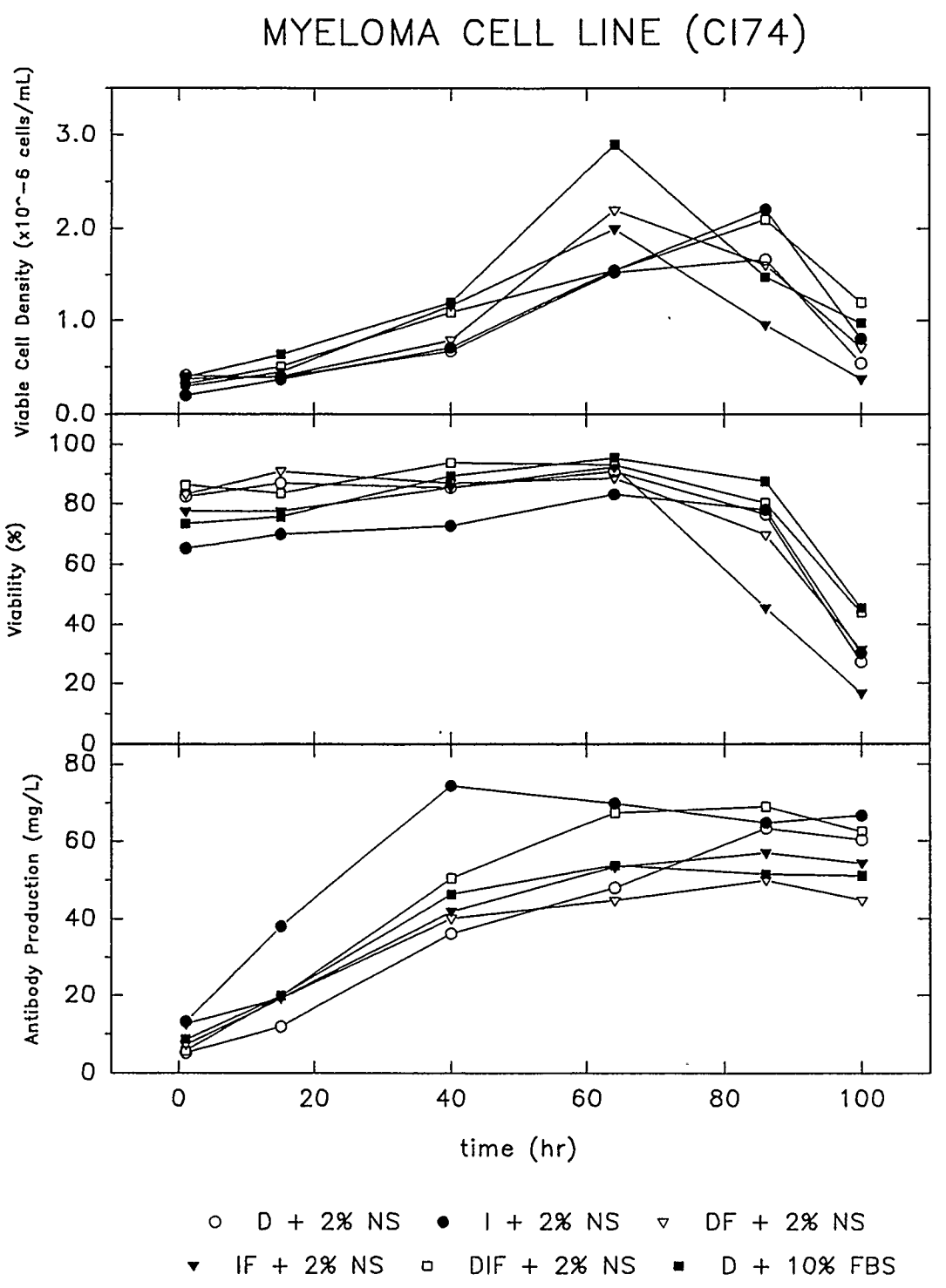


Figure 4.2: Viable Cell Density, Viability, and Mab production for the myeloma cell line in the basal media selection experiment. The experiment was performed in 150 mL Corning spinner flasks in an incubator with a water saturated atmosphere and 5 % CO_2.

- DIF was chosen as the basal medium for both cell lines.

Chapter 5: Cell Characterization

5.1 Introduction

This chapter details the important characteristics of the two cell lines. Growth rate, antibody production, and nutrient consumption are some of the aspects of the cells which were investigated. The importance of these experiments are that they develop a **baseline for comparing** the performance of the serum free medium developed. The most important comparison presented (chapter 10) is the one on an economic basis between commercially available media and our developed media. DIF (our selected basal medium comprised of DMEM, IMDM, and F12 in a 1:1:1 ratio) and DMEM both supplemented with 10 % FBS were used in these experiments. The DMEM represents the base case to which we will be making our comparison.

5.2 Experimental Setup

The experiment consisted of the following steps:

1. An inoculum was generated in 150 mL spinner flasks over a 3 day period.
2. Each cell line was inoculated into spinner flasks (at 4 x 10^5 cells/mL). One spinner flask contained DMEM + 10 % FBS and the other DIF + 10 % FBS.
3. Cell counts were performed periodically and samples were taken and frozen down for later analysis.

5.3 Results - Myeloma Cell Line (C174)

The viable cell density, viability, and antibody concentration for the myeloma cell line is shown in Figure 5.1. The graphs show that the maximum viable cell density reached in DMEM was 2.18 x 10^6 cells/mL and in DIF was 3.13 x 10^6 cells/mL. The major substrates (glucose and glutamine) were equalized in this experiment to the levels found in DMEM (4.5 g/L glucose and 4 mmol/L glutamine). One possible explanation for this large difference is that DIF provides a better nutrient base than does DMEM. Also of note is that there is significantly more glutamate (also a substrate) in the DIF medium

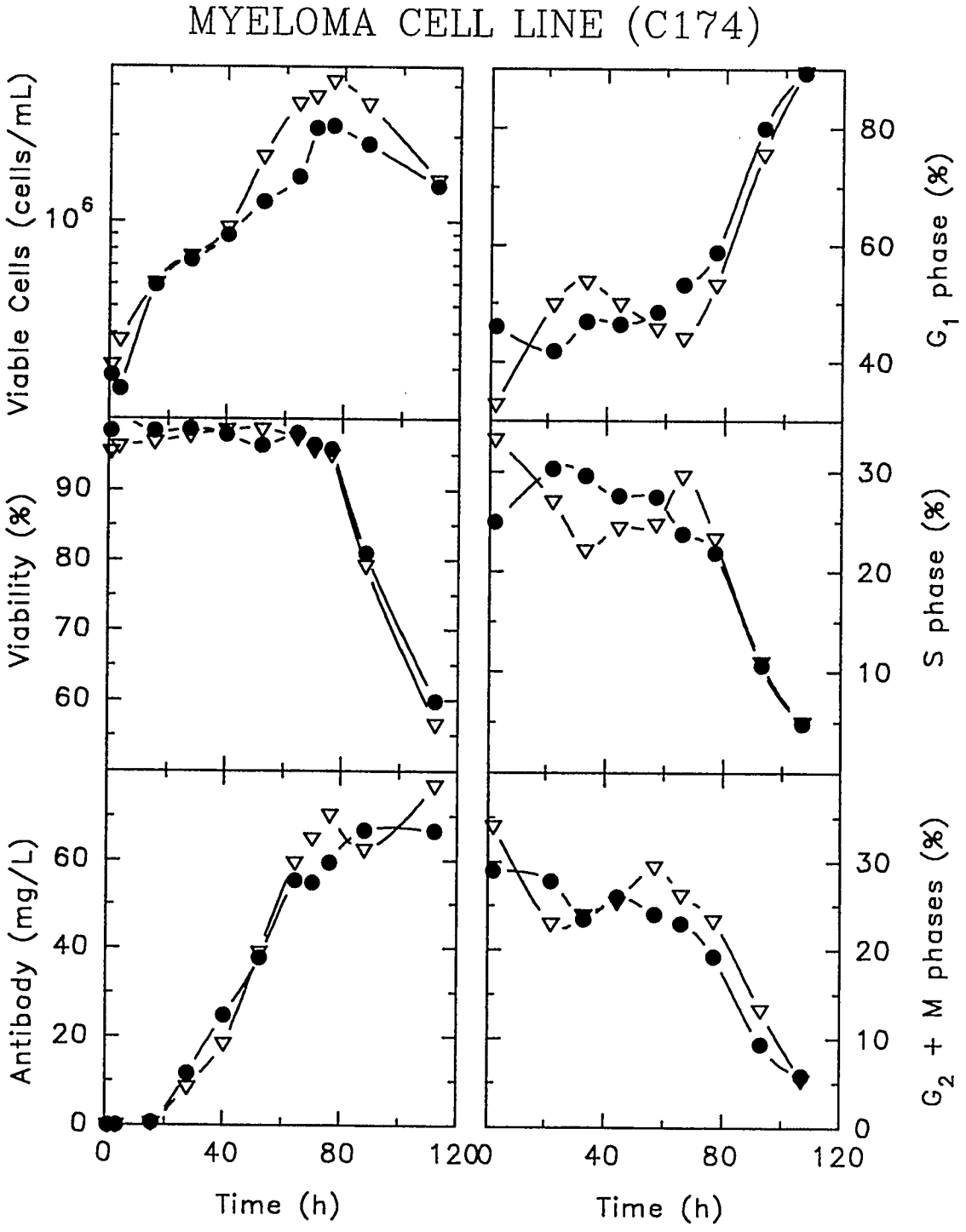


Figure 5.1: Various cell characteristics of the myeloma cell line (C174) obtained in two different media, namely DMEM (●) and DIF (▽) supplemented with 10 % FBS. Experiments were performed in 150 mL Corning spinner flasks in an incubator with a water saturated atmosphere and 5 % CO_2.

which is completely consumed by the myeloma cells and may account for the differences between the two media tested. The antibody concentration reached was about 70 mg/L for both the DIF and DMEM medium. The cell cycle distributions show as the cells reach the stationary phase more and more cells become arrested in the G_1 phase and consequently fewer cells are in the S and M phases.

Figure 5.2 shows the consumption of the major substrates (glucose and glutamine) and the production of the major metabolic toxins (ammonia and lactate). It is apparent that the depletion of glutamine is the limiting nutrient causing the death of the culture in both media. Therefore one could obviously increase the life of the culture and likely the final antibody concentration by supplementing the media with extra glutamine (or other substrates such as glutamate and glucose). This is normally a trial and error procedure to obtain the optimum concentration of glutamine and other components since effects such as overflow metabolism must be avoided. Overflow metabolism refers to the increased consumption of substrates such as glutamine and glucose (when added in excess), with little or no improved growth or antibody production. Generally with overflow metabolism there is an associated increase in metabolic byproducts such as lactate and ammonia. The production of lactate reaches a maximum of 2.6 g/L in DMEM and 2.2 g/L in DIF, and the ammonia concentration reaches a maximum of about 2.25 mmol/L for both media. The LDH activity was essentially zero for the batch experiment. Since the LDH is a measure of cell lysis this implies that no cell lysis occurred. Note that the LDH activity of the 10 % serum was subtracted from the LDH reading of the samples collected. Figure 5.2 also shows that a larger amount of aspartate should be added to the media since it is completely consumed by the myeloma cells in both media.

Figures 5.3, 5.4 and 5.5 show the concentration of some of the amino acids during the course of the batch run in 150 mL Corning spinner flasks for the two different media. Arginine/threonine and tryptophan/methionine were lumped together because their respective peaks could not be separated on the HPLC. Glutamate,

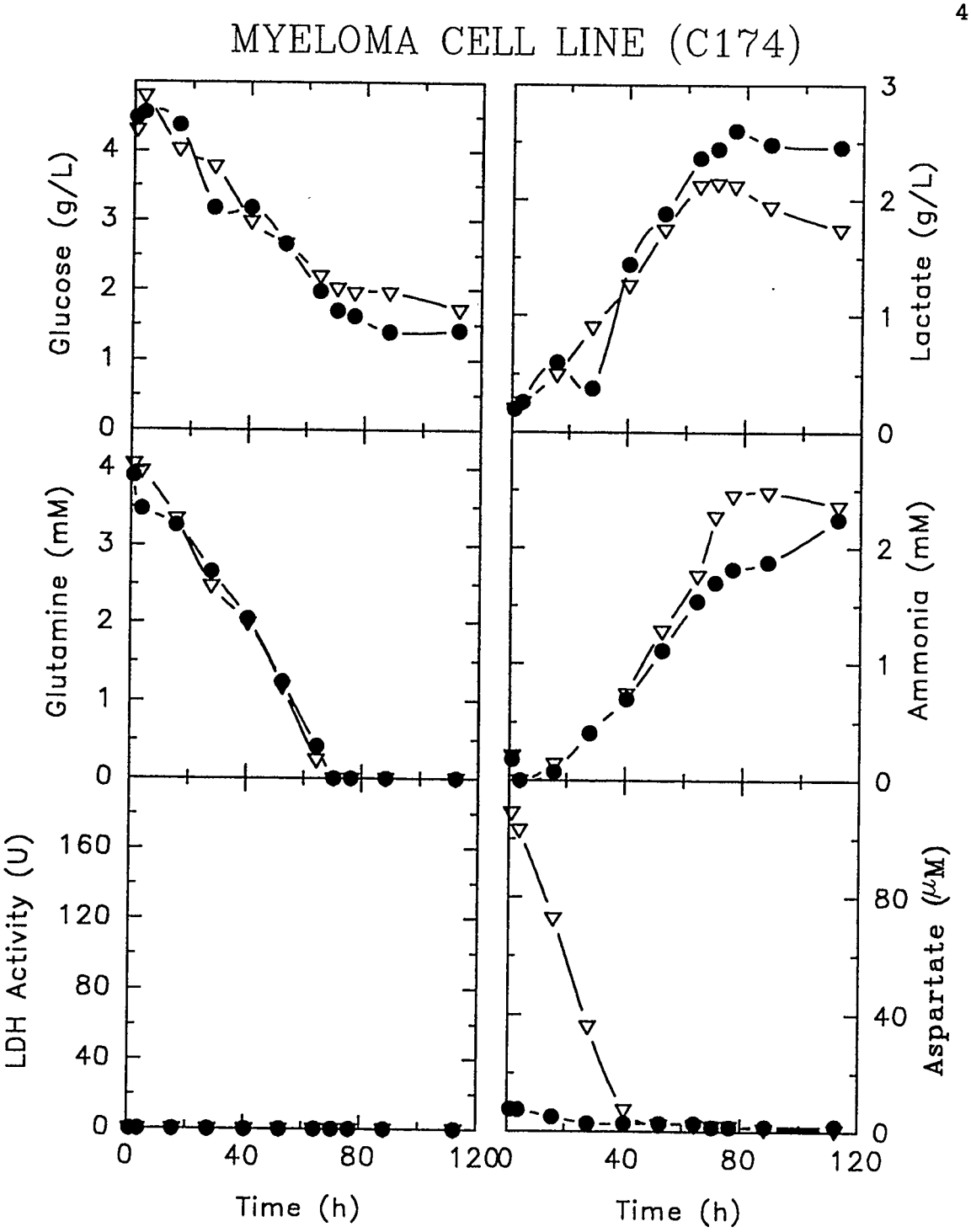


Figure 5.2: Glucose, glutamine, and aspartate consumption curves along with ammonia and lactate production curves for myeloma (C174) cell characterization experiment in DIF + 10% FBS (▽) and DMEM + 10% FBS (●). Also shown is the LDH activity showing the extent of cell lysis. Experiments were performed in an incubator with a water saturated atmosphere and 5 % CO_2.

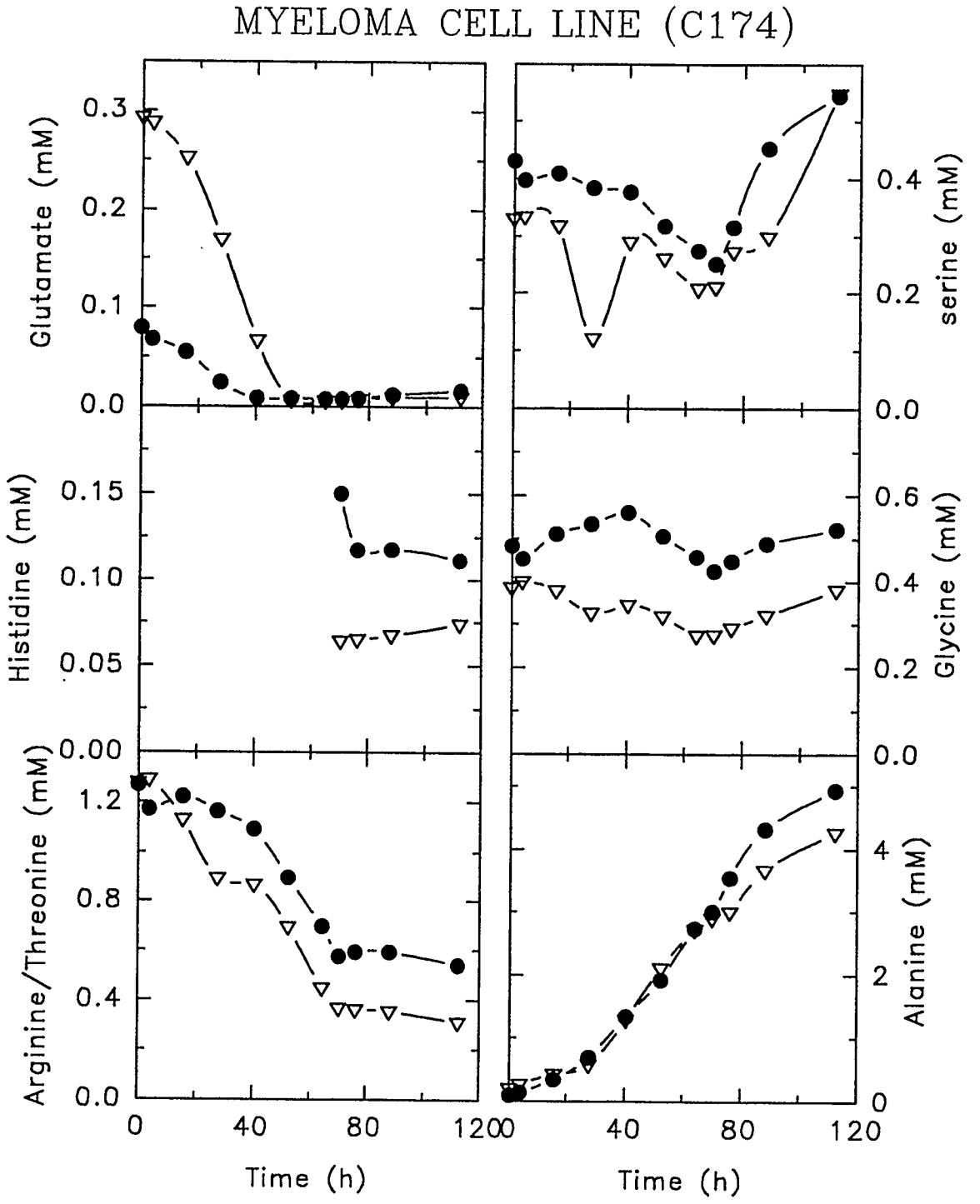


Figure 5.3: Various amino acid concentrations for myeloma (C174) cell characterization experiment in DIF + 10 % FBS (▽) and DMEM + 10 % FBS (●). Experiments were performed in 150 mL Corning spinner flasks in an incubator with a water saturated atmosphere and 5 % CO_2.

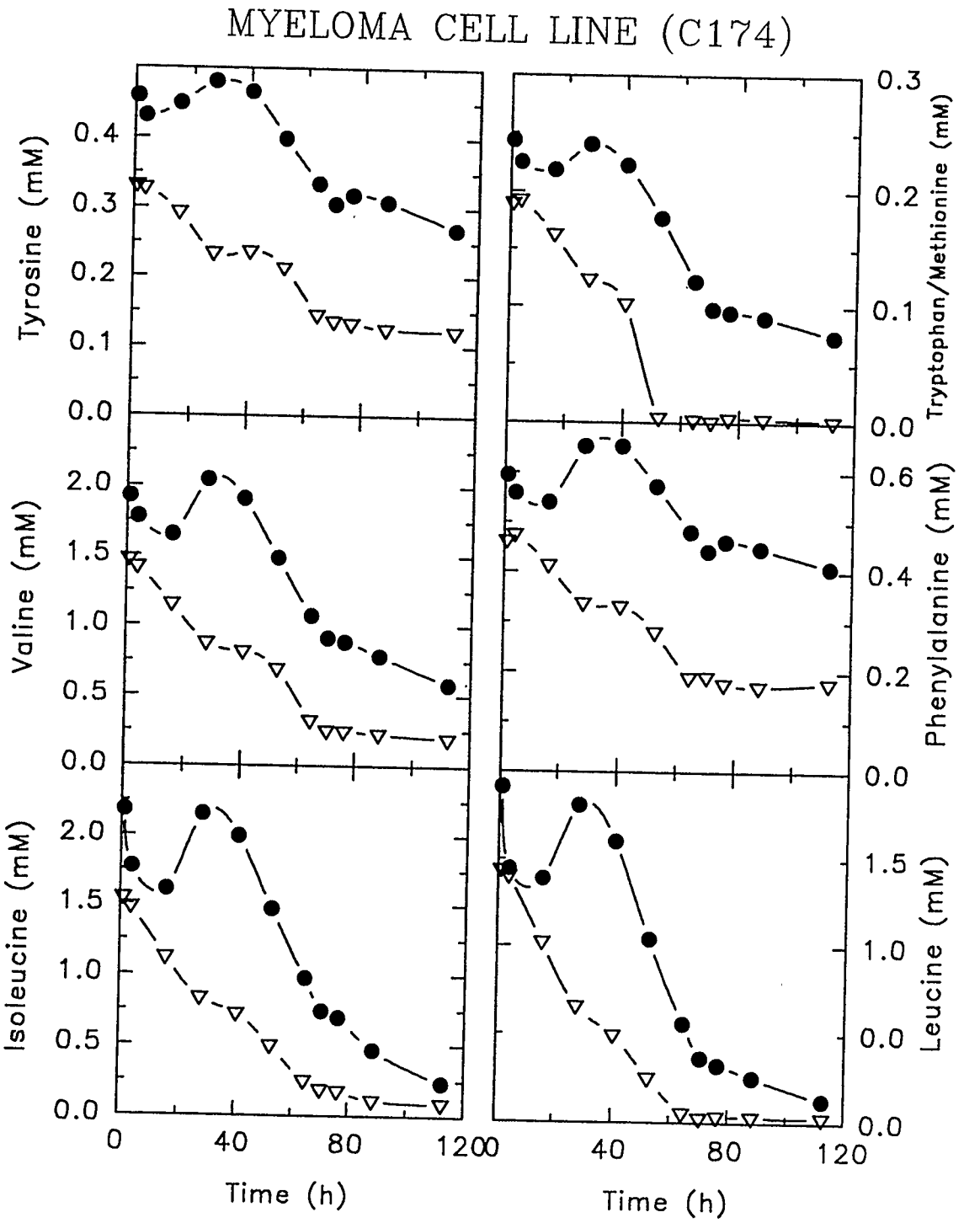


Figure 5.4: Various amino acid concentrations for myeloma (C174) cell characterization experiment in DIF + 10 % FBS (▽) and DMEM + 10 % FBS (●). Experiments were performed in 150 mL Corning spinner flasks in an incubator with a water saturated atmosphere and 5 % CO_2.

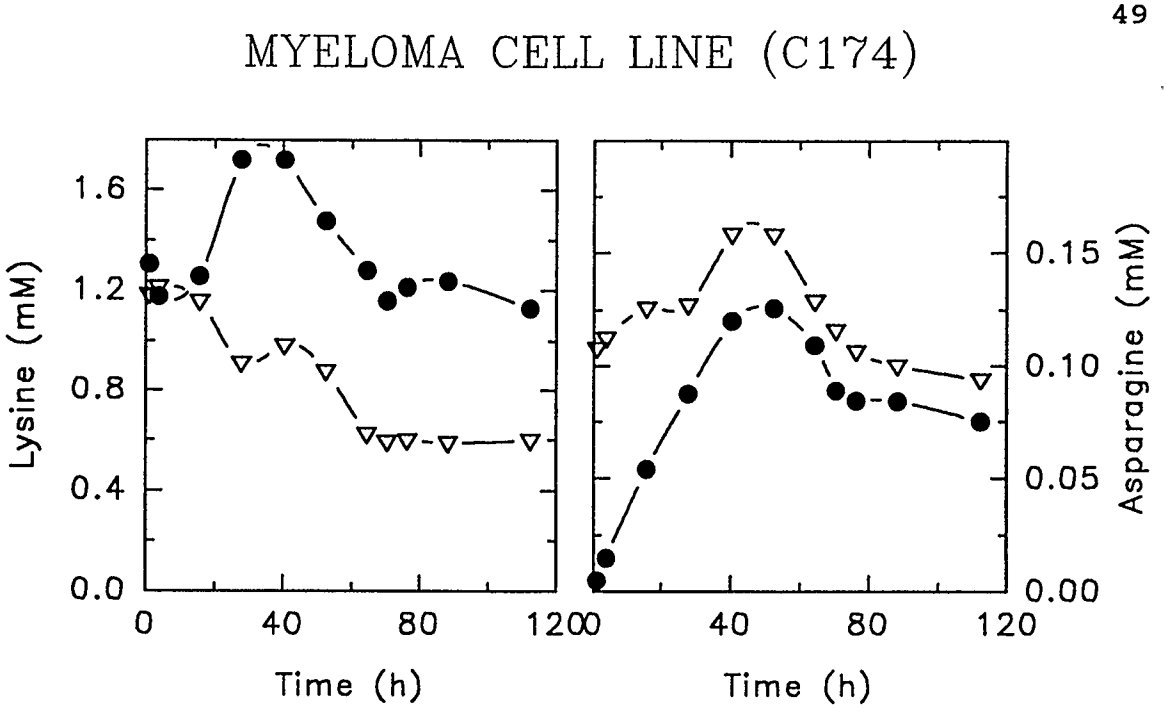


Figure 5.5: Lysine and aspargine concentrations for myeloma (C174) cell characterization experiment in DIF + 10 % FBS (▽) and DMEM + 10 % FBS (●). Experiments were performed in 150 mL Corning spinner flasks in an incubator with a water saturated atmosphere and 5 % CO_2.

tryptophan/methionine, leucine and isoleucine are completely consumed in both media, whereas other amino acids such as alanine and serine are produced. Therefore, the conclusion from these experiments is that these amino acids should be added to see if they have any effect on growth and antibody production. Other amino acids such as glycine, histadine, and lysine remain relatively constant. Due to the complex nature of cell culture biochemical reactions, very few conclusion can be drawn concerning fortification of amino acids to improve growth, the only option is to perform tedious factorial experiments to find the optimal media.

Figure 5.6 shows the calculation of specific growth rate, specific uptake and production rates, and some yield coefficients. The graphs show the relationships are fairly linear and thus average values were calculated using linear regression. The calculated values are summarized in Table 5.1. The table shows that the specific production of IgG_3 is slightly higher in the DMEM than in the DIF. However, the specific growth rate, final antibody concentration, and the maximum cell density is highest in the DIF medium. By comparing the doubling times we observe that the myeloma cells growing on DIF medium grow much faster. Overall the cells performed better in the DIF medium. There are two obvious reasons why this has occurred. The first is that the DIF medium clearly contains more glutamate and thus the cells have more substrate available, enabling the cells to grow to a higher cell density. The second reason is that the IMDM in the DIF medium contains HEPES buffer that can maintain the medium pH in the optimum region more effectively than sodium bicarbonate enabling the cell culture to generate more biomass. Overall DIF has a better nutrient base than DMEM and thus the cells perform better in the DIF medium.

5.4 Results - Hybridoma Cell Line (M174)

The results for the hybridoma cell line are shown in Figures 5.7 to 5.12. Figure 5.7 shows the antibody production, viability, viable cell density, and the cell cycle distribution during the batch characterization experiment. The maximum cell density reached was about 1.9×10^6 cells/mL and the corresponding antibody produced was

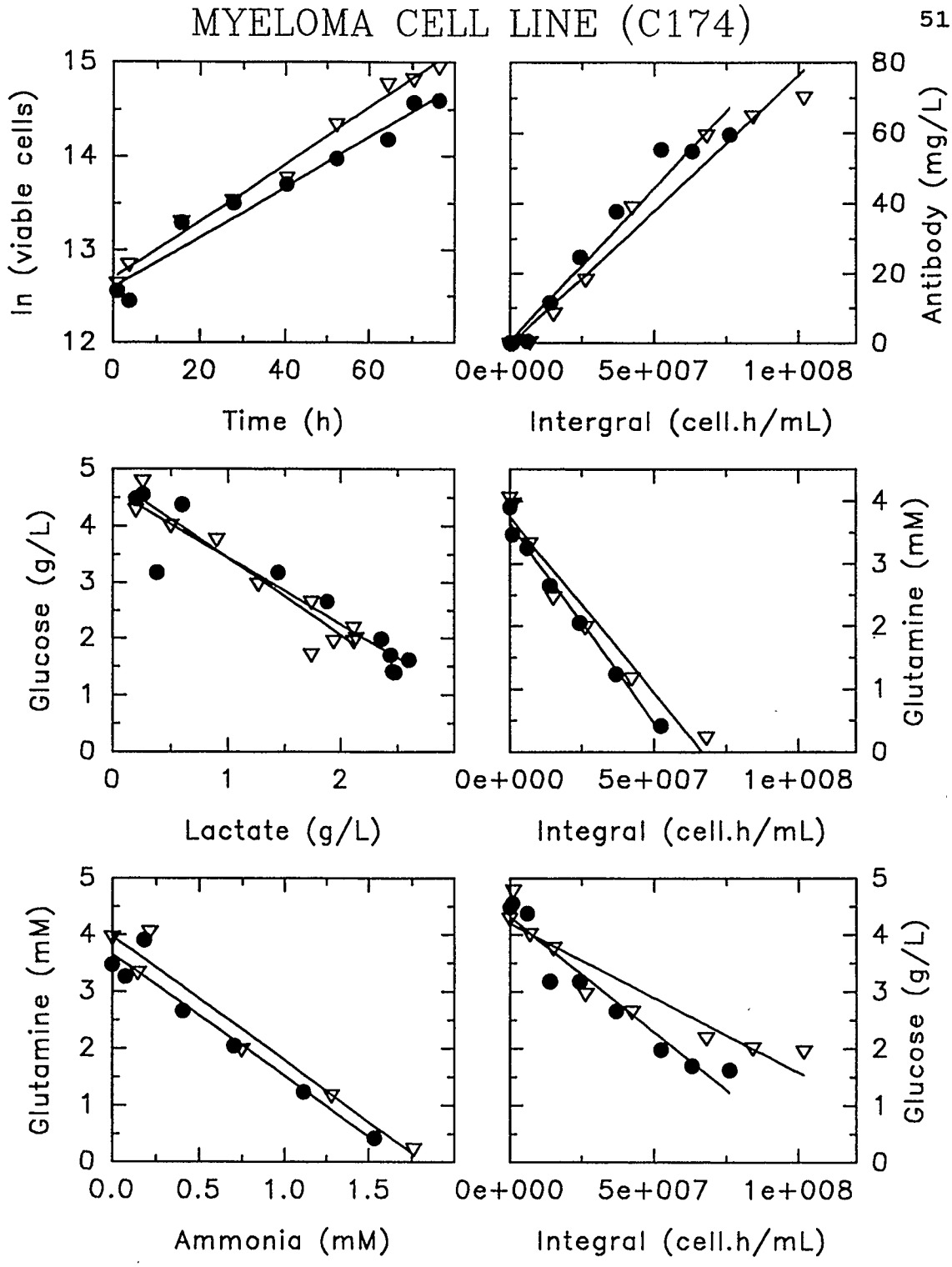


Figure 5.6: Batch data regressions required for determination of metabolic parameters for the myeloma (C174) cell line. Specific glutamine and glucose uptake, and antibody production rates were determined in the exponential growth phase. Symbols represent DIF + 10 % FBS (▽) and DMEM + 10 % FBS (●).

Table 5.1 - Myeloma Characteristics in Media supplemented with 10 % FBS Experiments Performed in 150 mL Corning Spinner Flasks

Parameter	DMEM	DIF
$X_{v\,max}$ (cells/mL)	2.18×10^{6}	3.13×10^{6}
μ_{max} (h^{-1})	0.0268	0.0304
t_d (h)	25.9	22.8
q_{gln} (mmol/(cell•h))	-6.37×10^{-11}	-5.62×10^{-11}
q_{glc} (g/(cell•h))	-4.06×10^{-11}	-2.62×10^{-11}
q_{MAb} (μg/(cell•h))	8.80×10^{-7}	7.70×10^{-7}
$Y_{amm/gln}$ (mmol/mmol)	0.46	0.46
$Y_{lac/glc}$ (g/g)	0.84	0.72

Note: X - cell density, μ - specific growth rate, q - specific production rate, Y - yield coefficient

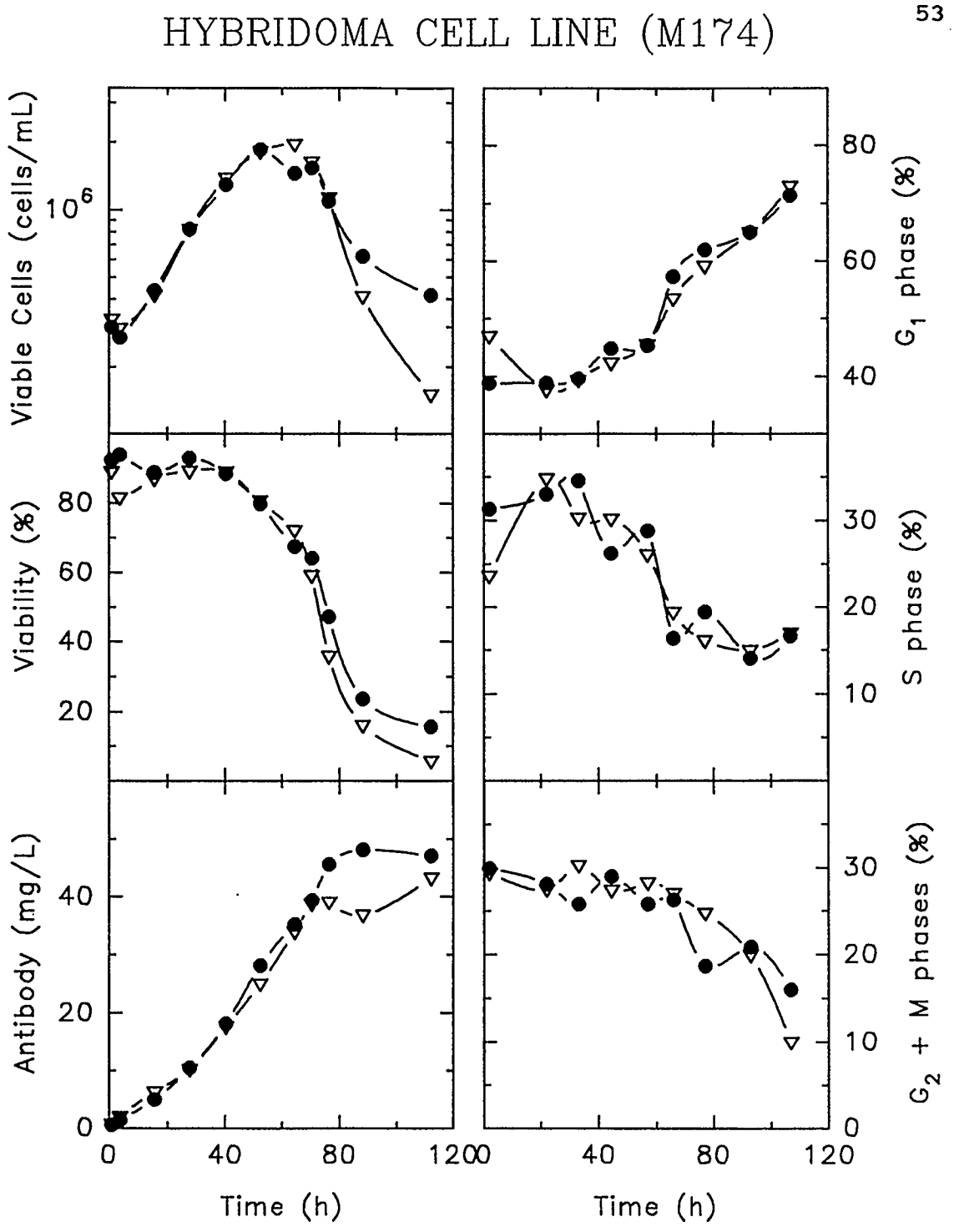


Figure 5.7: Various cell characteristics of the hybridoma cell line (M174) obtained in two different media, namely DMEM (●) and DIF (▽) both supplemented with 10 % FBS. Experiments were performed in 150 mL Corning spinner flasks in an incubator with a water saturated atmosphere and 5 % CO_2.

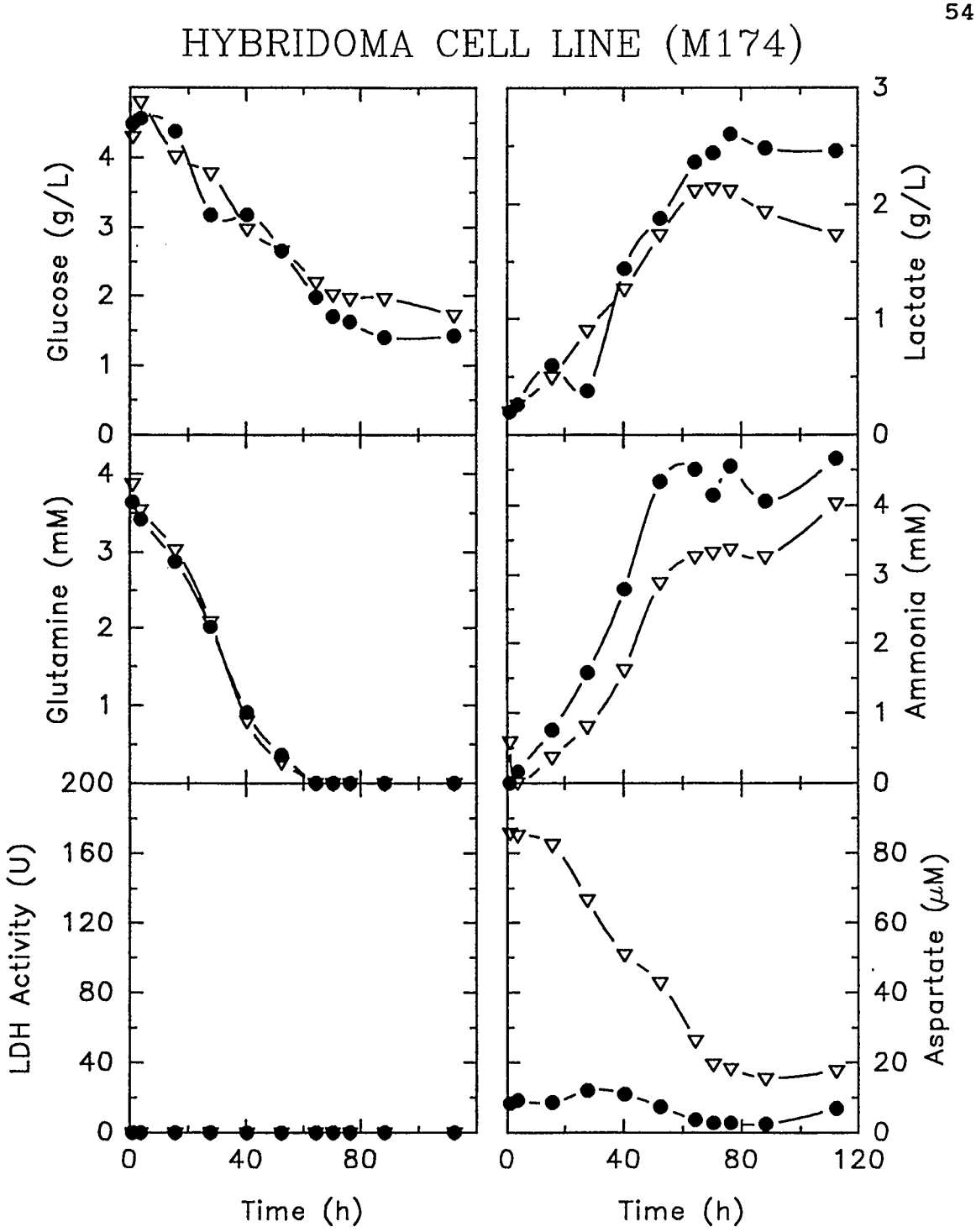


Figure 5.8: Glucose, glutamine, and aspartate consumption curves along with ammonia and lactate production curves for hybridoma (M174) cell characterization experiment in DIF + 10% FBS (▽) and DMEM + 10% FBS (●). Also shown is the LDH activity (extent of cell lysis). Experiments were performed in 150 mL Corning spinner flasks in an incubator with a water saturated atmosphere and 5 % CO_2.

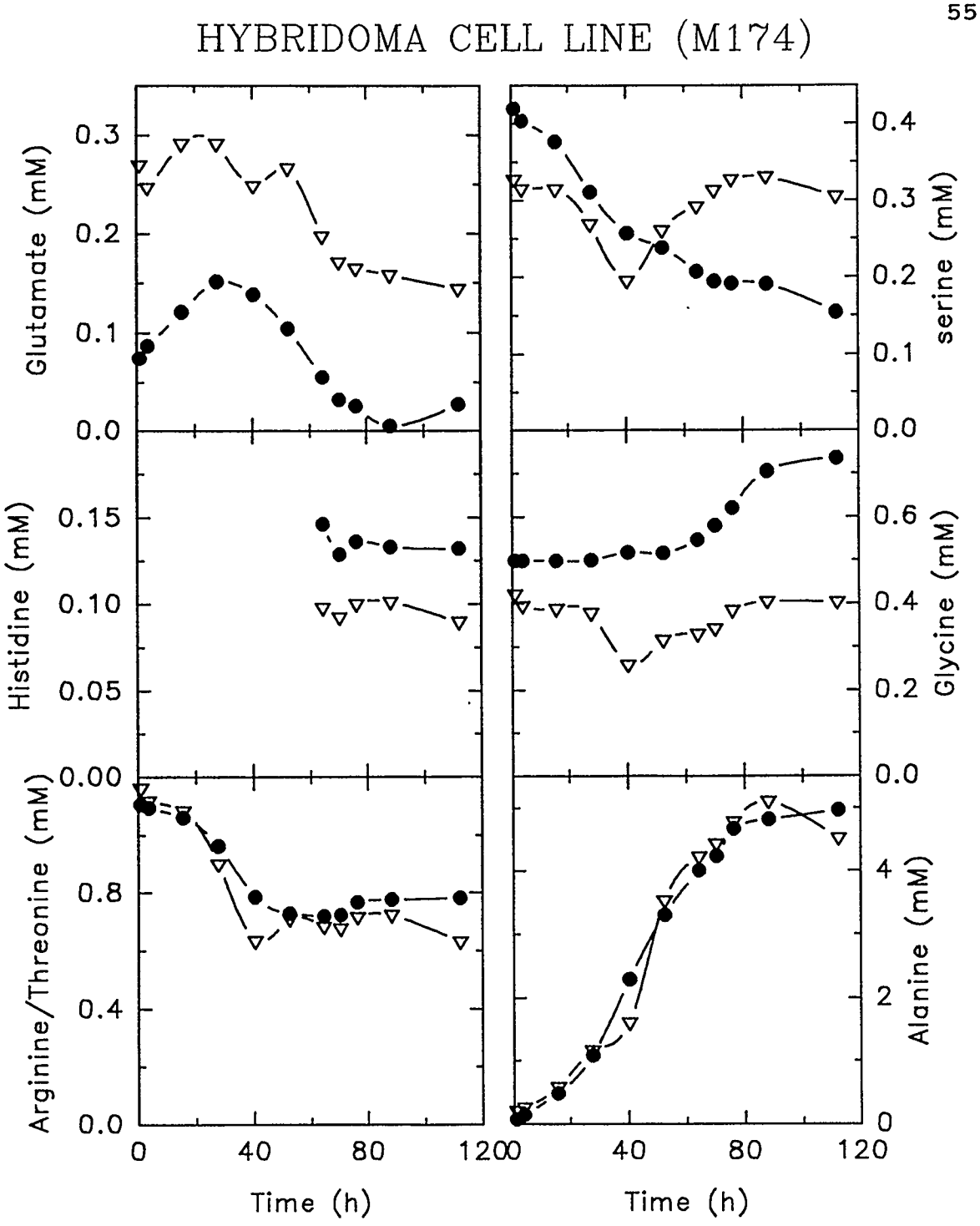


Figure 5.9: Various amino acid concentrations for hybridoma (M174) cell characterization experiment in DIF + 10 % FBS (▽) and DMEM + 10 % FBS (●). Experiments were performed in 150 mL Corning spinner flasks in an incubator with a water saturated atmosphere and 5 % CO_2.

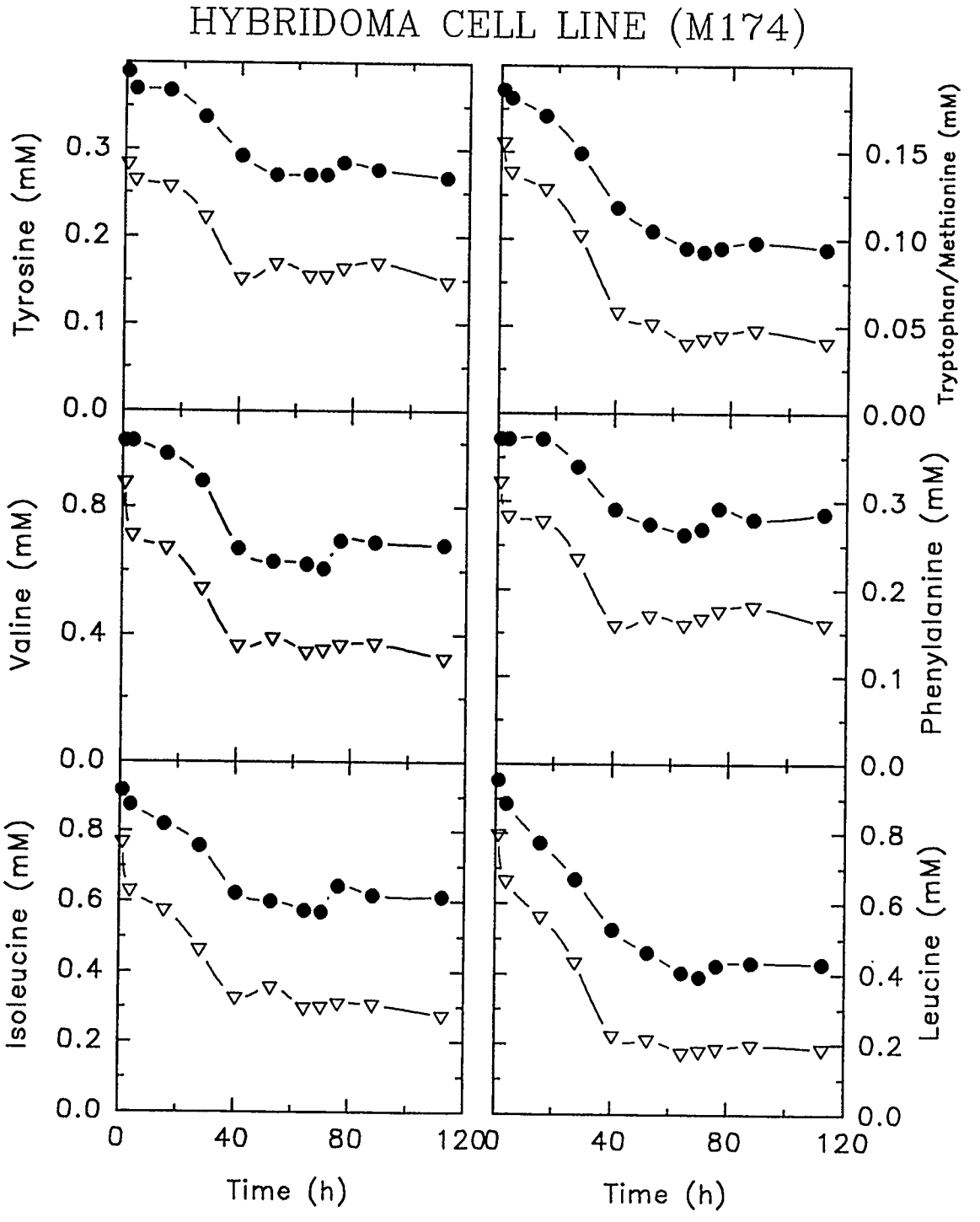


Figure 5.10: Various amino acid concentrations for hybridoma (M174) cell characterization experiment in DIF + 10 % FBS (▽) and DMEM + 10 % FBS (●). Experiments were performed in 150 mL Corning spinner flasks in an incubator with a water saturated atmosphere and 5 % CO_2.

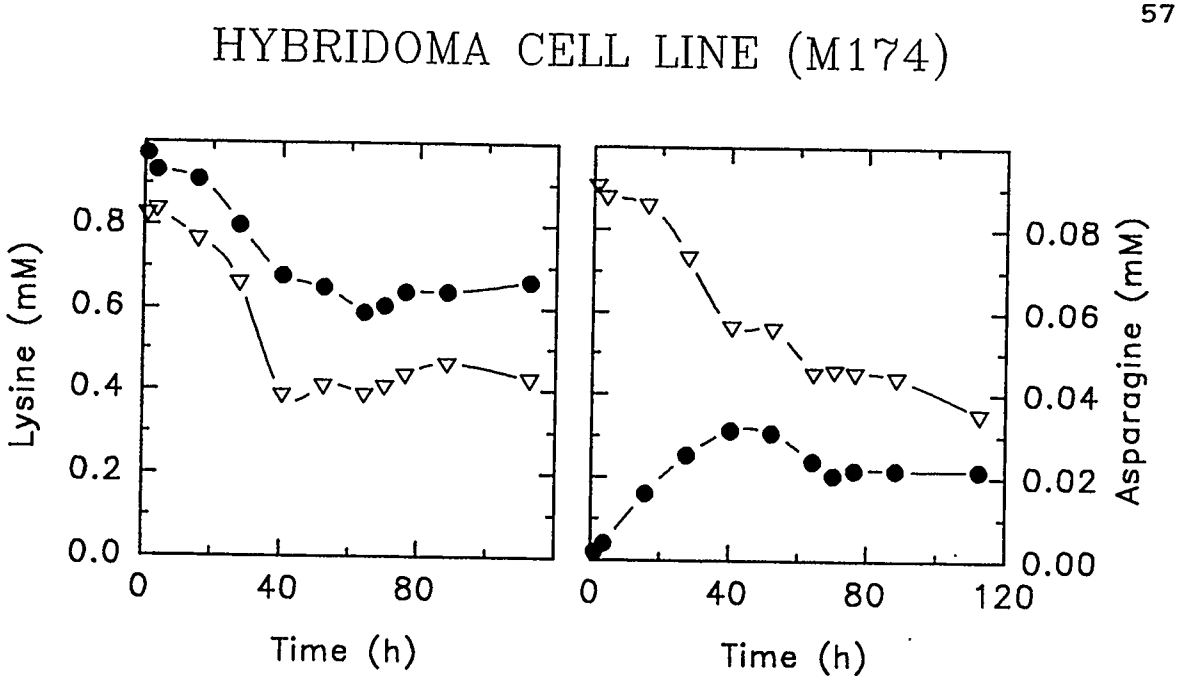


Figure 5.11: Lysine and aspargine concentrations for hybridoma (M174) cell characterization experiment in DIF + 10 % FBS (▽) and DMEM + 10 % FBS (●). Experiments were performed in 150 mL Corning spinner flasks in a water saturated incubator with 5 % CO_2.

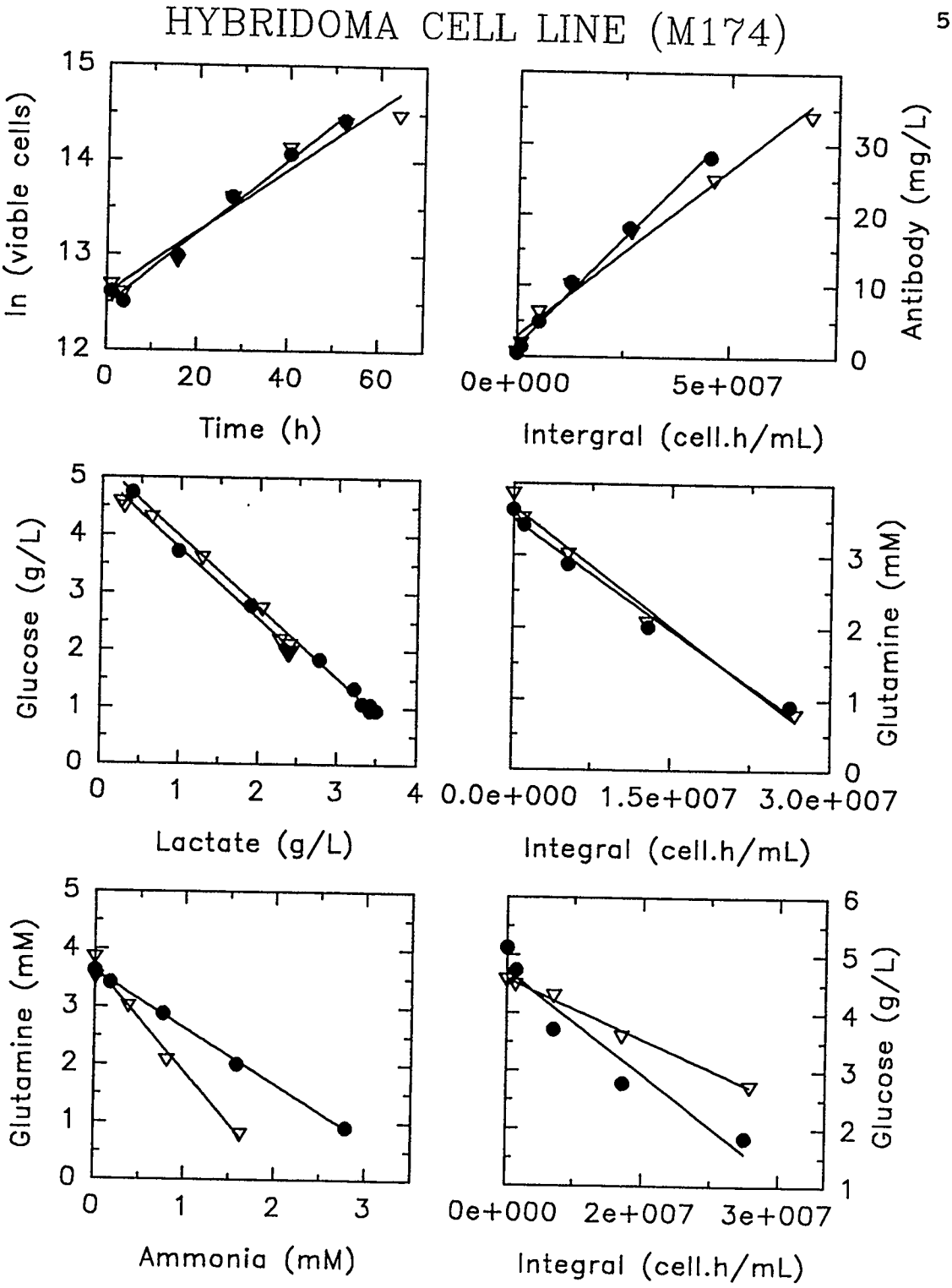


Figure 5.12: Batch data regressions used to determine of metabolic parameters for the hybridoma (M174) cell line. Specific glutamine and glucose uptake, and antibody production rates were determined in the exponential growth phase. The different symbols refer to DIF + 10 % FBS (∇) and DMEM + 10 % FBS (●).

about 45 mg/L for both the DIF and DMEM media. Since the media was supplemented with 10 % FBS, the viability was somewhat lower than expected. Overall we experienced difficulty in the thawing and maintenance of this cell line due to its inherent instability. The instability refers to poor growth (generally viability under 80 %), loss of antibody productivity, and difficulties in thawing of cells. We observed the loss of antibody production occurred after about a month which translates to approximately 70 generations based on the maximum growth rate. Again the cell cycle distribution shows that the cells become arrested in the G_1 after the cells reach the stationary phase.

Figure 5.8 shows the nutrient uptake rates and the corresponding byproduct production rates for the hybridoma cell line. The following conclusions can be drawn.

(a) Glutamine is shown to be a limiting nutrient as the concentration drops to zero at about 60 hours.

(b) Glucose is consumed much slower after 60 hours when the glutamine is completely depleted.

(c) Lactate reaches a maximum concentration of 2 g/L in the DIF medium and 2.5 g/L in the DMEM medium.

(d) The ammonia concentration has a maximum of 4.5 mmol/L in the DMEM and 4 mmol/L in the DIF medium.

(e) The LDH activity is again zero throughout the batch run mainly due to the shear protection provided by the serum.

(f) Aspartate is consumed and may be further supplemented in the medium

Figures 5.9 to 5.11 show the concentration of various amino acids for the two different media. The graphs clearly show that none of the amino acids are limiting and therefore cell death can be completely attributed to the lack of glutamine present. By increasing the initial concentration of glutamine likely increased growth and antibody production will result. Amino acids such as isoleucine and leucine which are generally consumed by hybridoma cells are not being used to their fullest extent in this case. Alanine was the only amino acid produced in the batch run. Figure 5.11 also shows that asparagine

is consumed in the DIF medium while it is produced in the DMEM medium. Asparagine is not available in basal DMEM and is obviously required by the cells since it is consumed in the DIF medium. One hypothesis would be that the hybridoma cells are triggered to produce asparagine to satisfy certain metabolic needs. A net production of asparagine results because more asparagine is produced than required. Figure 5.12 shows the calculation of specific growth rate, specific uptake and production rates, and ammonia and lactate yield coefficients using the integral method. Again the graphs show the relationships are fairly linear, therefore average values were calculated using linear regression. The calculated values are summarized in Table 5.2.

Table 5.2 shows that the specific productivity (q_{MAb}) of IgG [6.12×10^{-7} μg/(cell·h)] is significantly higher in the DMEM than in the DIF, possibly as a result of the high ammonia concentrations causing the cells to become stressed in the DMEM medium. However, the specific growth rate, final antibody concentration, and the maximum cell density are very similar when comparing the two different media. By comparing the doubling times we observe that the hybridoma cells growing in DMEM medium grow slightly faster. Overall there is really no difference in performance between the two media. The excess glutamate in the DIF medium does not enable the hybridoma cells to grow better as was the case for the myeloma cells. It appears that the hybridoma cell line is more dependent on a glutamine source than on other substrate sources.

Table 5.2 - Hybridoma Characteristics in Media supplemented with 10 % FBS Experiments Performed in 150 mL Corning Spinner Flasks

Parameter	DMEM	DIF
$X_{v\,max}$ (cells/mL)	1.85×10^{6}	1.96×10^{6}
μ_{max} (h^{-1})	0.0385	0.0329
t_d (h)	18.0	21.0
q_{gln} (mmol/(cell•h))	-1.02×10^{-10}	-1.11×10^{-10}
q_{glc} (g/(cell•h))	-1.21×10^{-10}	-1.028×10^{-10}
q_{MAb} (μg/(cell•h))	6.12×10^{-7}	4.74×10^{-7}
$Y_{amm/gln}$ (mmol/mmol)	1.02	0.55
$Y_{lac/glc}$ (g/g)	0.82	0.82

Note: X - cell density, μ - specific growth rate, q - specific production rate, Y - yield coefficient

Chapter 6: Cell Adaptation

6.1 Introduction

Many medium and short term methods to adapt cells successfully from high serum to low serum or serum free conditions are reported in the literature. These methods fall into three basic categories, namely

A. Balanced Adaptation (medium term)

B. Gradual Serum Reduction (medium term)

C. Shock Adaptation (short term)

No long term adaptation methods (generally 6 months and more) were attempted because of the instability of the two cell lines being considered (see section 2.6). The balanced adaptation method slowly decreases the serum content while at the same time gradually increasing the media supplements (BITES; see Table 7.1 or Section 3.9 for details) until at the end of this procedure the serum is completely eliminated and the serum free formulation is at the final desired concentration. In the gradual serum reduction protocol the media supplements (BITES) are added at full strength at the beginning of the adaptation. The serum content of the media is slowly reduced over a period of time until the cells become completely adapted to serum free conditions. The shock adaptation method attempts to adapt the cells by putting them directly in the serum free formulation (BITES) with little (0.1 % to 1 %) or no serum. The cells are then subcultured at high cell densities (usually greater than 2.5 x 10^5 cells/mL and as high as 5 x10^5 cells/mL) until the cells become adapted. The exact protocols that were used in this study are summarized in the following section.

6.2 Adaptation Protocols

A. Balanced Adaptation Procedure (medium term)

Week #1: Cells are grown initially in basal media + 10 % FBS. The serum content of the culture is reduced from 10 % to 7.5 %. At the same time the BITES (BSA, insulin, transferrin, ethanolamine, selenium) concentration is increased to 20 % of the final concentration for the specified

formulation (i.e. 15 μg/mL, 150 μg/mL, and 450 μg/mL total protein content) is added to the medium.

Week #2: The serum content is reduced from 7.5 % to 5 %. At the same time the BITES concentration is increased to 40 % of final concentration required in the medium.

Week #3: The serum content is reduced from 5 % to 2.5 % and the BITES content is increased to 75 % of desired final concentration.

Week #4: The serum content is reduced from 2.5 % to 1 % and the BITES concentration is increased to 90 % of desired final concentration.

Week #5: The serum content is reduced from 1 % to 0 % and the BITES content is increased to 100 % of desired final concentration.

Week #6: Subculture to adapt cells more fully.

Week #7: Subculture to adapt cells more fully.

B. Gradual Serum Reduction (medium term)

Week #1: Reduce serum content of culture from 10 % to 7.5 %. At this pont BITES (BSA, insulin, transferrin, ethanolamine, and selenium) are added at the desired final concentration and kept at this level at all times in the adaptation.

Week #2: Reduce serum content of culture from 7.5 % to 5 %.

Week #3: Reduce serum content of culture from 5 % to 2.5 %.

Week #4: Reduce serum content of culture from 2.5 % to 1 %.

Week #5: Reduce serum content of culture from 1 % to 0 %.

Week #6: Subculture to allow cells to adapt more fully.

Week #7: Subculture to allow cells to adapt more fully.

C. Shock Adaptation (short term)

Week #1: Cells cultured with basal media + 10 % FBS. Reduce serum content from 10 % to 0.1 % and subculture for one week. BITES are not added

at this point.

Week #2: Centrifuge cells removing all supernatant (i.e. this removes all serum present) and resuspend cells in serum free media at final BITES concentration desired. Culture cells for two weeks in media using serial dilution.

Week 3-5: Allow cells to fully adapt to new conditions.

Note: When cells are subcultured in the above protocols they were always seeded at a concentration of 4 x 10^5 cells/mL.

6.3 Experimental Setup

Three different formulations (based on protein content) were considered for the adaptation experiments. The serum free media formulations consists of 1X BITES solution in DIF basal media and are shown in Table 6.1. The three different adaptation protocols were applied to each cell line using each of the three serum-free media formulations. This resulted in 9 adaptation experiments for each cell line. For each formulation and protocol, the cells were subcultured into two separate T-flasks in order to select for the better adapted cells. The cells produce certain amino acids and other chemicals which may be required for cell growth or antibody production. Therefore 1.0 mL of supernatant was added when the cells were subcultured in order to condition the media. A schematic representation of the experimental adaptation protocols for the hybridoma and recombinant myeloma cell lines are shown in Figures 6.1 and 6.2 respectively.

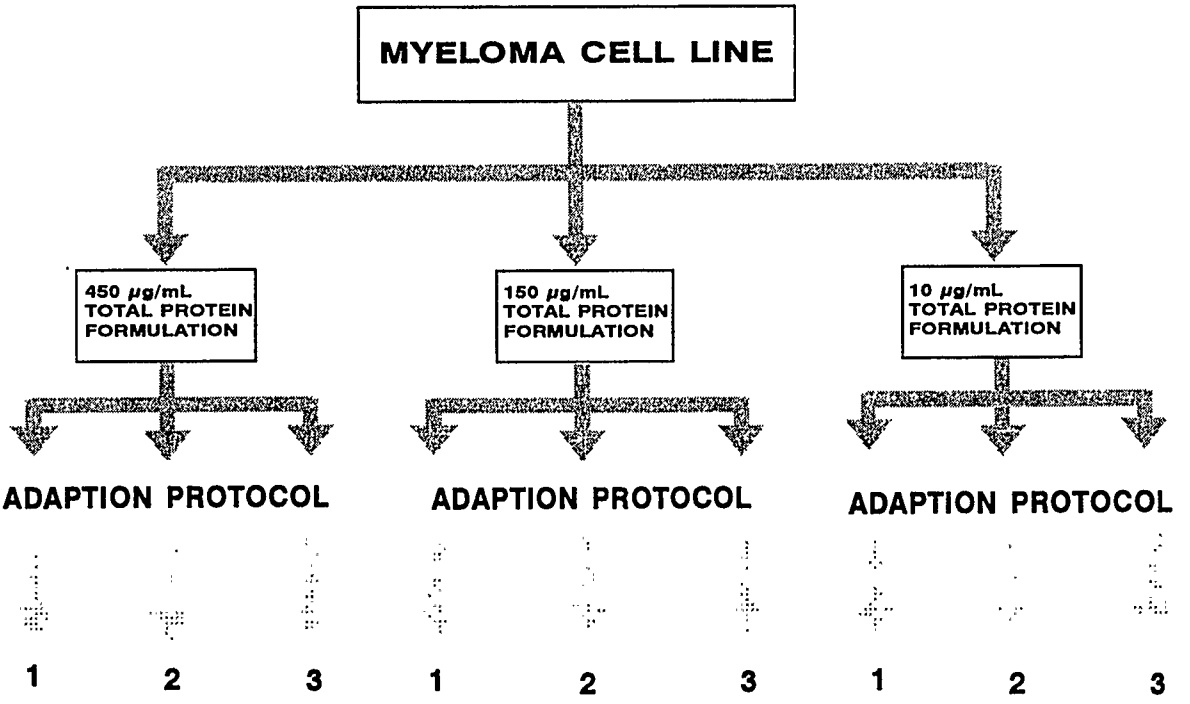
6.4 Results - Myeloma (C174) Cell Line

Figures 6.3 to 6.5 show the viable cell density and antibody production for the myeloma cell lines being adapted to the different formulations. The graphs show slight differences between the different protocols in the early stages, however as the adaptations progressed the cells reach the same stage. The graphs show that, with the formulation being used, the myeloma cells are not improving as time progresses (not adapting). During the

Table 6.1 - Three Serum Free Media Formulation Considered Based on Protein Content

Substance	**450 μg/mL total protein formulation**	**150 μg/mL total protein formulation**	**15 μg/mL total protein formulation**
Ethanolamine	30 μmol/L	30 μmol/L	30 μmol/L
Na-Selenite	10 nmol/L	10 nmol/L	10 nmol/L
Insulin	5 μg/mL	5 μg/mL	5 μg/mL
Transferrin	10 μg/mL	10 μg/mL	10 μg/mL
2-Mercaptoethanol	30 μmol/L	30 μmol/L	30 μmol/L
Trace Elements	1 X	1 X	1 X
Cholesterol	1 μg/mL	1 μg/mL	1 μg/mL
BSA-Oleic	435	135	0

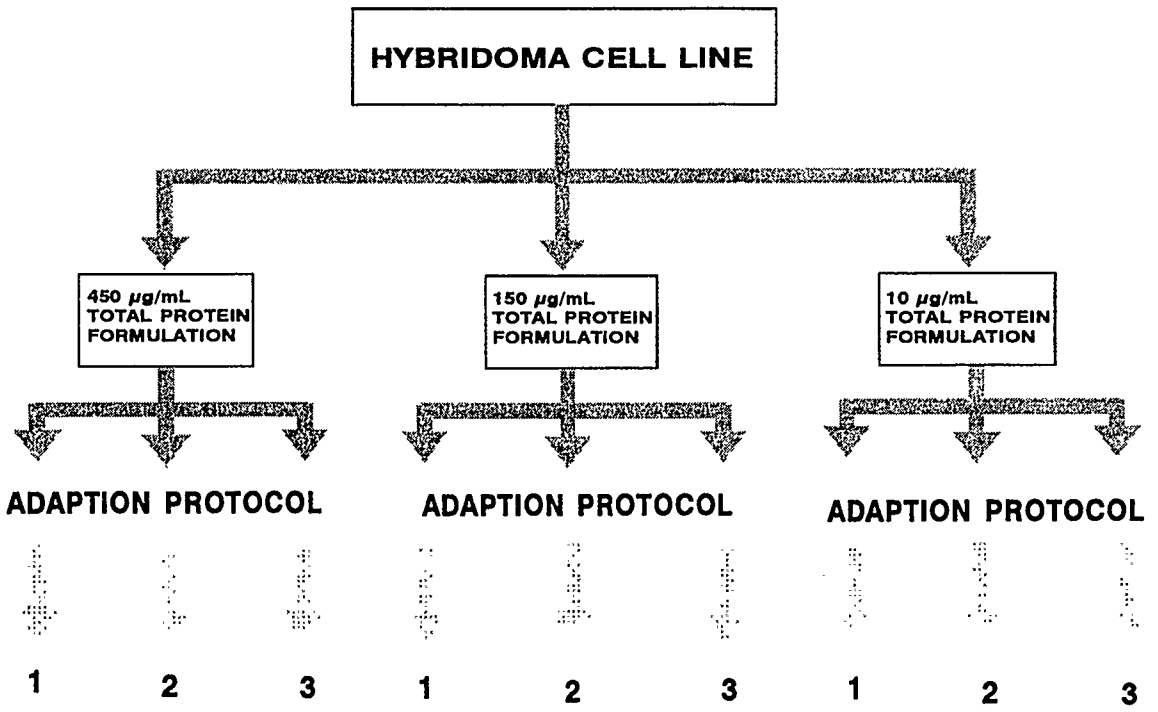
Note: The target protein concentration requested by Biomira for the new medium was 150 μg/mL as specified in the contract.



protocol 1 - gradual serum reduction
protocol 2 - balanced serum reduction
protocol 3 - shock adaption

For each of the adaption protocols (1,2, and 3) we will have two separate T-flasks. At each subculturing the healthiest T-flask will be chosen and resuspended in fresh media with 1 mL of supernatant added.

Figure 6.1: Schematic representation of the experiment adaptation protocols for the myeloma cell line using three different media formulations and three different adaptation protocols.



protocol 1 - gradual serum reduction
protocol 2 - balanced serum reduction
protocol 3 - shock adaption

For each of the adaption protocols (1,2, and 3) we will have two separate T-flasks. At each subculturing the healthiest T-flask will be chosen and resuspended in fresh media with 1 mL of supernatant added.

Figure 6.2: Schematic representation of the experiment adaptation protocols for the hybridoma cell line using the three media formulation and three different adaptation protocols.

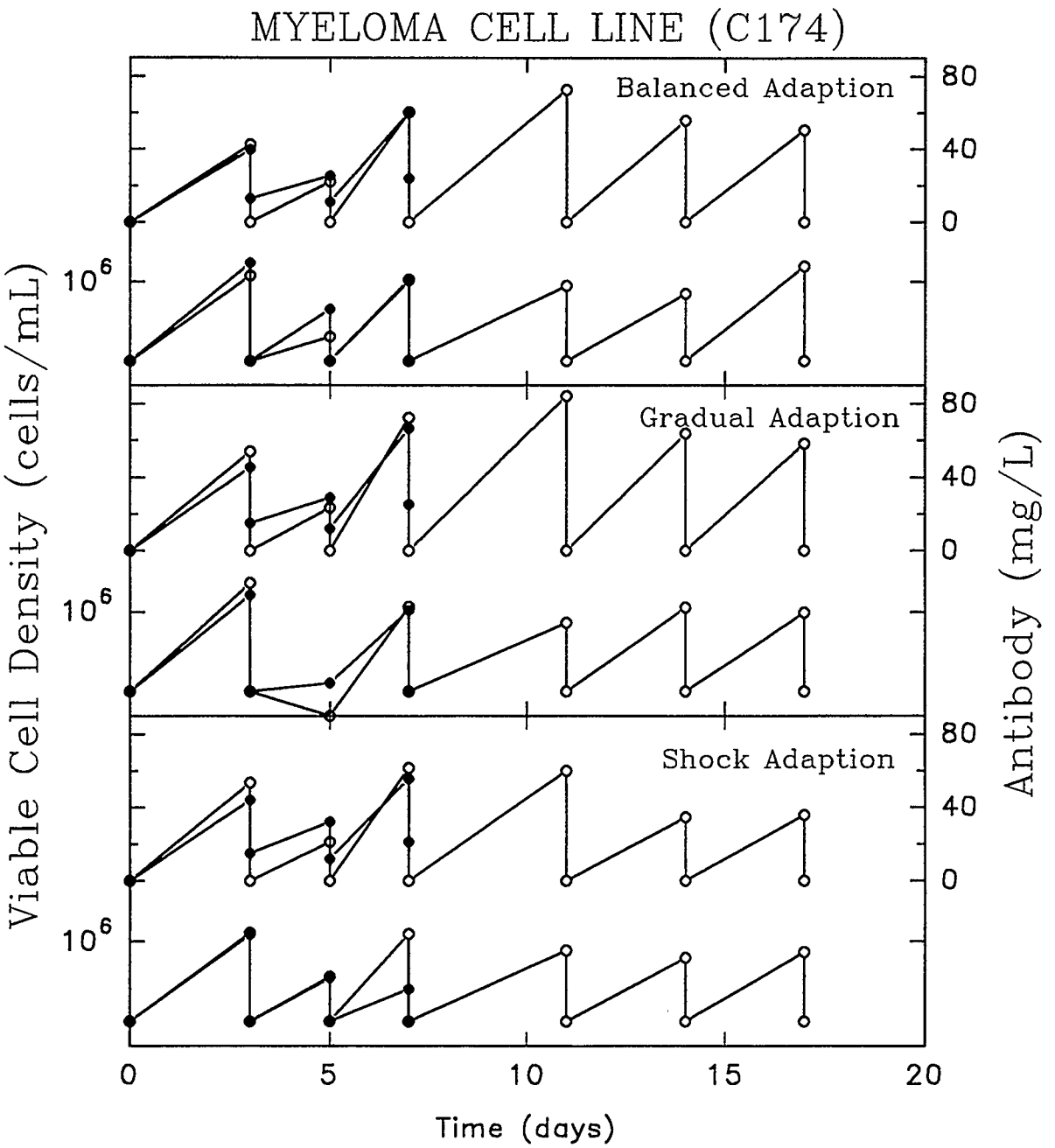


Figure 6.3: Graphs of adaptation experiment for the myeloma cell line using the 450 μg/mL media formulation and three different adaptation protocols. Experiments were performed in 25 cm^2 T-flasks (Nunc) in an incubator with a water saturated atmosphere at 37 °C and 5 % CO_2. Initially two T-flasks were counted for each experiment; T-flask **A** (○) and T-flask **B** (●), after 1 week only T-flask **A** was counted.

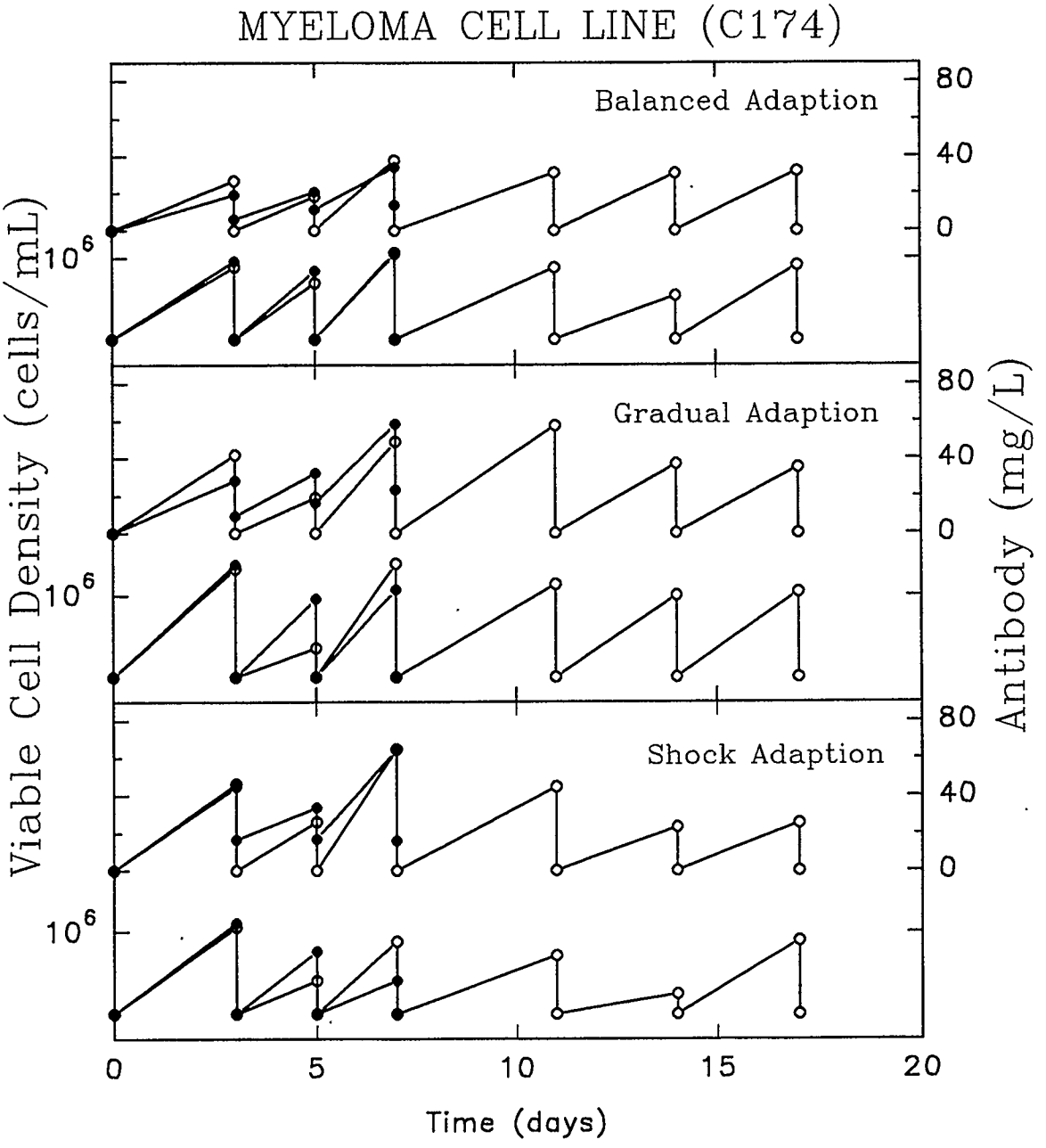


Figure 6.4: Graphs of adaptation experiment for the myeloma cell line using the 150 μg/mL media formulation and three different adaptation protocols. Experiments were performed in 25 cm^2 T-flasks (Nunc) in an incubator with a water saturated atmosphere at 37 °C and 5 % CO_2. Initially two T-flasks were counted for each experiment; T-flask **A** (○) and T-flask **B** (●), after 1 week only T-flask **A** was counted.

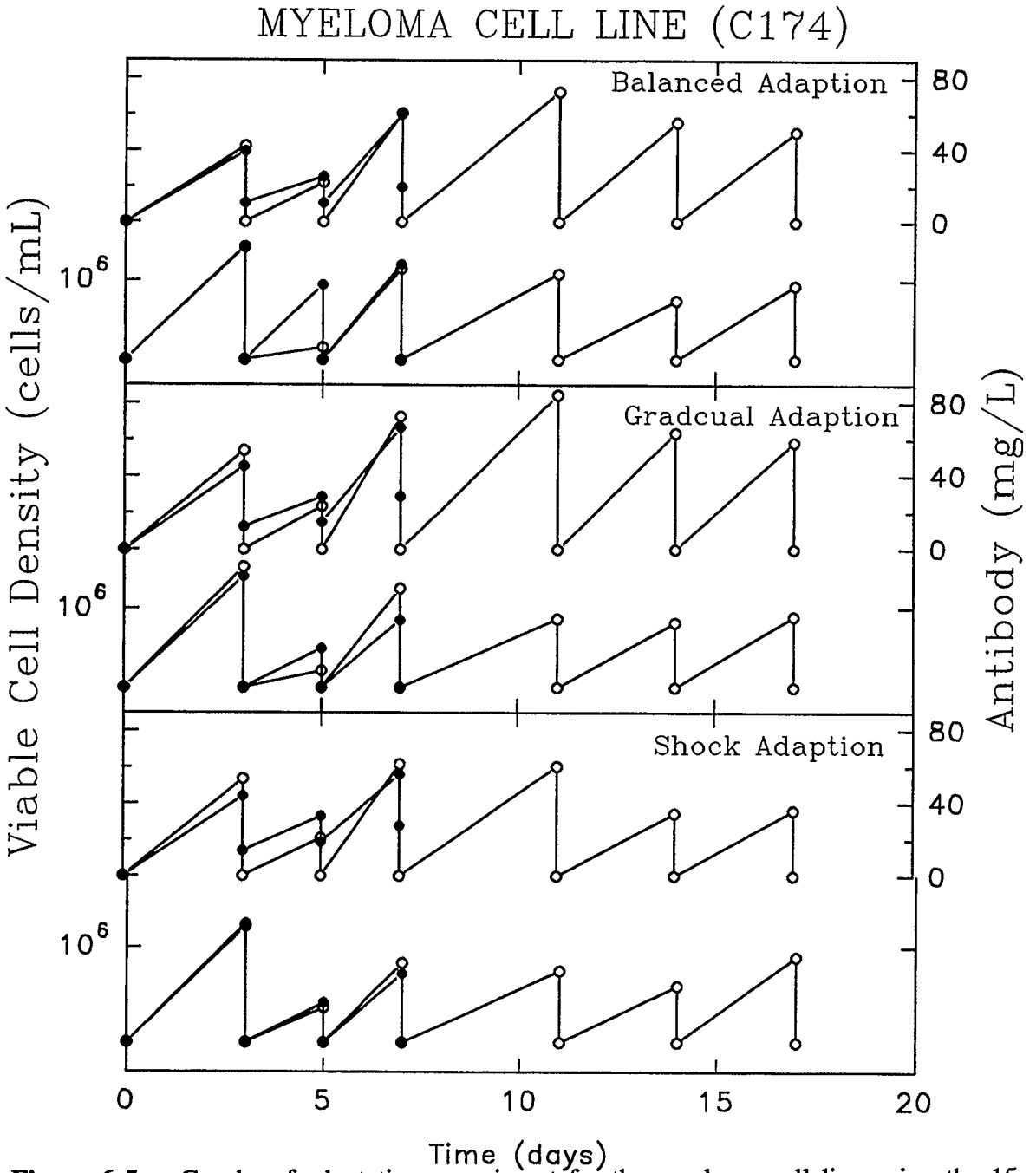


Figure 6.5: Graphs of adaptation experiment for the myeloma cell line using the 15 μg/mL media formulation and three different adaptation protocols. Experiments were performed in 25 cm^2 T-flasks (Nunc) in an incubator with a water saturated atmosphere at 37 °C and 5 % CO_2. Initially two T-flasks were counted for each experiment; T-flask **A** (○) and T-flask **B** (●), after 1 week only T-flask **A** was counted.

course of the experiment, we would take cells from the different protocols and attempt to grow them in serum free conditions. Although we could grow the myeloma cells at low serum levels (approximately 1 %) supplemented with the BITES formulation, at no time were we able to grow the myeloma cells in serum free conditions. The viability of the cells remains fairly high (above 70 %) when the medium was supplemented with 1.0 % serum and the cells looked healthy when examined under a microscope. After these experiments ended, we discovered that the myeloma cells required their source of cholesterol in the form of a lipoprotein (cholesterol alone did not help growth). By supplementing the media with ICN's low density lipoprotein the myeloma cells were able to grow in serum free conditions for a short time (approximately 7 subcultures or 10 days). This enabled us to pursue experiments to test other possible supplements which will be described in the next section.

6.5 Results - Hybridoma (M174) Cell Line

Figures 6.6 to 6.8 show the viable cell density and antibody production for the hybridoma cell line. Again the conclusion is that all protocols performed equally well. Unlike the myeloma cell line, the hybridoma cell line was able to grow in serum free conditions, although at a reduced growth rate and viability (approximately 50 to 60 % viability). It is clearly evident from the data collected that these cells cannot be adapted in a short time period. This is important because we have recently confirmed in our own PPRF laboratory that the hybridoma cells lose all antibody productivity after approximately 60-70 generations (about 1 month). Therefore the only real option is to adapt the cells quickly (not possible) or to use our serum free formulation as a production medium for the cells. The latter option will be discussed in Chapter 9 - "Scale-up".

6.6 Conclusions

The following conclusions can be drawn from the adaptation experiments

- The myeloma cell line could not be adapted to the serum free medium. Addition of egg yolk low density lipoprotein to the serum free medium enabled the cells

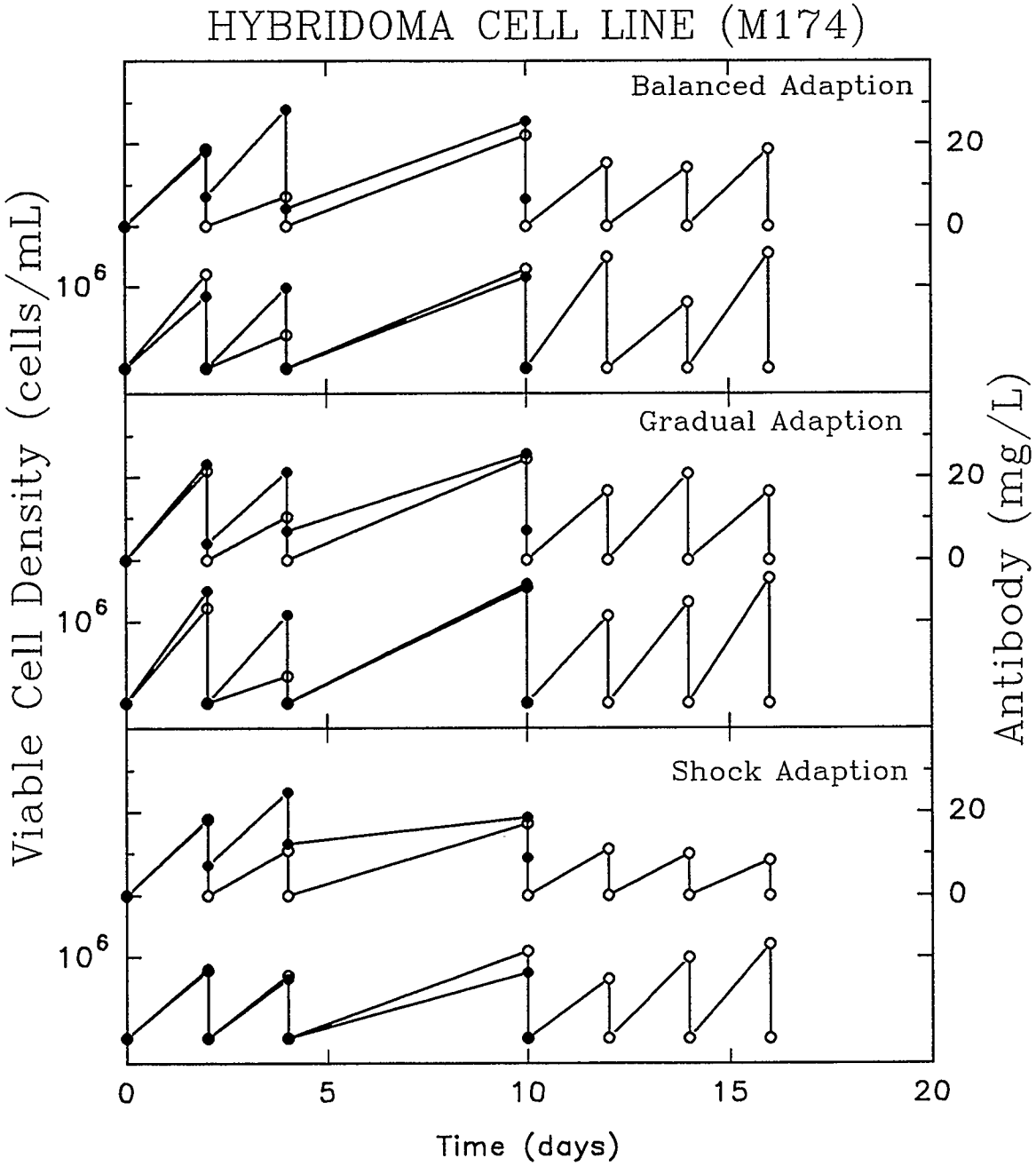


Figure 6.6: Graphs of adaptation experiment for the hybridoma cell line using the 450 μg/mL media formulation and three different adaptation protocols. Experiments were performed in 25 cm^2 T-flasks (Nunc) in an incubator with a water saturated atmosphere at 37 °C and 5 % CO_2. Initially two T-flasks were counted for each experiment; T-flask **A** (○) and T-flask **B** (●), after 1 week only T-flask **A** was counted.

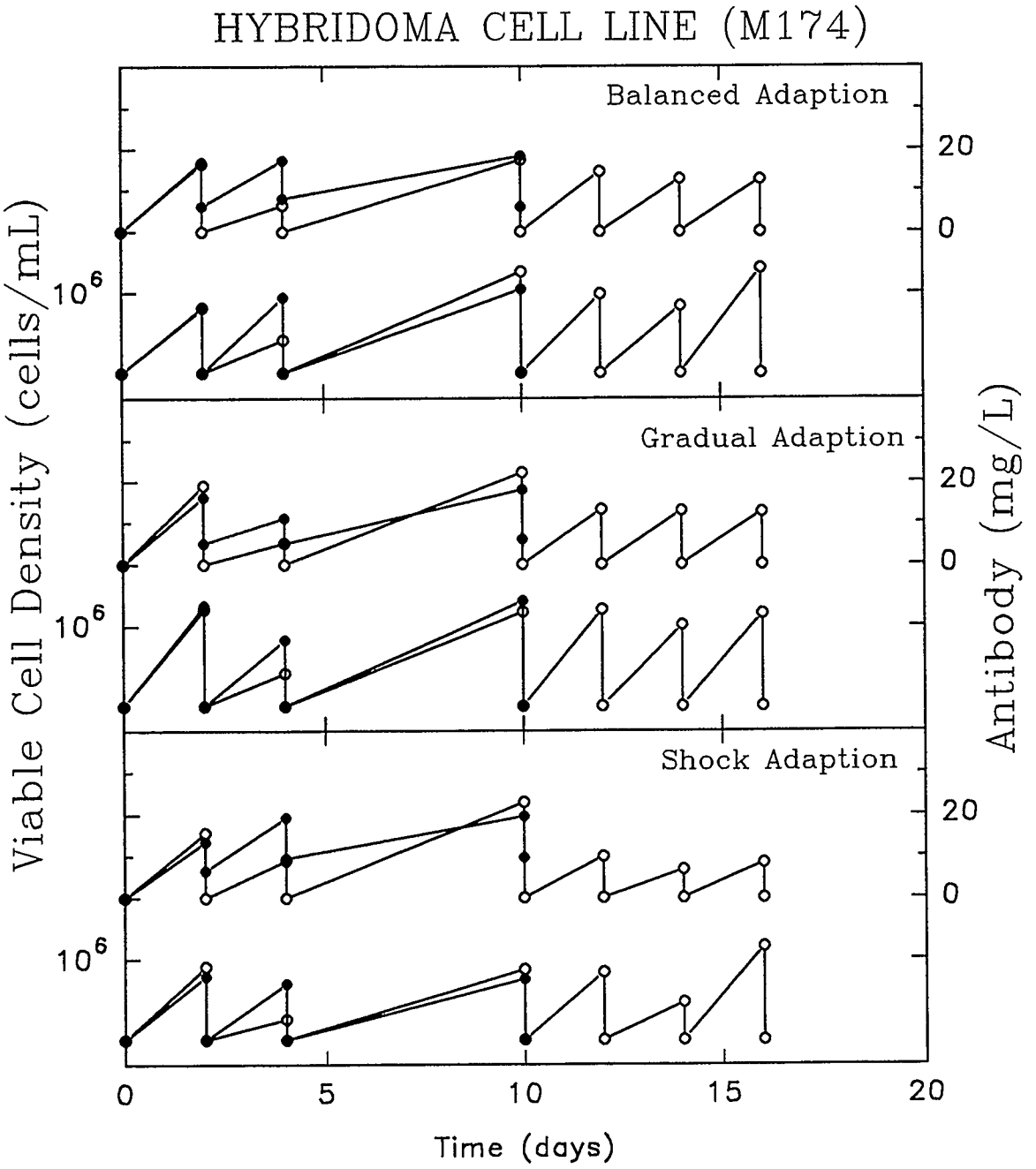


Figure 6.7: Graphs of adaptation experiment for the hybridoma cell line using the 150 μg/mL media formulation and three different adaptation protocols. Experiments were performed in 25 cm^2 T-flasks (Nunc) in an incubator with a water saturated atmosphere at 37 °C and 5 % CO_2. Initially two T-flasks were counted for each experiment; T-flask **A** (○) and T-flask **B** (●), after 1 week only T-flask **A** was counted.

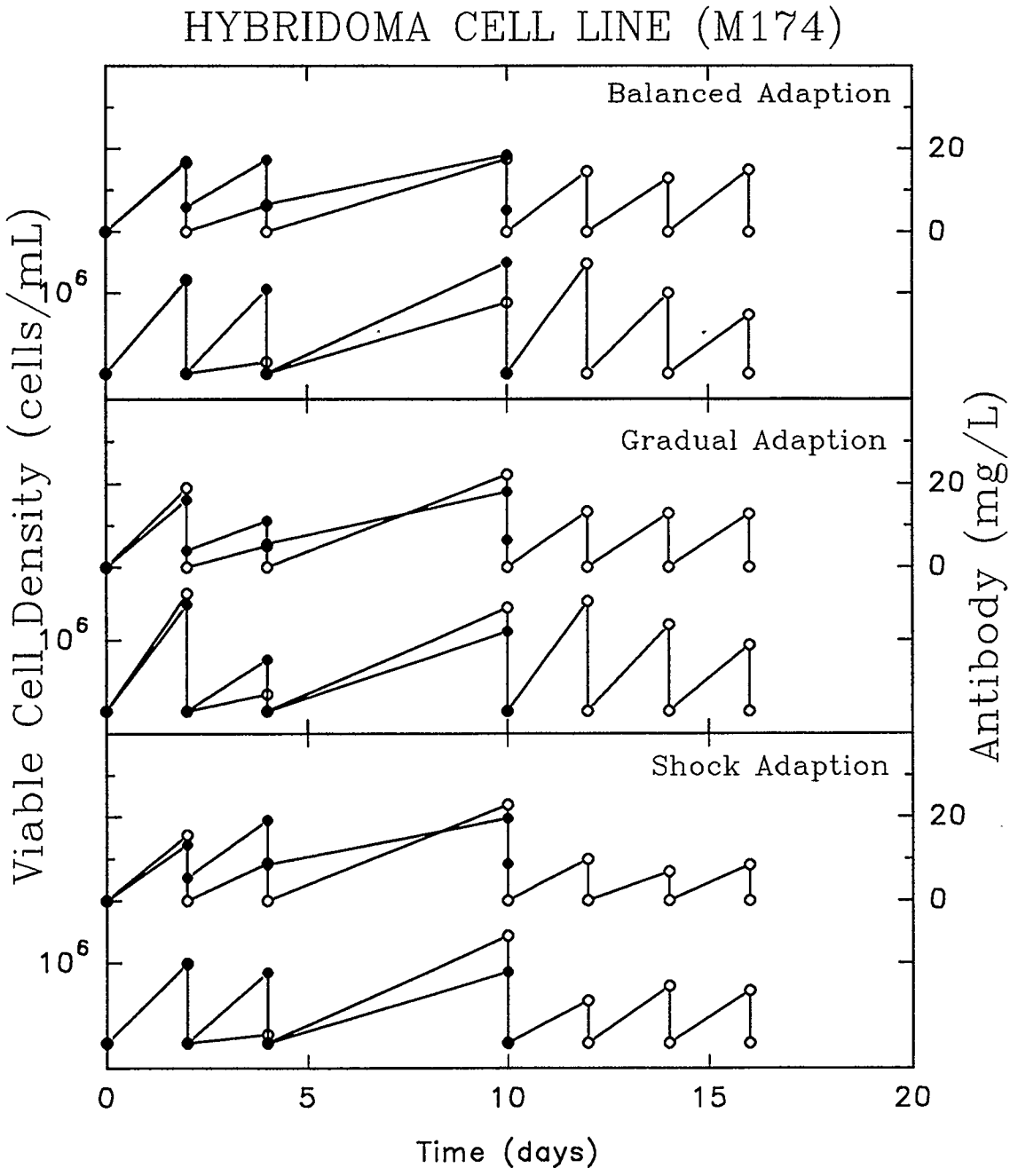


Figure 6.8: Graphs of adaptation experiment for the hybridoma cell line using the 15 μg/mL media formulation and three different adaptation protocols. Experiments were performed in 25 cm^2 T-flasks (Nunc) in an incubator with a water saturated atmosphere at 37 °C and 5 % CO_2. Initially two T-flasks were counted for each experiment; T-flask **A** (○) and T-flask **B** (●), after 1 week only T-flask **A** was counted.

to grow only for approximately 1 week (with subculturing) in serum free medium.

- The hybridoma cell line was able to grow in the serum free medium, but at significantly lower growth rates and viabilities than might be acceptable for any large scale production of antibody. Also the hybridoma cells lose their antibody production capability after about *60-70 generations*.

- Since both cell lines grow fairly well in our formulation for a short time period if directly seeded from media supplemented with 10 % FBS, the inoculum for the production bioreactor can be generated in 10 % FBS. The production of antibody can then be done in our developed serum-free medium. By using this method the monoclonal antibody concentration can be increased relative to the other proteins, which will decrease the cost of antibody purification.

Chapter 7: Testing of Supplements

7.1 Introduction

The development of a serum free medium can become very complicated when faced with the task of testing chemicals (hormones, growth factors, etc.) which have been reported to stimulate the growth and antibody production of hybridoma and myeloma cell lines. Not only are a large number of potential components, but many of the components may only be active when others are also present (synergistic effects). For these reasons a systematic approach must be used in the testing of possible components for use in a serum free medium. From our literature survey we concluded that the following chemicals were the most likely to improve the performance of the two cell lines under consideration:

> Caffeine, Hydrocortisone, Estradiol, Progesterone, Prostaglandin-E1, d-α-tocopherol, o-phenyldiamine, Egg yolk LDL, Casein, Locust Bean Gum, Citric Pectin, Interleukin-2, Interleukin-4, Interleukin-6.

In order to reduce the number of experiments, similar chemicals were grouped together and then tested using a factorial experiment. The concentrations of each chemical tested were based on our estimates of optimal values (1X), from past serum development programs for animal cells. The 1X notation refers to the concentration actually used and reported in the literature, and similarly 10X denotes that we are using 10 times the concentration in that particular experiment.

7.2 Experimental Setup

The factorial experiment was performed in 25 cm^2 T-flasks (Nunclon) using the same serum free formulation described in the previous chapter enriched with excess BSA-Oleic and transferrin. The formulation for the myeloma (C174) cell line was also supplemented with ICN low density lipoprotein which provides cholesterol in the form of a lipoprotein. This formulation shown in Table 7.1, gives the optimal values found in the literature for the base medium to be used. The supplements will be tested by adding them to the base medium as groups as discussed in the next section. The protein

Table 7.1 - Enriched Formulation used for Quick Adaptation of Cells to Test Chemicals Reported to Stimulate Growth and Antibody Production

Substance	Concentration
Ethanolamine	30 μmol/L
Na-Selenite	10 nmol/L
Insulin	5 mg/L
Transferrin	15 mg/L
2-Mercaptoethanol	30 μmol/L
Trace Elements (100X)	10 mL/L
Cholesterol	1 mg/L
BSA-Oleic	1000 mg/L
ICN LDL*	300 mg/L

* ICN LDL (low density lipoprotein) was used only for the myeloma (C174) cell line.

content of ICN low density lipoprotein at the concentration shown in the table is approximately 4 μg/mL. The two cell lines grown initially on DIF + 10 % FBS were adapted to the serum free formulations by slowly diluting out the serum over the period of a week. The cells were then subcultured (completely resuspended by centrifuging) twice in the serum free medium to remove all traces of serum and then the T-flasks were inoculated at 4 x 10^5 cells/mL. The groups of supplements tested are discussed in the next section.

7.3 Grouping of Supplements

The chemicals tested were divided into four different groups with each group expected to have similar effects. Tables 7.2 (a) to 7.2 (d) summarize the groups. Group A consists of 3 hormones and caffeine which have all been reported to stimulate growth. This groups represents the most likely of the groups to give significant results. Group B contains a prostaglandin, a lipid, and some specific reducing agents. Groups C is an inexpensive group reported to stimulate the production of antibody. Finally, Group D contains only interluekins which have many different intracellular functions and have been reported to stimulate growth and antibody production of mammalian cells.

7.4 Factorial Experiment

The factorial experiments were performed in T-flasks, and the groups were tested at three different concentrations (0.1X, 1X, 10X) shown in the Table 7.3. Note that each entry in the table refers to one T-flask experiment, and the cells which survived from the first experiment were subcultured again to check the passage effect, which may have been positive or negative. Also the 1X concentration represents what we deem to be optimal concentrations based on many literature references and previous animal cell media development experiments performed at PPRF.

7.5 Hybridoma Cell Line (M174) Results

Figures 7.1 to 7.3 show the growth rate and viability recorded for the hybridoma cell line. Based on the obtained experimental data, it is evident that, except for the

Table 7.2 - Supplements Reported to stimulate Growth and/or antibody production for Hybridoma cell lines

Table 7.2 (a) - Group A - Hormones/stimulants

Substance	1X Concentration
Caffeine	5 μmol/L
Hydrocortisone	2 μg/L
Estradiol	10 nmol/L
Progesterone	0.01 mg/L

Table 7.2 (b) - Group B - Growth Enhancing

Substance	1X Concentration
Prostaglandin E1	10 μg/L
Dexamethasone	25 μg/L
d-α-tocopherol	10 mg/L
o-phenyldiamine	10 mg/L
Egg Yolk LDL	30 mg/L

Table 7.2 (c) - Group C - Antibody Stimulants

Substance	1X Concentration
Citric Pectin	1 mg/L
Casein	5 μg/L
Chitosan	1 mg/L
Locust Bean Gum	1 mg/L

Table 7.2 (d) - Group D - Interleukins

Substance	1X Concentration
Interleukin-2	1 μg/L
Interleukin-4	1 μg/L
Interleukin-6	1 μg/L

Chemicals in these table were tested in DIF medium supplemented with BITES (see Table 6.1)

Table 7.3 - Combinations of Chemicals tested in Factorial Experiment using T-flasks. Experiments were performed for both the Hybridoma and Myeloma Cell Lines

0.1X Concentration	1X Concentration	10X Concentration
A	A	A
B	B	B
C	C	C
D	D	D
A+B	A+B	A+B
A+B+C	A+B+C	A+B+C
A+D	A+D	A+D
B+C	B+C	B+C

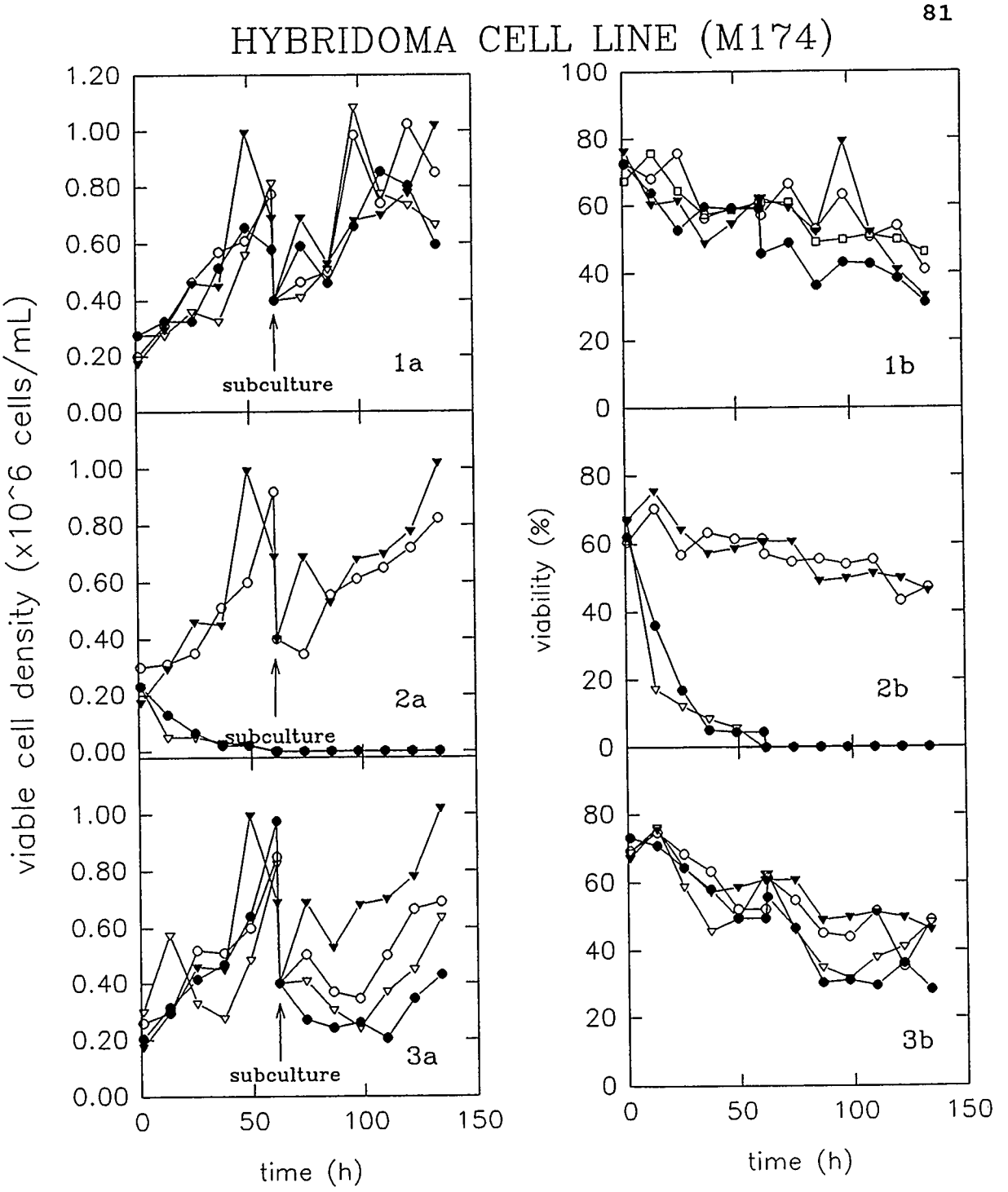


Figure 7.1: Growth and viability curves for the hybridoma (M174) cell line tested in different groups, Group A (Fig 1a,b), Group B (FIG 2a,b), and Group C (Fig 3a,b). The symbols represents the different group concentrations 0.1X (○), 1X (●), 10X (▽), and the control (▾) which is DIF supplemented with BITES only. The experiment was performed in a 25 cm^2 T-flasks at 37 °C and 5% CO_2.

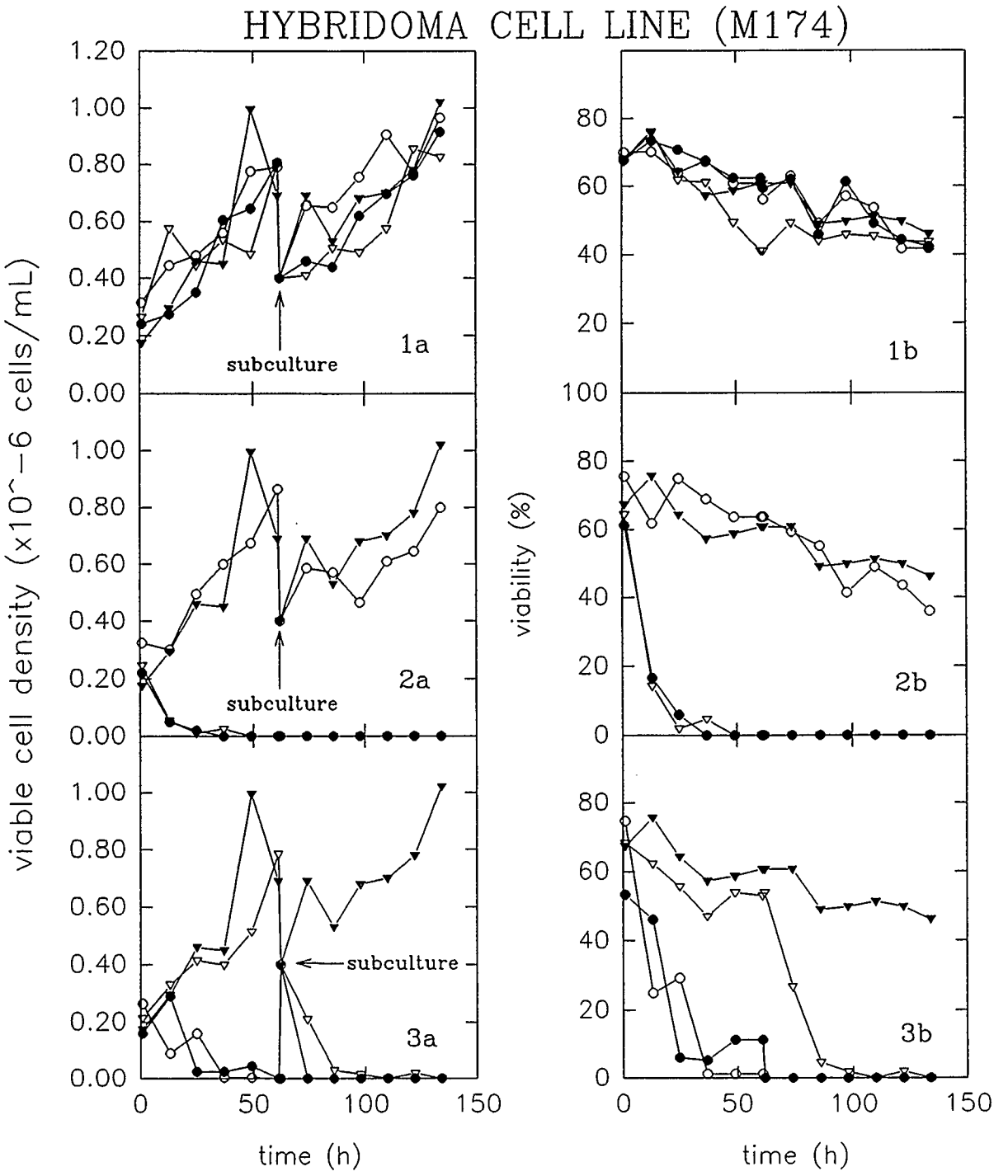


Figure 7.2: Growth and viability curves for the hybridoma (M174) cell line tested in different groups, Group D (Fig 1a,b), Group A+B+C (Fig 2a,b), and Group A+B (Fig 3a,b). The symbols represents the different group concentrations 0.1X (○), 1X (●), 10X (▽), and the control (▾) which is DIF supplemented with BITES only. The experiment was performed in 25 cm^2 T-flasks at 37 °C and 5% CO_2.

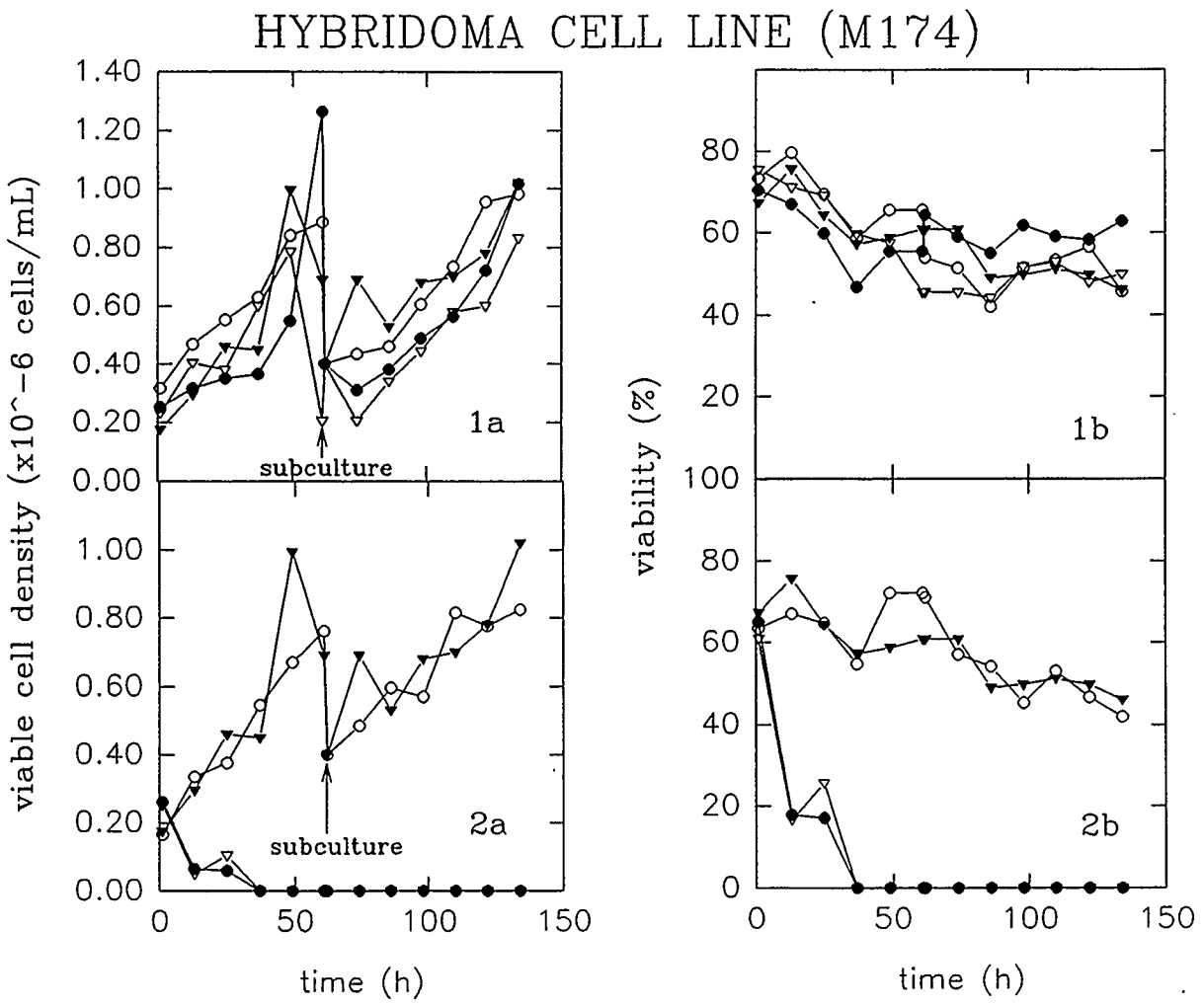


Figure 7.3: Growth and viability curves for the hybridoma (M174) cell line tested in different groups, Group A+D (Fig 1a,b), and Group B+C (Fig 2a,b). The symbols represents the different group concentrations 0.1X (○), 1X (●), 10X (▽), and the control (▾) which is DIF supplemented with BITES only . The experiment was performed in 25 cm^2 T-flasks at 37 °C and 5% CO_2.

interleukins the supplement groups did not enhance growth or viability. This was found to be the case for both the first and second subculture. Overall the cells looked healthier (rounder in shape) when the Group D (interleukins) was added to the medium. Figure 7.3 (1a) shows that the maximum peak of the 10X concentration of the Group D obtained a cell density of about 1.25 x 10^6 cells/mL while the control reached only 1.0 x 10^6 cells/mL. As well, the viability of the 10X concentration was found to be slightly higher than the control. The only drawback to using interleukins in the medium is the high cost. The antibody production for each passage for the hybridoma cell line is given in Figure 7.4. The control performed the best for all the groups in both the first and second subculture reaching a concentration of 35 mg/L in the second subculture. The supplements had no positive effect especially in the area of antibody production leading to the conclusion that none of the supplements tested should be added to the medium.

In the experiments for the hybridoma cell line, Group B was found to have a strong "toxic" effect on the cells and did not perform as expected. We found by trial and error that the supplement o-phenlydiamine in group B was very "toxic". Hence, we eliminated it from the supplement groups in all subsequent experiments. It should be noted however, that this chemical has been reported in literature to stimulate the growth of hybridoma cells.

7.6 Results Myeloma Cell Line (C174)

The growth and viability curves for the myeloma cell line are represented in Figures 7.5 to 7.7. Essentially these graphs show that the cells supplemented with the groups and groups combinations at the 1X concentration level obtained the highest cell density and viability in every case except for the case where the medium was supplemented with A+D (10X). The antibody production curves (Figure 7.8) indicate that the control had the highest final antibody concentration. Because of this result, we concluded again that no supplements should be added to the medium. In fact most of the supplements significantly reduced the antibody concentration, and specific antibody production of the myeloma cells. Most likely the cells redirected their energy into producing more cell

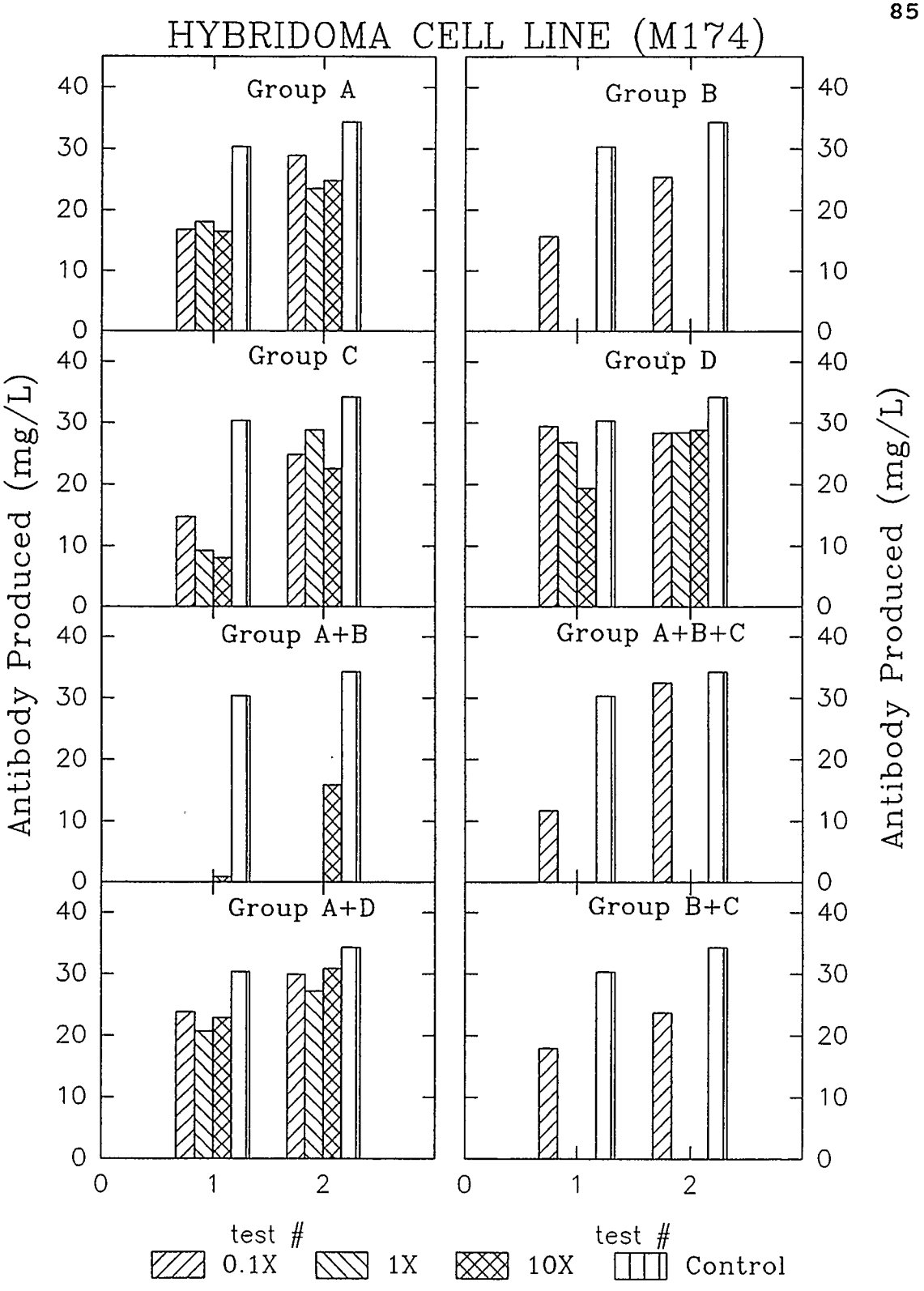


Figure 7.4: Antibody production for the hybridoma (M174) cell line tested in different groups at different concentrations. Test 1 refers to the first subculture and Test 2 to the second. The experiments were performed in 25 cm^2 T-flasks at 37°C with 5 % CO_2.

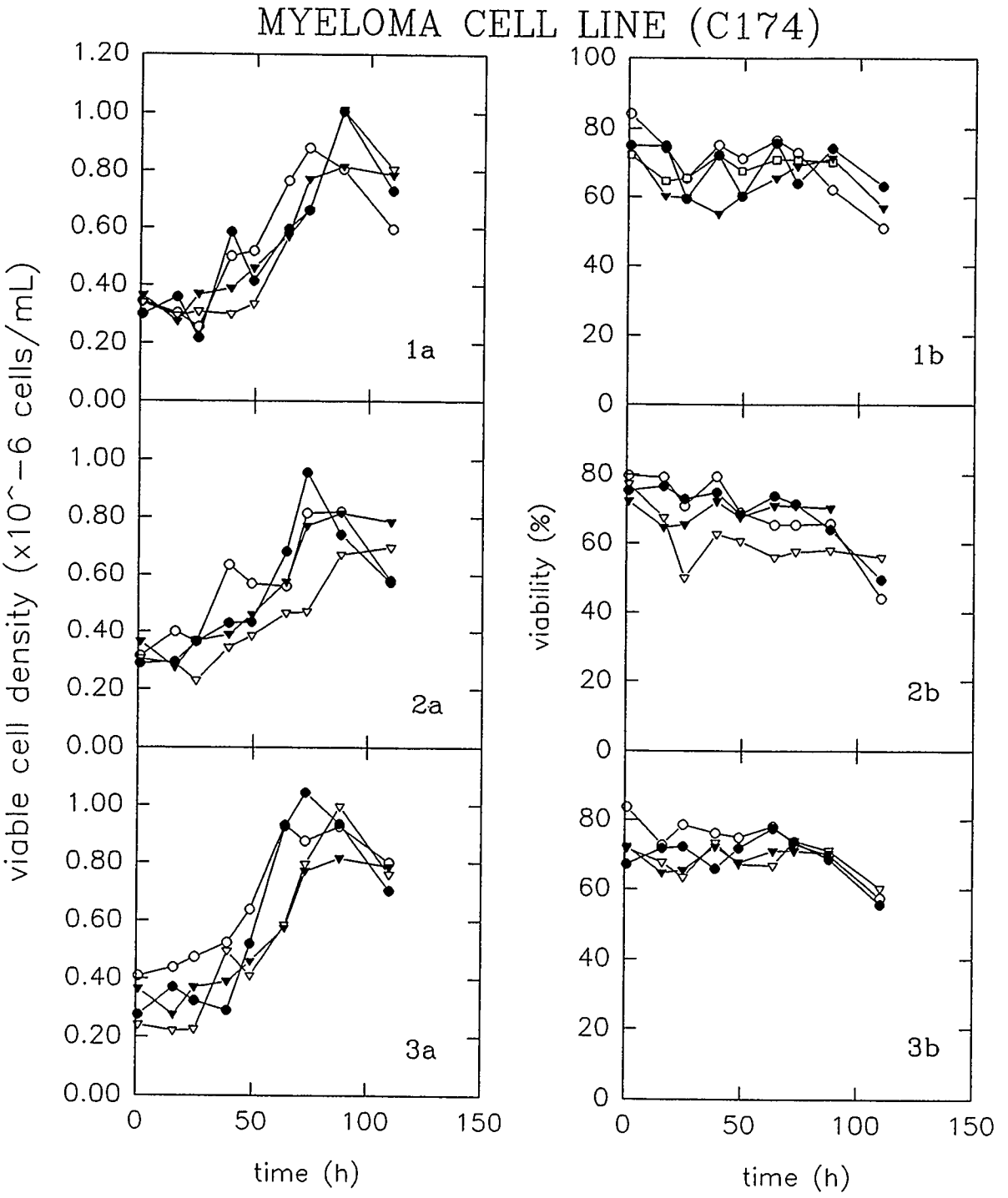


Figure 7.5: Growth and viability curves for the myeloma (C174) cell line tested in different groups, Group A (Fig 1a,b), Group B (Fig 2a,b), and Group C (Fig 3a,b). The symbols represents the different group concentrations 0.1X (○), 1X (●), 10X (▽), and the control (▾) which is DIF supplemented with BITES only. Experiments were performed in 25 cm^2 T-flasks at 37°C with 5 % CO_2.

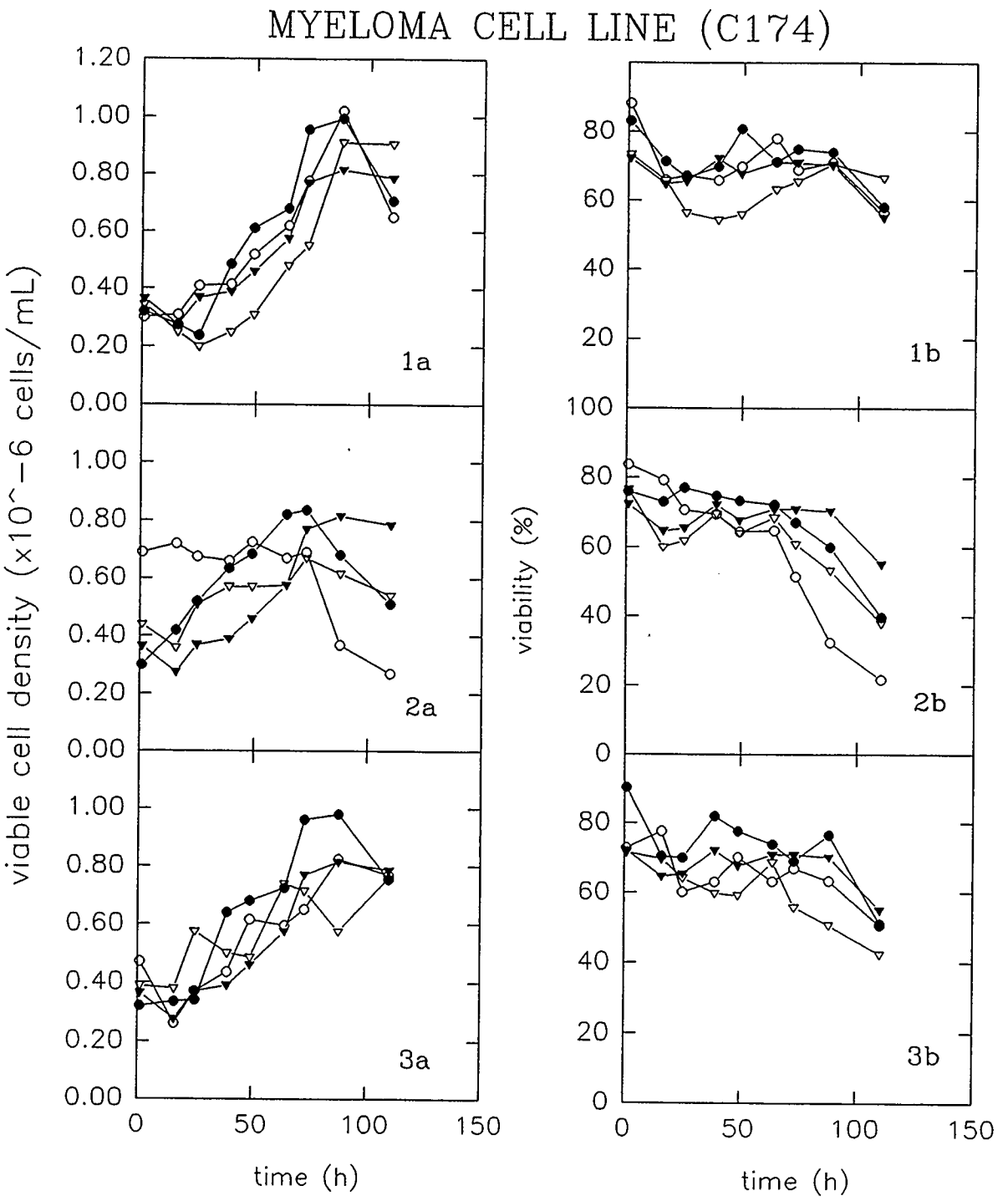


Figure 7.6: Growth and viability curves for the myeloma (C174) cell line tested in different groups, Group D (Fig 1a,b), Group A+B+C (Fig 2a,b), and Group A+B (Fig 3a,b). The symbols represents the different group concentrations 0.1X (○), 1X (●), 10X (▽), and the control (▾) which is supplemented with BITES only. Experiments were performed in 25 cm^2 T-flasks at 37°C with 5 % CO_2.

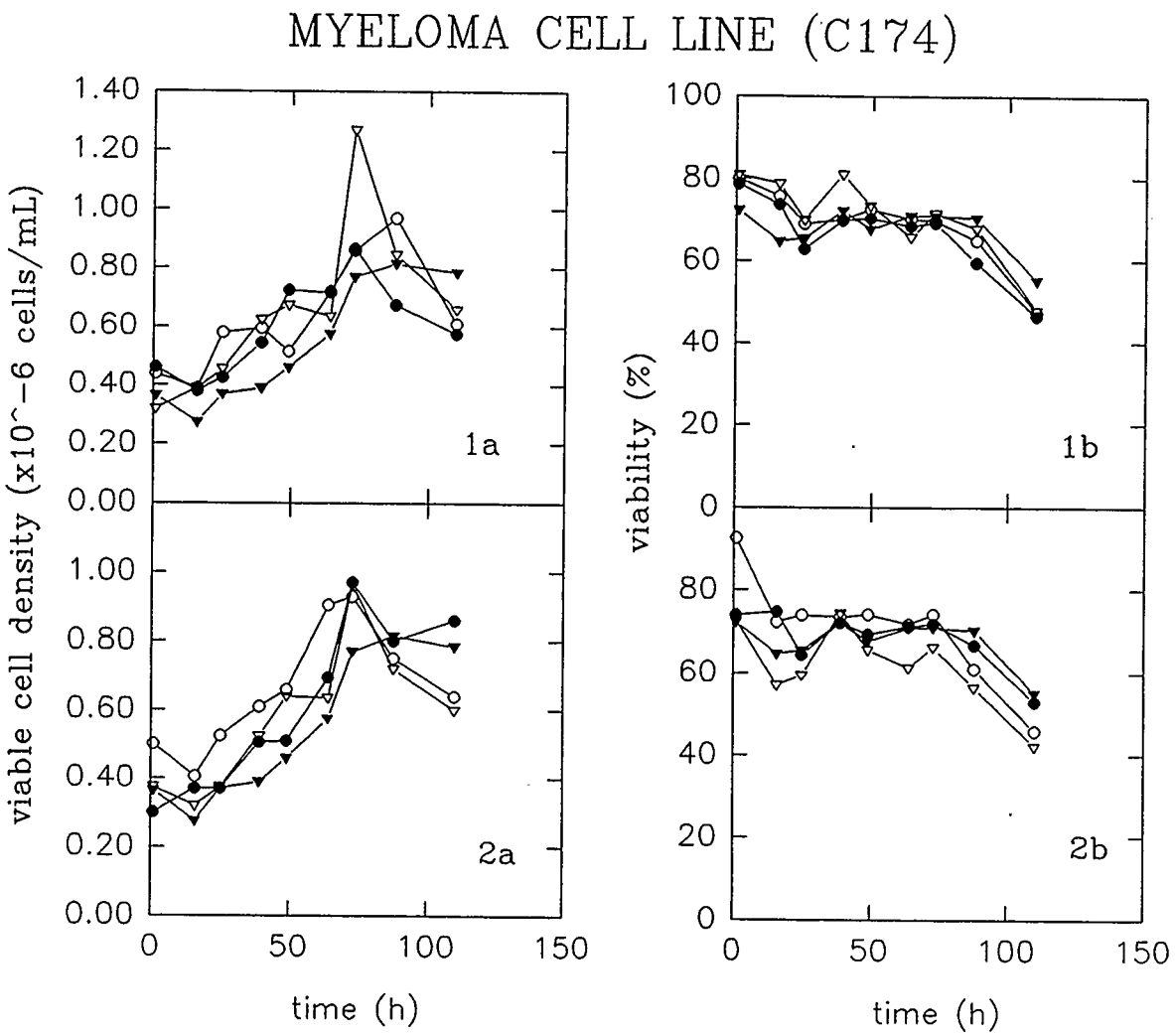


Figure 7.7: Growth and viability curves for the hybridoma (M174) cell line tested in different groups, Group A+D (Fig 1a,b), and Group B+C (Fig 2a,b). The symbols represents the different group concentrations 0.1X (○), 1X (●), 10X (▽), and the control (▾) which is supplemented with BITES only. Experiments were performed in 25 cm^2 T-flasks at 37°C with 5 % CO_2.

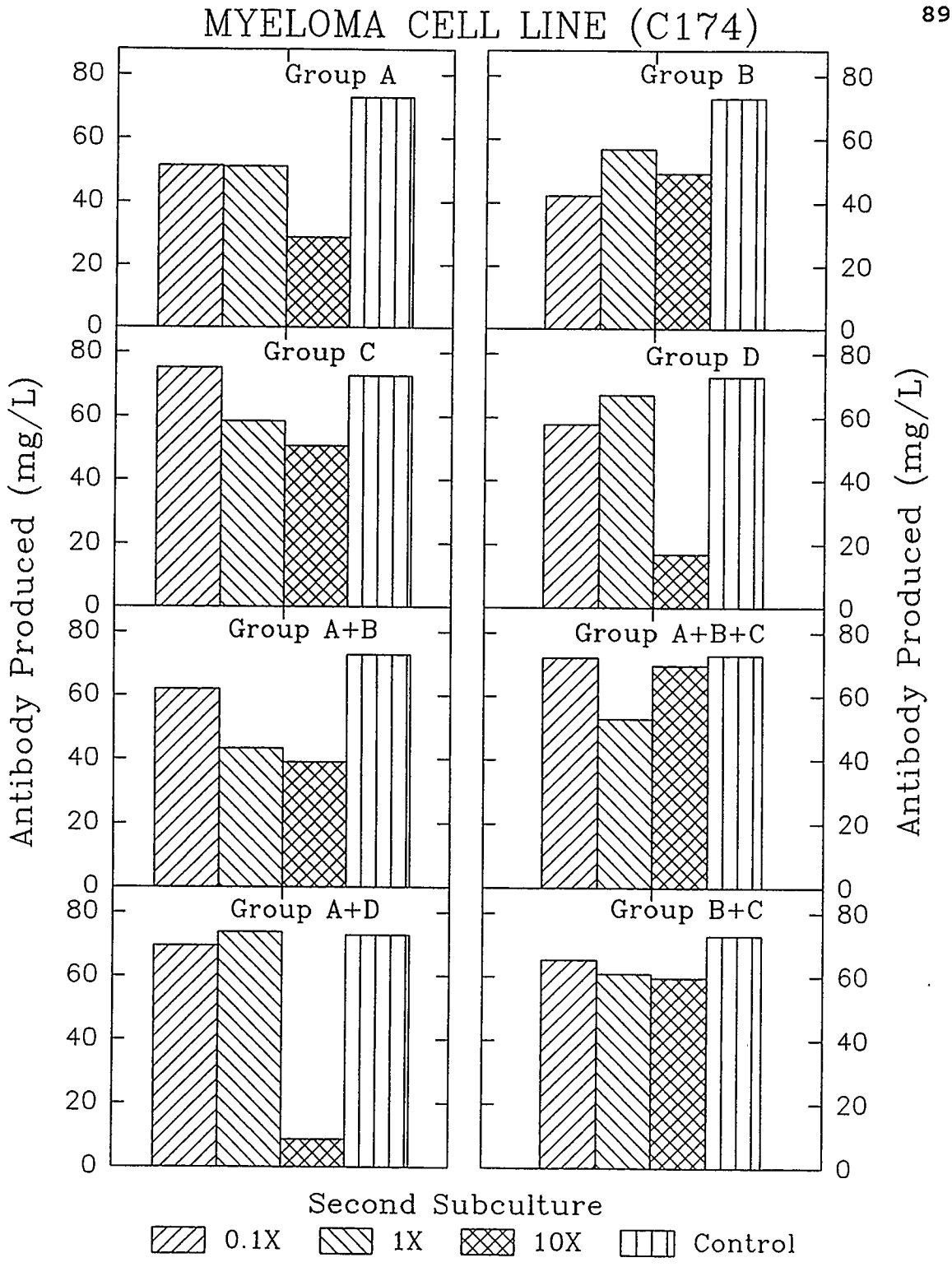


Figure 7.8: Antibody production for the myeloma (C174) cell line tested in different groups at different concentrations. The experiments were performed in 25 cm^2 T-flasks at 37°C with 5 % CO_2.

structures (such as cell walls etc.) and thus less antibody due to the supplements added. This explains the improved growth rate as well as the reduced antibody production.

7.7 Conclusions

Based on the factorial experiments performed the following conclusions can be drawn,

- no supplements were found to enhance the antibody productivity of either cell line since the control performed the best in all cases in terms of antibody production.
- the 1X (optimal concentration from literature review) enhanced the growth of the myeloma cell line, but not the antibody production.
- further testing with the myeloma cell line should be done to see the long term effects of the supplements on the growth, viability, and antibody production since currently we cannot subculture the myeloma cells for more than six passages.

Chapter 8: Media Optimization

8.1 Introduction

Experiments for the hybridoma and myeloma cell line were performed to determine the optimal concentrations (in terms of growth and antibody production) for BSA-oleic, transferrin, insulin, and serum. These components are expensive and important for cell proliferation. The lipoprotein source purchased from ICN for the myeloma cell line was also optimized. By performing these experiments it is not only hoped to improve cell growth but also to make the media more economically feasible. However, because obtaining good growth in serum free media is difficult, the growth aspect for the cells is the primary concern.

8.2 Experimental Setup

The organization of the experimental program is as follows,

1. Cells were grown in 150 mL Corning spinner flasks containing DIF medium supplemented with 10 % FBS. After a large enough cell density was built up the cells were subcultured twice in the BITES base medium (shown in Tables 8.1 and 8.2 for the hybridoma and myeloma cell line respectively). Subculturing consisted of centrifuging the cell culture (in order to remove all traces of serum) and then resuspending the cell pellet in BITES base medium.
2. Various media (shown below) were prepared to test different concentration ranges of the supplements discussed above namely, BSA-oleic, insulin, transferrin, serum, and egg yolk lipoprotein. The cells were then inoculated into the different media to be tested and cell counts and ELISA assays (to measure the antibody concentration) were performed.

The following sections describe the experiments performed for the two cell lines. For each cell line the experiments will be set up to test a range of concentrations for the chemical being optimized. The BITES base medium consists of all the components of

Table 8.1 - The BITES Base Medium Composition for the Hybridoma Cells (M174) (Total Protein Content = 145 mg/L)

Substance	Base Concentration	Symbol in BITES
BSA - Oleic	130 μg/mL	B
Insulin	5 μg/mL	I
Transferrin	10 μg/mL	T
Serum	0 %	----------
Ethanolamine	30 μmol/L	E
Na-Selenite	10 nmol/L	S
2-mercaptoethanol	30 μmol/L	----------
Trace elements (100X)	10mL/L	----------
Cholesterol	1 μg/mL	----------

Table 8.2 - BITES Base Medium Composition for the Myeloma Cells (C174)
(Total Protein Content = 149 mg/L)

Substance	Base Concentration	Symbol in BITES
BSA - Oleic	130 μg/mL	B
Insulin	5 μg/mL	I
Transferrin	10 μg/mL	T
Serum	0 %	----------
Ethanolamine	30 μmol/L	E
Na-Selenite	10 nmol/L	S
2-mercaptoethanol	30 μmol/L	----------
Trace elements (100X)	10mL/L	----------
Cholesterol	1 μg/mL	----------
ICN Lipoprotein	150 μg/mL	*LDL*

the serum free medium except the ones being tested in that particular experiment. For example, BTES refers to all the components listed in Table 8.1 except insulin all dissolved in DIF medium. In order to test the effect of insulin three separate experiments are performed in T-flasks in which 0, 5 and 10 mg/L of insulin are added to the BTES dissolved in DIF. Cell counts were recorded and samples were taken and stored at -20 °C. ELISA assays were performed at a later date to determine the final antibody concentrations for each T-flask.

Hybridoma Cell Line (M174)

BITES + SERUM

1. BITES (base conc.) + 0.5 % serum (protein: 330 mg/L)
2. BITES (base conc.) + 0.1 % serum (protein: 186 mg/L)
3. BITES (base conc.) + 0 % serum (protein: 150 mg/L)

ITES + BSA-OLEIC

1. ITES(base conc.) + 430 μg/mL BSA-OLEIC (protein:450 mg/L)
2. ITES(base conc.) + 130 μg/mL BSA-OLEIC (protein:150 mg/L)
3. ITES(base conc.) + 0 μg/mL BSA-OLEIC (protein:20 mg/L)

BTES + INSULIN

1. BTES(base conc.) + 10 μg/mL insulin (protein:150 mg/L)
2. BTES(base conc.) + 5 μg/mL insulin (protein:145 mg/L)
3. BTES(base conc.) + 0 μg/mL insulin (protein:140 mg/L)

BIES + TRANSFERRIN

1. BIES(base conc.) + 10 μg/mL transferrin (protein: 150 mg/L)
2. BIES(base conc.) + 5 μg/mL transferrin (protein: 145 mg/L)
3. BIES(base conc.) + 10 μg/mL transferrin (protein: 140 mg/L)

Myeloma Cell Line (C174)

BITES + SERUM

1. BITES (base conc.) + 0.5 % serum (protein:330 mg/L)
2. BITES (base conc.) + 0.1 % serum (protein:186 mg/L)
3. BITES (base conc.) + 0 % serum (protein:150 mg/L)

ITES*LDL* + BSA-OLEIC

1. ITES(base conc.) + 430 μg/mL BSA-OLEIC (protein:454 mg/L)
2. ITES(base conc.) + 130 μg/mL BSA-OLEIC (protein:154 mg/L)
3. ITES(base conc.) + 0 μg/mL BSA-OLEIC (protein:24 mg/L)

BTES*LDL* + INSULIN

1. BTES(base conc.) + 10 μg/mL insulin (protein:154 mg/L)
2. BTES(base conc.) + 5 μg/mL insulin (protein:149 mg/L)
3. BTES(base conc.) + 0 μg/mL insulin (protein:144 mg/L)

BIES*LDL* + TRANSFERRIN

1. BIES(base conc.) + 10 μg/mL transferrin (protein:154 mg/L)
2. BIES(base conc.) + 5 μg/mL transferrin (protein:149 mg/L)
3. BIES(base conc.) + 10 μg/mL transferrin (protein:144 mg/L)

BITES + *LDL*

1. BITES(base conc.) + 300 μg/mL LDL (protein:158 mg/L)
2. BITES(base conc.) + 150 μg/mL LDL (protein:154 mg/L)
3. BITES(base conc.) + 0 μg/mL LDL (protein:150 mg/L)

8.3 Results - Hybridoma Cell Line

The growth and viability of the hybridoma cell line for the different experiments are depicted in Figures 8.1 and 8.2. The antibody production for these same experiments is shown in Figure 8.3. These graphs show that the optimal values for the experiments performed are 430 mg/L BSA, 5 mg/L insulin, and 5 mg/L transferrin. The optimal value for BSA is 430 mg/L because the highest antibody production and maximum cell

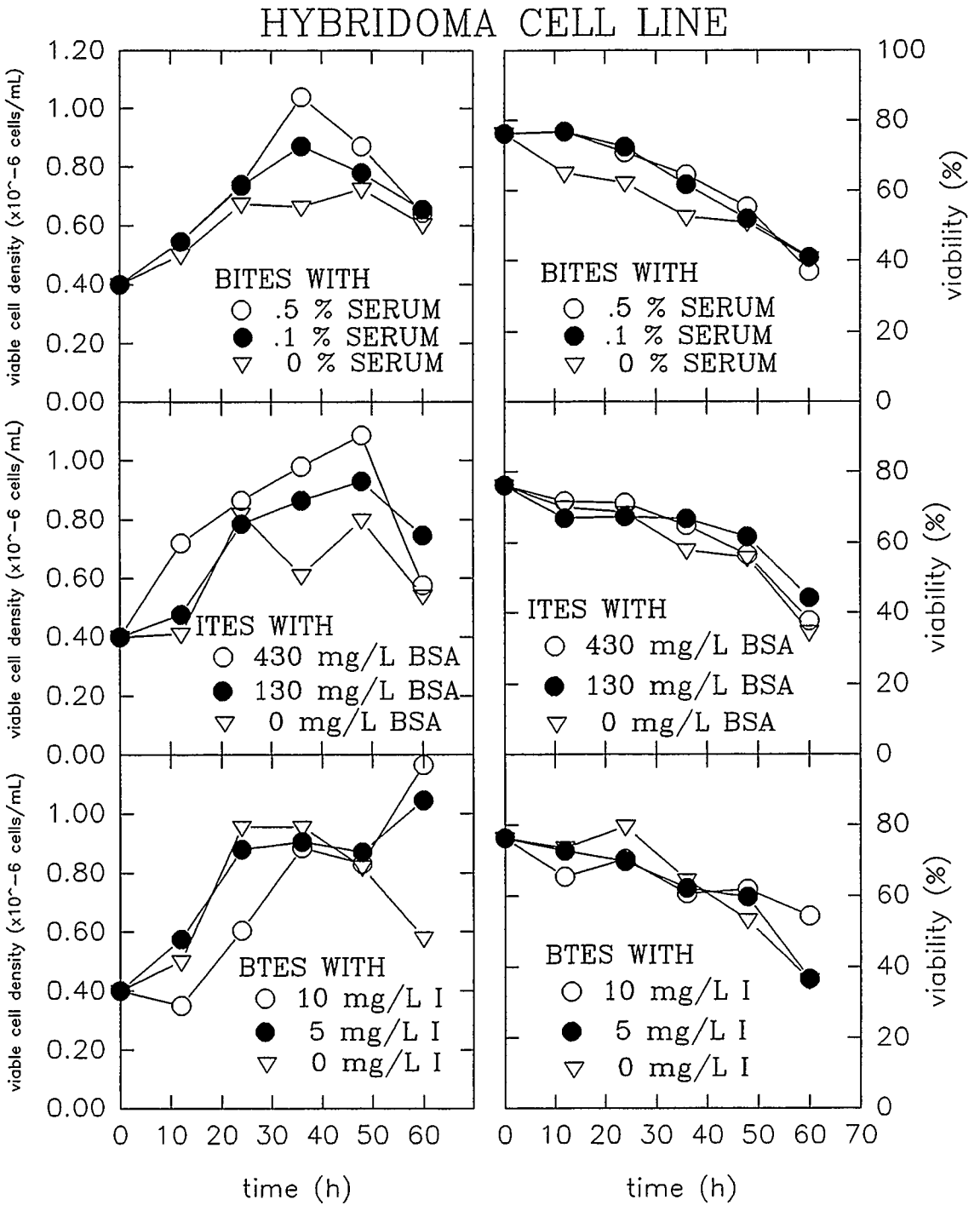


Figure 8.1: Hybridoma cell line optimization experiments. Graphs show growth rate and viability for varying concentrations of serum, BSA (bovine serum albumin), and I (insulin). Experiments were performed in T-flasks in an incubator with a water saturated atmosphere at 37°C with 5 % CO_2.

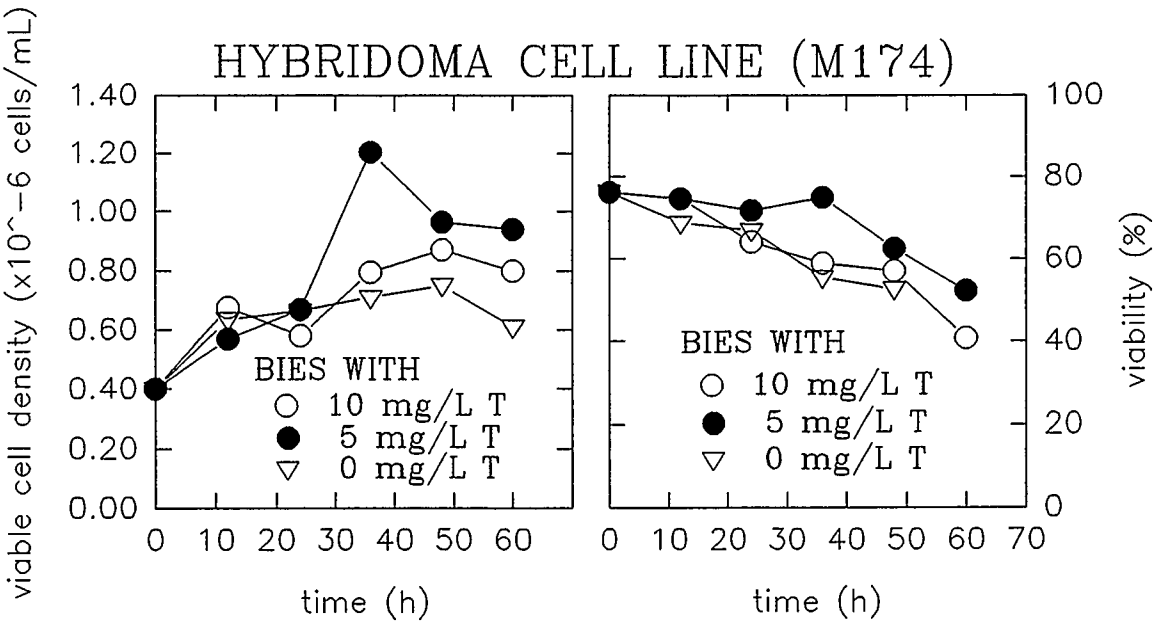


Figure 8.2: Hybridoma cell line optimization experiments showing the effect of varying concentrations of T (transferrin) on cell growth and viability. Experiments were performed in T-flasks in an incubator with a water saturated atmosphere at 37°C and 5 % CO_2.

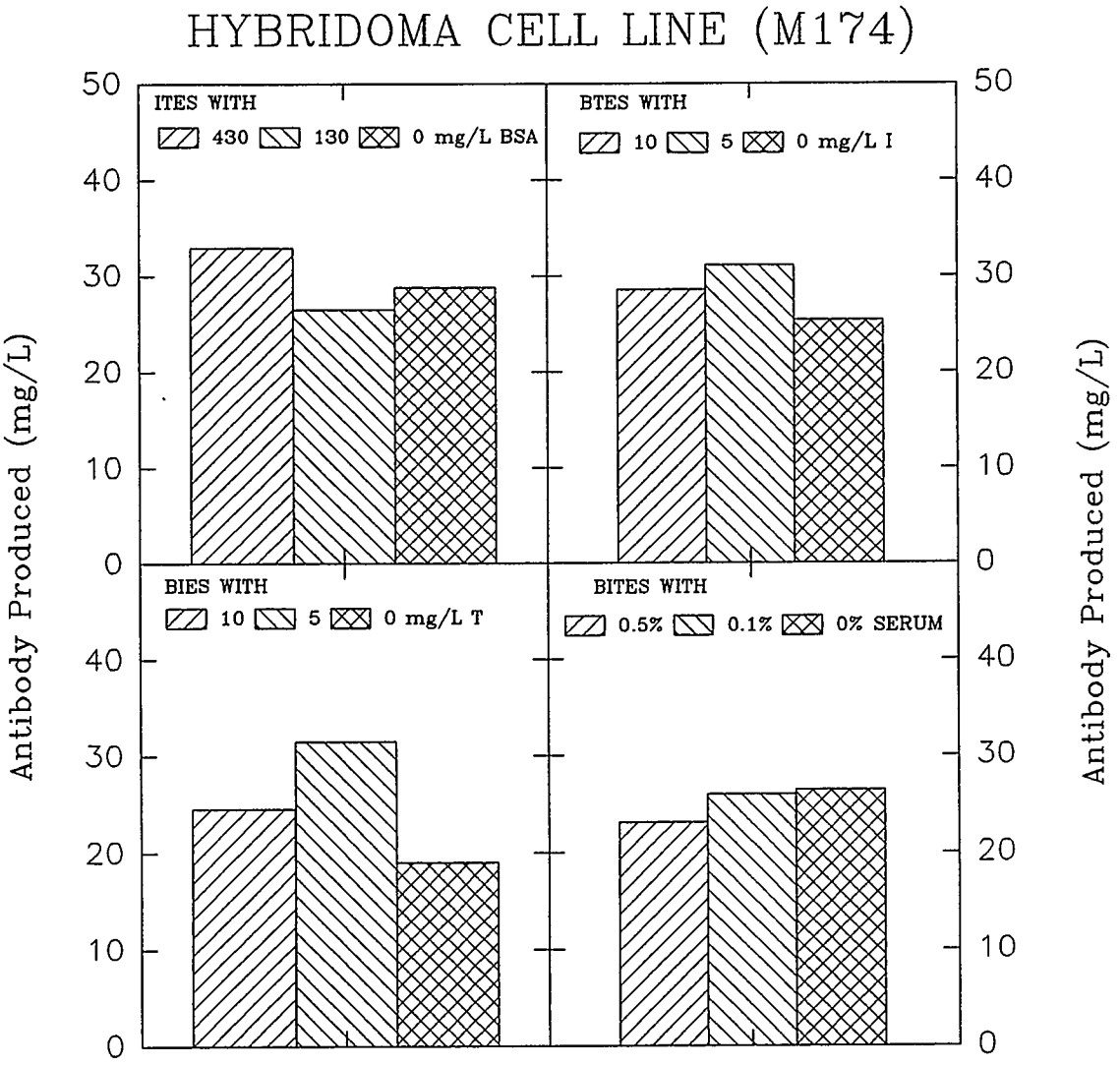


Figure 8.3: Hybridoma cell line optimization experiments showing the effect of BSA (bovine serum albumin), I (insulin), T (transferrin), and serum on the final antibody concentration. Experiments were performed in T-flasks in an incubator with a water saturated atmosphere at 37°C and 5 % CO_2.

density was achieved for this BSA level. For insulin the 10 mg/L and 5 mg/L cases are almost identical so the least expensive scenario was chosen, which is the 5 mg/L case. Also 5 mg/L of transferrin gives the highest antibody and maximum viable cell density.

8.4 Results - Myeloma Cell Line

Figures 8.4 and 8.5 show the growth and viability curves for the myeloma cell line. Figure 8.6 shows the associated antibody concentration. The optimal values for BSA, insulin, transferrin, and ICN low density lipoprotein are 130 mg/L BSA, 5 mg/L insulin, 10 mg/L transferrin, and 150 mg/L ICN LDL. Figure 8.6 shows that the highest antibody production occurs for 130 mg/L BSA consistently (i.e. from subculture to subculture). It also shows that antibody production increases with increasing ICN low density lipoprotein, however a lower value of 150 mg/L ICN LDL was chosen because of the negligible increase in antibody produced and because of the high cost associated ICN low density lipoprotein. It can also be seen that the myeloma cell line does not grow at all when ICN low density lipoprotein is not present in the medium. Also the final antibody concentration is the highest for 10 mg/L insulin and 5 mg/L transferrin. The optimal values for these components were chosen as 5 mg/L insulin and 10 mg/L transferrin because the associated antibody concentrations were almost as high as for 10 mg/L insulin and 5 mg/L transferrin. The growth for these cases is also very good.

8.5 Conclusions

Based on the experimental results described previously, the following conclusions arise,

- The optimum values for the BSA (bovine serum albumin), I (insulin), and T (transferrin) for the hybridoma cell line are 430 mg/L, 5 mg/L, and 5 mg/L respectively with a total protein content of 440 mg/L.
- The myeloma cell line needs serum or ICN LDL to grow. Based on cost considerations, the optimum concentration of ICN LDL is 150 mg/L.
- The optimum values for the myeloma cell line for BSA, insulin, and transferrin are 130 mg/L, 5 mg/L, and 10 mg/L respectively with a corresponding total protein content of approximately 150 mg/L.

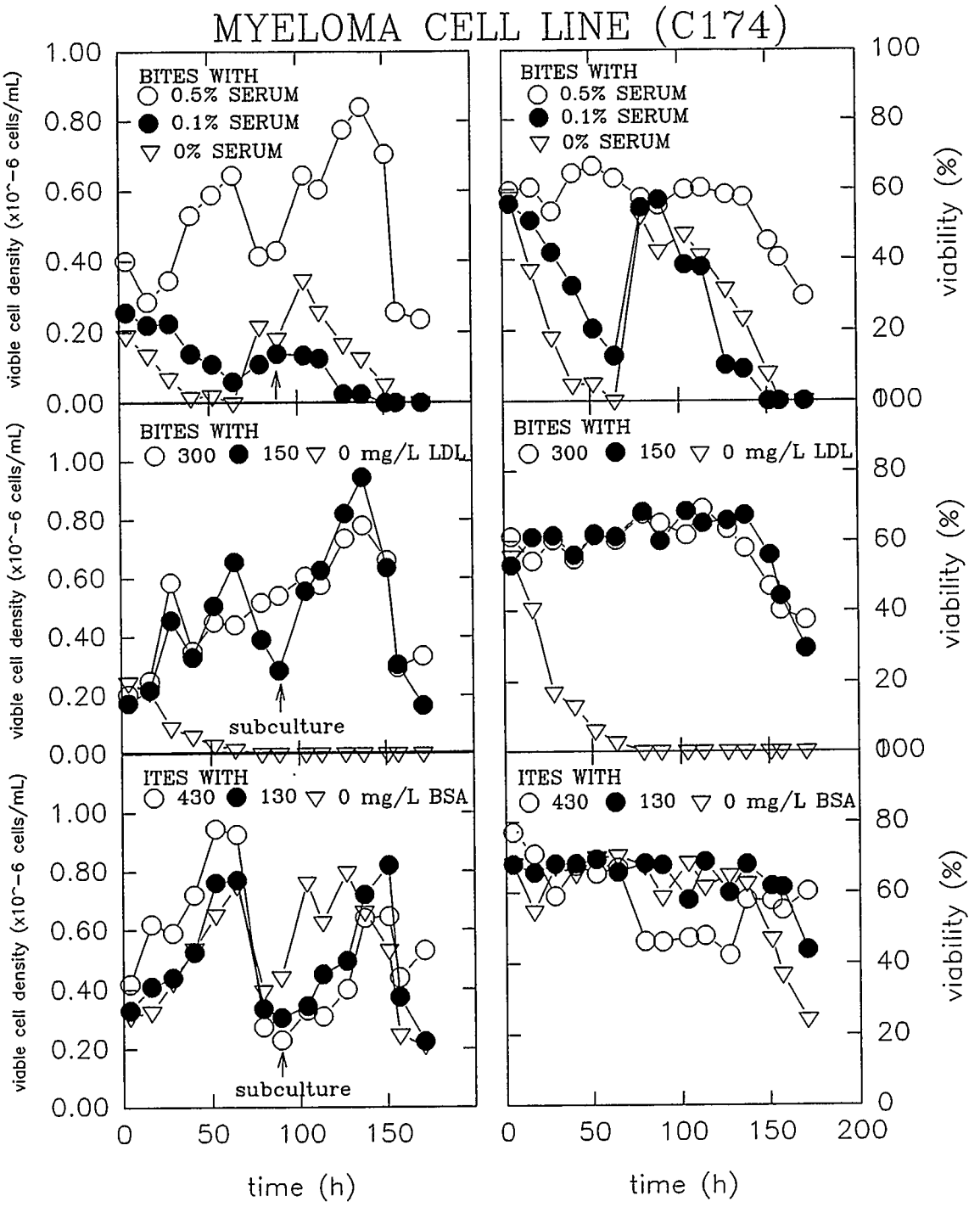


Figure 8.4: Myeloma cell line optimization experiments. Graphs show growth rate and viability for varying concentrations of serum, BSA (bovine serum albumin), and LDL (ICN low density lipoprotein). Experiments were performed in T-flasks in an incubator with a water saturated atmosphere at 37°C and 5 % CO_2.

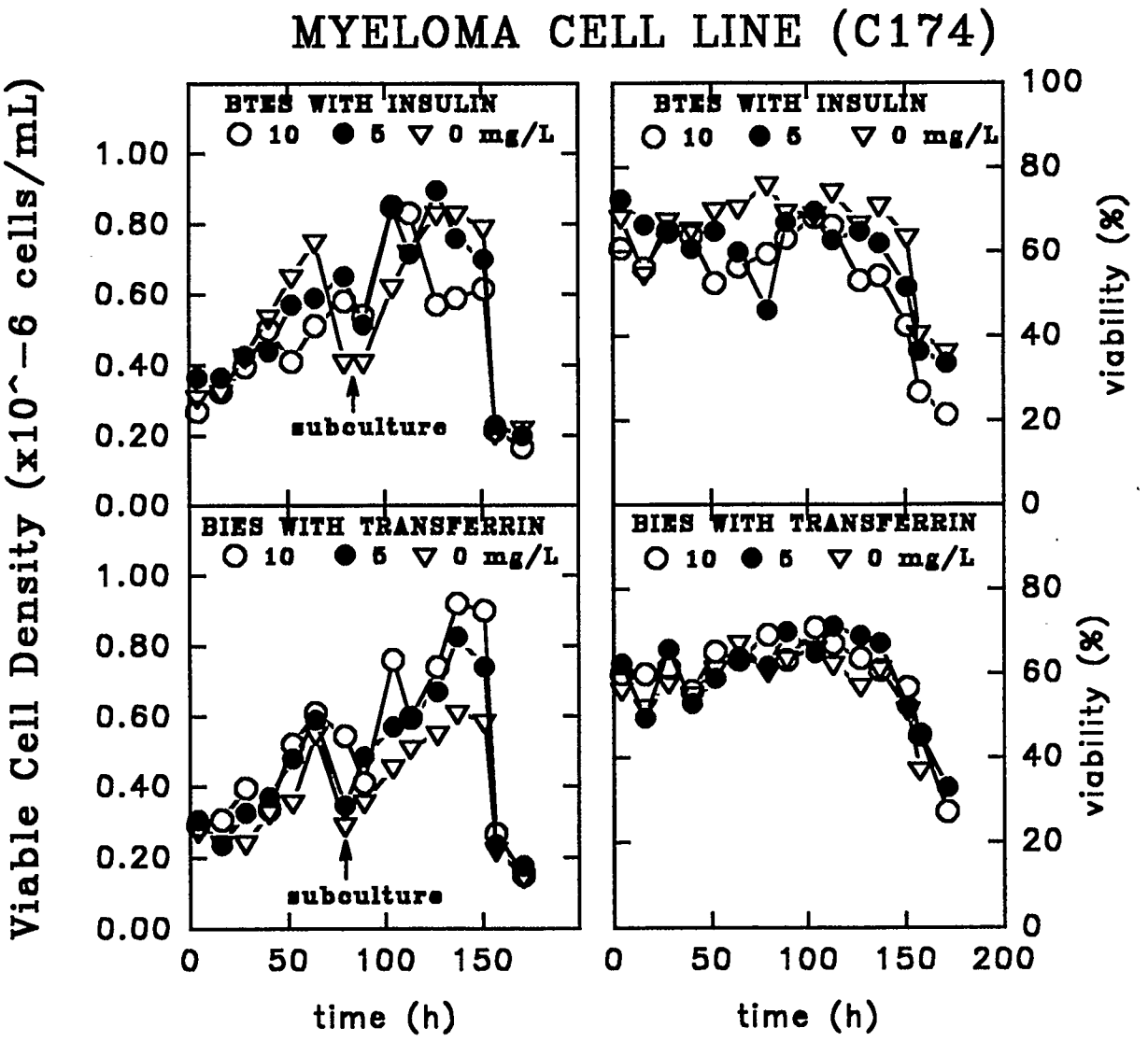


Figure 8.5: Myeloma cell line optimization experiments. Graphs show growth rate and viability for varying concentrations of T (transferrin), and I (insulin). Experiments were performed in T-flasks in an incubator with a water saturated atmosphere at 37°C and 5 % CO_2.

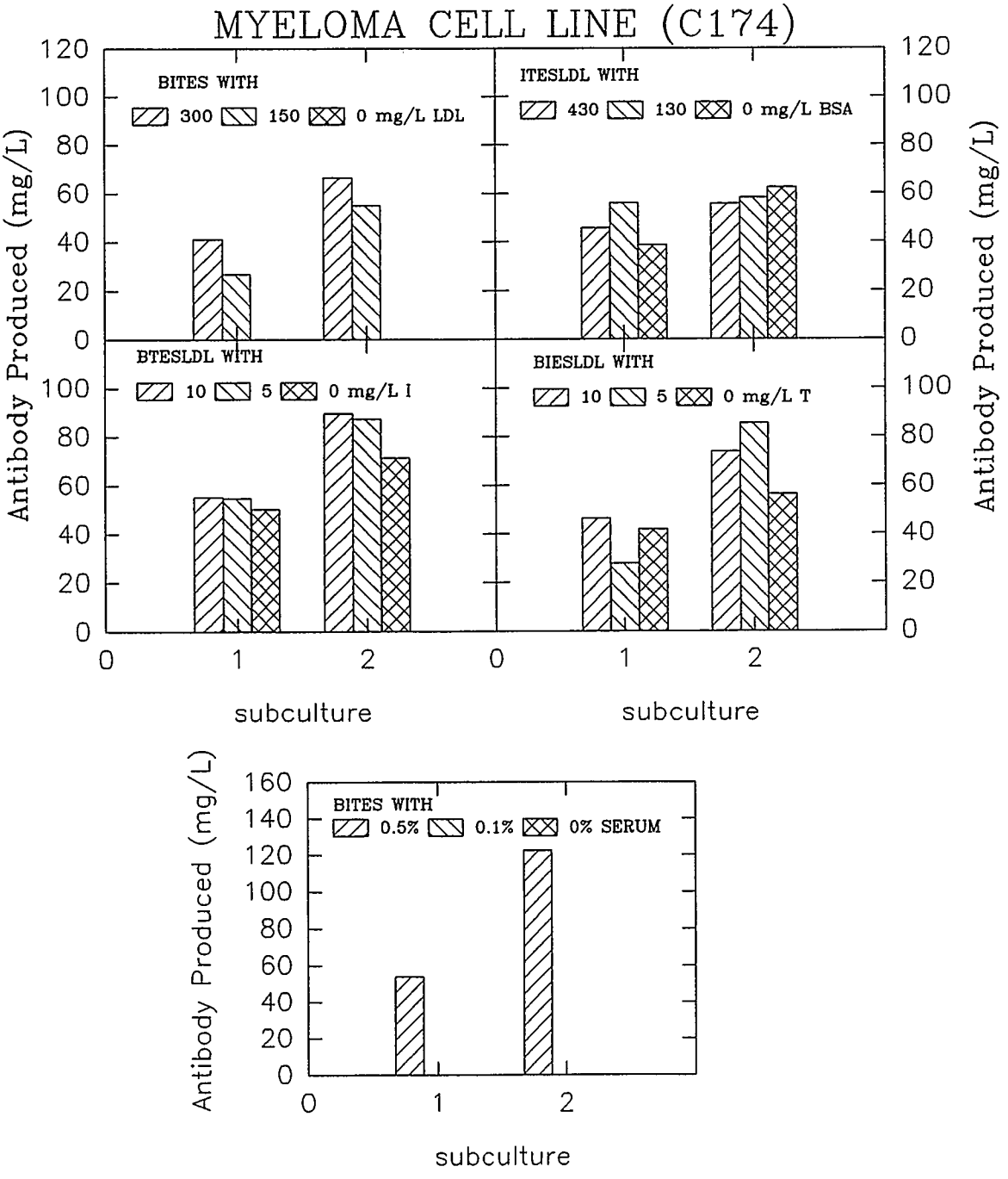


Figure 8.6: Myeloma cell line optimization experiments showing the effect of BSA (bovine serum albumin), I (insulin), T (transferrin), LDL (ICN low density lipoprotein), and serum on the final antibody concentration. Experiments were performed in T-flasks in an incubator with a water saturated atmosphere at 37°C and 5 % CO_2.

Chapter 9: Scale-up

9.1 Introduction

This chapter deals with the scale-up of the developed serum free medium (SFM) into a 1.5 L Celligen bioreactor. The growth, antibody production, and nutrient consumption rates of both cell lines were measured and compared to the growth curves generated using DIF supplemented with 10 % FBS given in Chapter 4. The cell characteristics obtained in Chapter 4 were performed in order to establish a reference base case to which we could compare our developed serum free medium. The importance of these experiments is to establish that the cells can be inoculated at a lower initial cell density (approx 1 x 10^5 cells/mL) and still follow a normal growth curve, as well as to ensure that the cells produce similar quantities of antibody as in medium supplemented with serum. DIF (DMEM, IMDM, and F12 in a 1:1:1 ratio) supplemented with the BITES formulation shown in Table 9.1 was used as the SFM medium for these experiments. In addition, Pluronic F68 was added at 0.2 %, a level we normally use in order to protect animal cells from shear damage (Zhang,1993). The pluronic is a polymer which is used to increase the viscosity of the medium and thus reduce the shear forces occurring in the bioreactor. We used Excite Lipoprotein instead of ICN Low Density Lipoprotein in the formulation for the myeloma cell line because it gives the same result and is less expensive. Excite lipoprotein is extracted from the blood of cows. At present the myeloma cell line cannot be subcultured for more than seven passages, and the hybridoma cell line cannot be adapted short term and loses antibody productivity when a long term adaptation is attempted. Hence, because of these constraints, the SFM developed must be used in the production of antibody in batch culture, since it can only support the very short term growth of cells. The advantage of doing this would be to reduce medium costs and/or simplify the purification process by reducing the protein content in the final production phase.

9.2 Experimental Setup

The experiment consisted of the following steps:

Table 9.1 - DIF Supplemented with BITES (serum free formulation) used in the 1.5 L Celligen Bioreactor Experiments for the Hybridoma and Myeloma Cell Lines

Substance	Concentration
BSA-Oleic	135 μg/mL
Insulin	5 μg/mL
Transferrin	10 μg/mL
Ethanolamine	30 μmol/L
Na-Selenite	10 μmol/L
2-Mercaptoethanol	30 μmol/L
Trace Elements (100X)	10mL/L
Cholesterol	1 μg/mL
Excite Lipoprotein *	125 μg/mL
Total Protein - Myeloma	**225 μg/mL**
Total Protein - Hybridoma	**150 μg/mL**

* ***Note*** Excite Lipoprotein is only used for the myeloma cell line and has a protein content of 75 mg/L at the concentration used.

1. An inoculum was generated in spinner flasks over a 3 day period in DIF supplemented with 10 % FBS serum.
2. On the day of the inoculation, enough cells to inoculate the 1.5 L Celligen bioreactor at 1 x 10^5 cells/mL were resuspended in a spinner flask in the serum free formulation.
3. After preparation of the bioreactor, which included sterilization and warming of the medium, the reactor was inoculated with the cells from step 2.

9.3 Results - Hybridoma Cell Line (M174)

The viable cell density, viability, and antibody concentration for the hybridoma cell line is shown in Figure 9.1 for the hybridoma cells given in serum free medium (Protein Concentration = 150 mg/L) and in DIF + 10 % FBS. The maximum viable cell density reached was approximately 2.0 x 10^6 cells/mL while the viability was virtually identical for both media. The major substrates (glucose and glutamine) were equalized in this experiment to the levels found in DMEM (4.5 g/L glucose and 4 mmol/L glutamine). The antibody concentration reached was about 45 mg/L for the medium supplemented with serum, unfortunately the serum free medium did not produce any antibody. It has previously been noted that after approximately a month of culturing (70 generations) these cells lose the ability to produce antibody. Unfortunately these cells had been cultured for approximately 70 generations before the Celligen experiment was performed. The loss of antibody may occur because of genetic drift (i.e. loss of chromosomes) in which a new non-producing clone develops and takes over the culture. Another possibility is that a non-producing clone may exist within the culture (i.e. poor HAT selection). The clone slowly takes over the culture and eventually after a month dominates it. It is important to note that these non-producing cells grew very well in the serum free formulation. The lactic acid produced was about the same for both media at just over 2 g/L. The glucose consumption occurred somewhat faster in the serum free medium and reached a level of about 1.0 g/L unlike the serum supplemented medium in which the final glucose concentration was about 2.0 g/L. Another important finding was

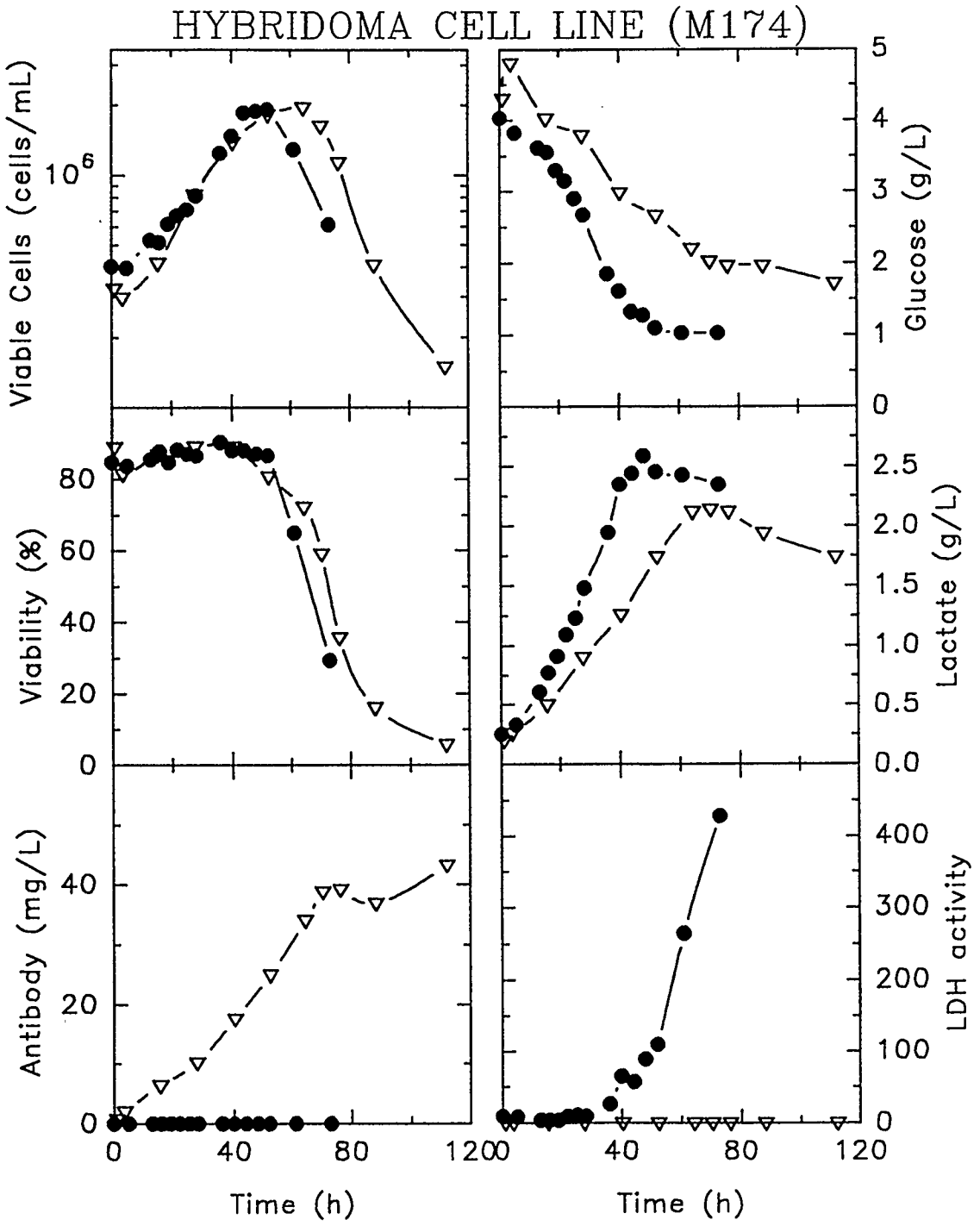


Figure 9.1: Graphs showing various hybridoma (M174) cell characteristics. The symbols indicate a batch Celligen bioreactor run (1.5 L) in DIF medium supplemented with BITES and Pluronic F68 (●), and a batch run in a 150 mL Corning spinner flask in DIF medium supplemented with 10 % FBS (▽). Experiments in the Celligen bioreactor were performed with controlled pH (7.2) and temperature (37°C). Experiments in the spinner flask were performed in an incubator with a water saturated atmosphere at 37°C and 5 % CO_2.

that the LDH activity (measure of the cell lysis) was quite high in the serum free medium. From examining the growth curve, the lysis begins to occur in the death phase and thus it is reasonable to assume that lysis of dead cells is occurring. Pluronic F68 was used at 0.2 % in the serum free medium to inhibit the effects of shear on the cells for the Celligen runs only. By increasing the Pluronic F68 we feel that the lysis due to shear can be eliminated altogether.

Figure 9.2 shows the consumption of the major substrate (glutamine) and the associated production of ammonia. The consumption of glutamine is the limiting nutrient causing the death of the culture in both media. The life of the culture and the final antibody concentration may be increased by supplementing the media with extra glutamine. The ammonia concentration reaches a maximum of about 1.75 mmol/L and 2.5 mmol/L for the SFM and serum supplemented media respectively. Figure 9.2 also shows that a larger amount of aspartate and serine could be added to the serum free media since it is completely consumed by the hybridoma cells. These amino acids can either be added at the beginning of the batch run (i.e. fortify medium) or they can be added at a later time (fed batch) during the batch run. Glutamate is completely consumed in the serum supplemented media whereas it is produced (from glycolysis of glutamine) in the serum free media. The glycine concentration remain relatively constant for both media.

Figures 9.3, and 9.4 give the concentration of various amino acids at different times during the batch run. Again, arginine/threonine and tryptophan/methionine were lumped together because their respective peaks could not be separated on the HPLC. Tryptophan/methionine, leucine, isoleucine, valine, and phenylanaline were completely consumed in the serum free media formulation and should probably be added if the media is used for production purposes. As stated previously, due to the complex nature of cell culture biochemical reactions, the limiting nutrients in the serum free medium may or may not be important. Further experiments are needed in order to determine the effect on growth and antibody production.

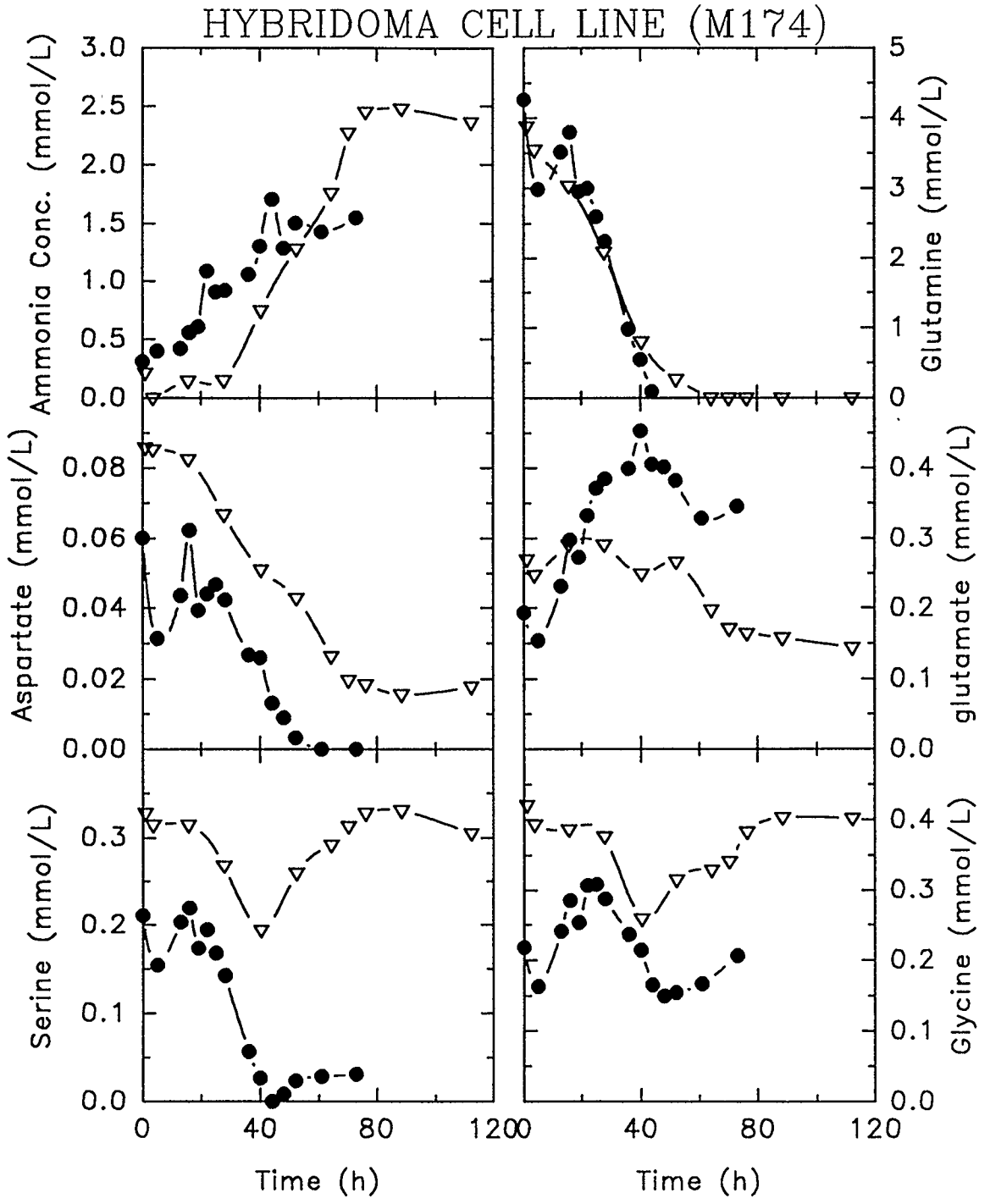


Figure 9.2: Graphs showing various hybridoma (M174) cell characteristics. The symbols indicate a batch Celligen bioreactor (1.5 L) run in DIF medium supplemented with BITES and Pluronic F68 (●), and a batch run in a 150 mL Corning spinner flask in DIF medium supplemented with 10 % FBS (▽). Experiments in the Celligen bioreactor were performed with controlled pH (7.2) and temperature (37°C). Experiments in the spinner flask were performed in an incubator with a water saturated atmosphere at 37°C and 5 % CO_2.

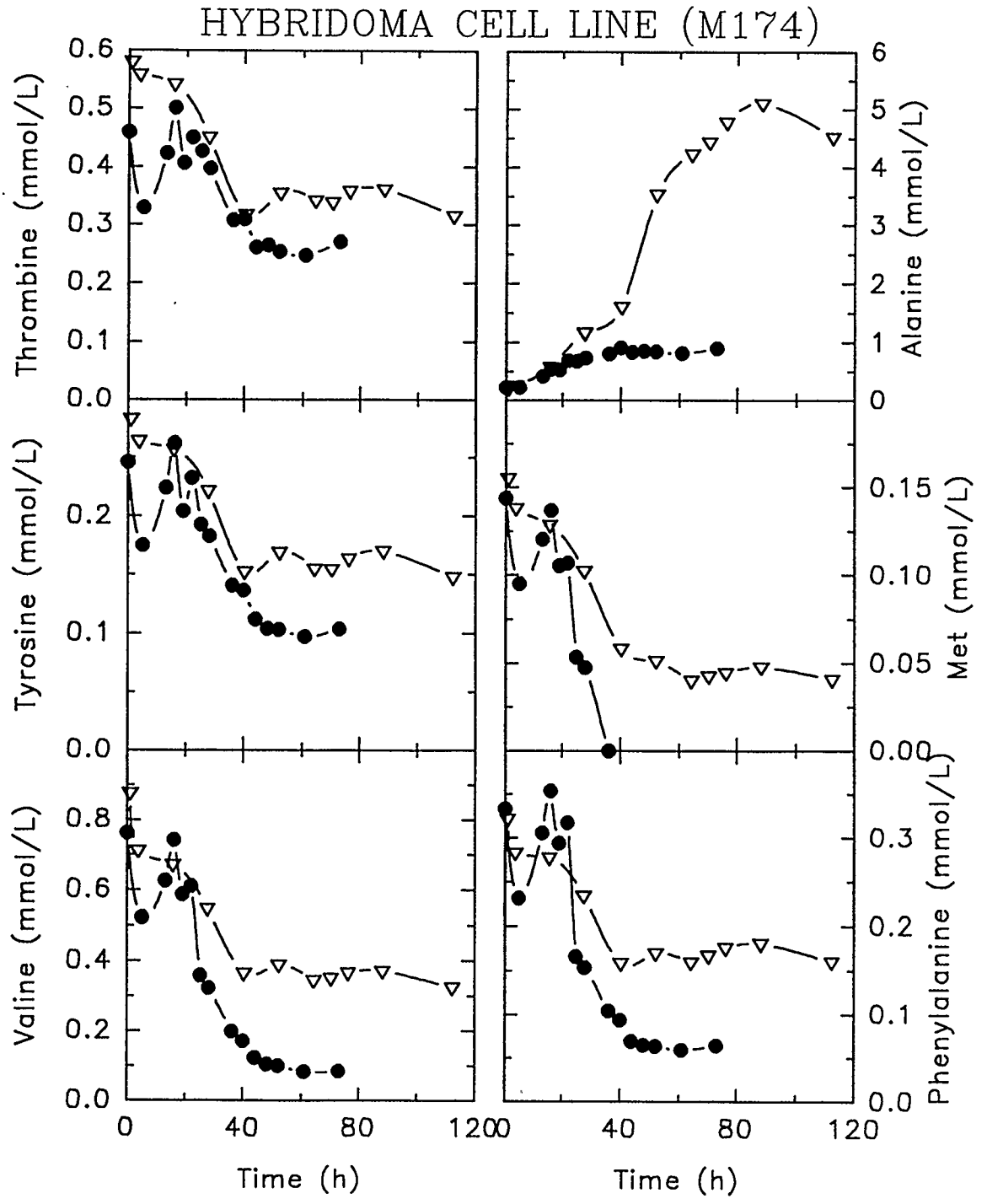


Figure 9.3: Graphs showing various hybridoma (M174) cell characteristics. The symbols indicate a batch Celligen bioreactor (1.5 L) run in DIF medium supplemented with BITES and Pluronic F68 (●), and a batch run in a 150 mL Corning spinner flask in DIF medium supplemented with 10 % FBS (▽). Experiments in the Celligen bioreactor were performed with controlled pH (7.2) and temperature (37°C). Experiments in the spinner flask were performed in an incubator with a water saturated atmosphere at 37°C and 5 % CO_2.

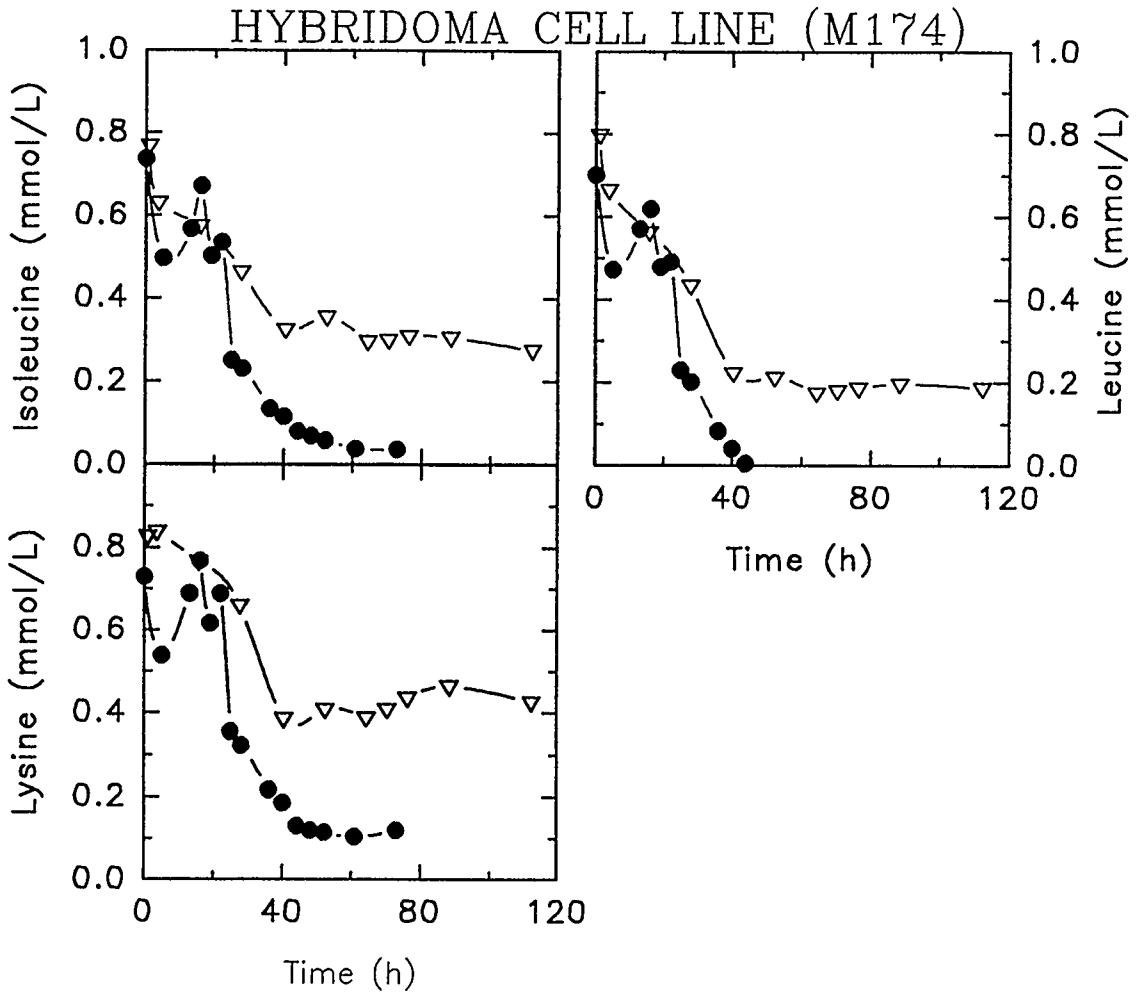


Figure 9.4: Graphs showing various hybridoma (M174) cell characteristics. The symbols indicate a batch Celligen bioreactor (1.5 L) run in DIF medium supplemented with BITES and Pluronic F68 (●), and a batch run in a 150 mL Corning spinner flask in DIF medium supplemented with 10 % FBS (▽). Experiments in the Celligen bioreactor were performed with controlled pH (7.2) and temperature (37°C). Experiments in the spinner flask were performed in an incubator with a water saturated atmosphere at 37°C and 5 % CO_2.

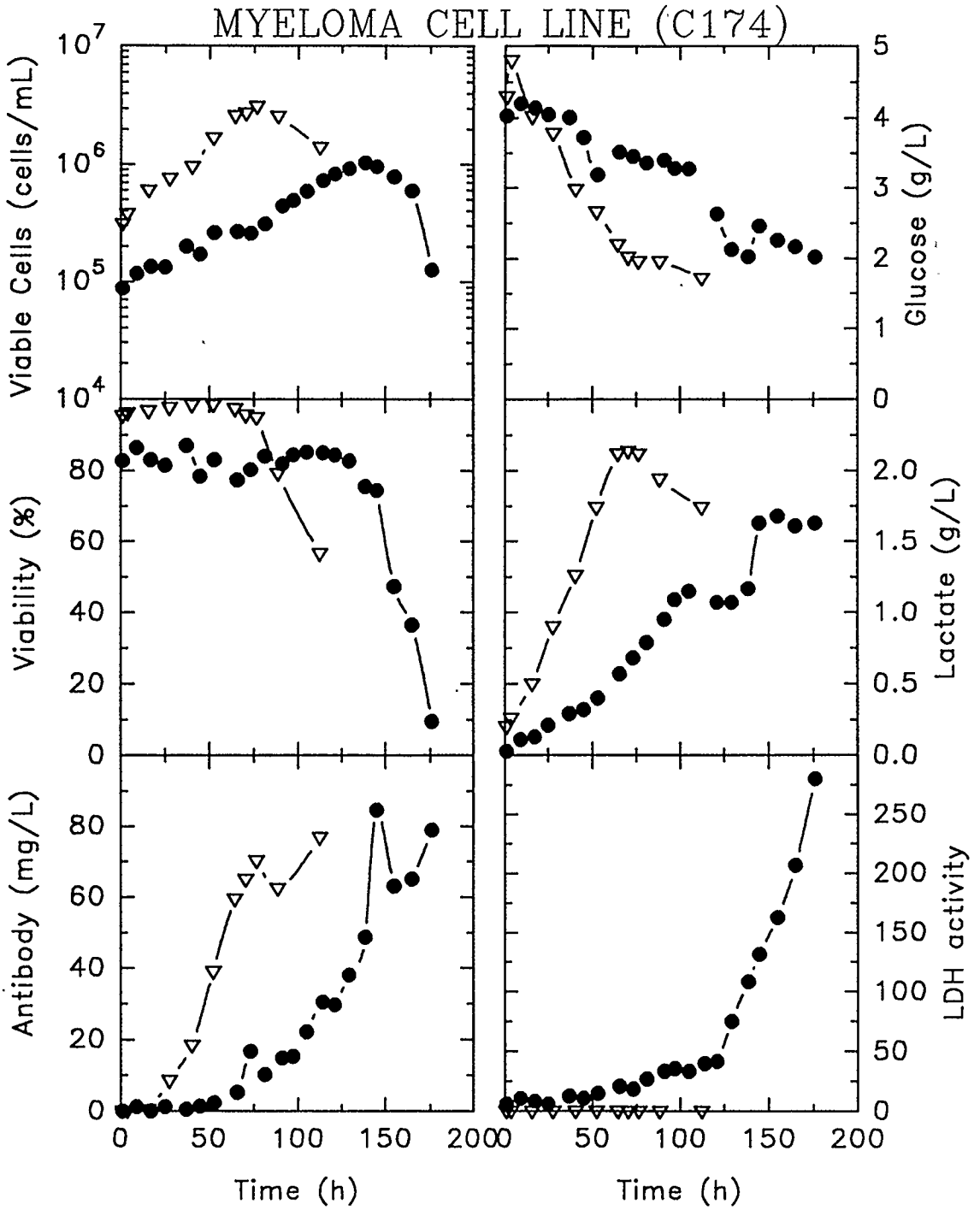


Figure 9.5: Graphs showing various recombinant myeloma (C174) cell characteristics. The symbols indicate a batch Celligen bioreactor run (1.5 L) in DIF medium supplemented with BITES and Pluronic F68 (●), and a batch run in a 150 mL Corning spinner flask in DIF medium supplemented with 10 % FBS (▽). Experiments in the Celligen bioreactor were performed with controlled pH (7.2) and temperature (37°C). Experiments in the spinner flask were performed in an incubator with a water saturated atmosphere at 37°C and 5 % CO_2.

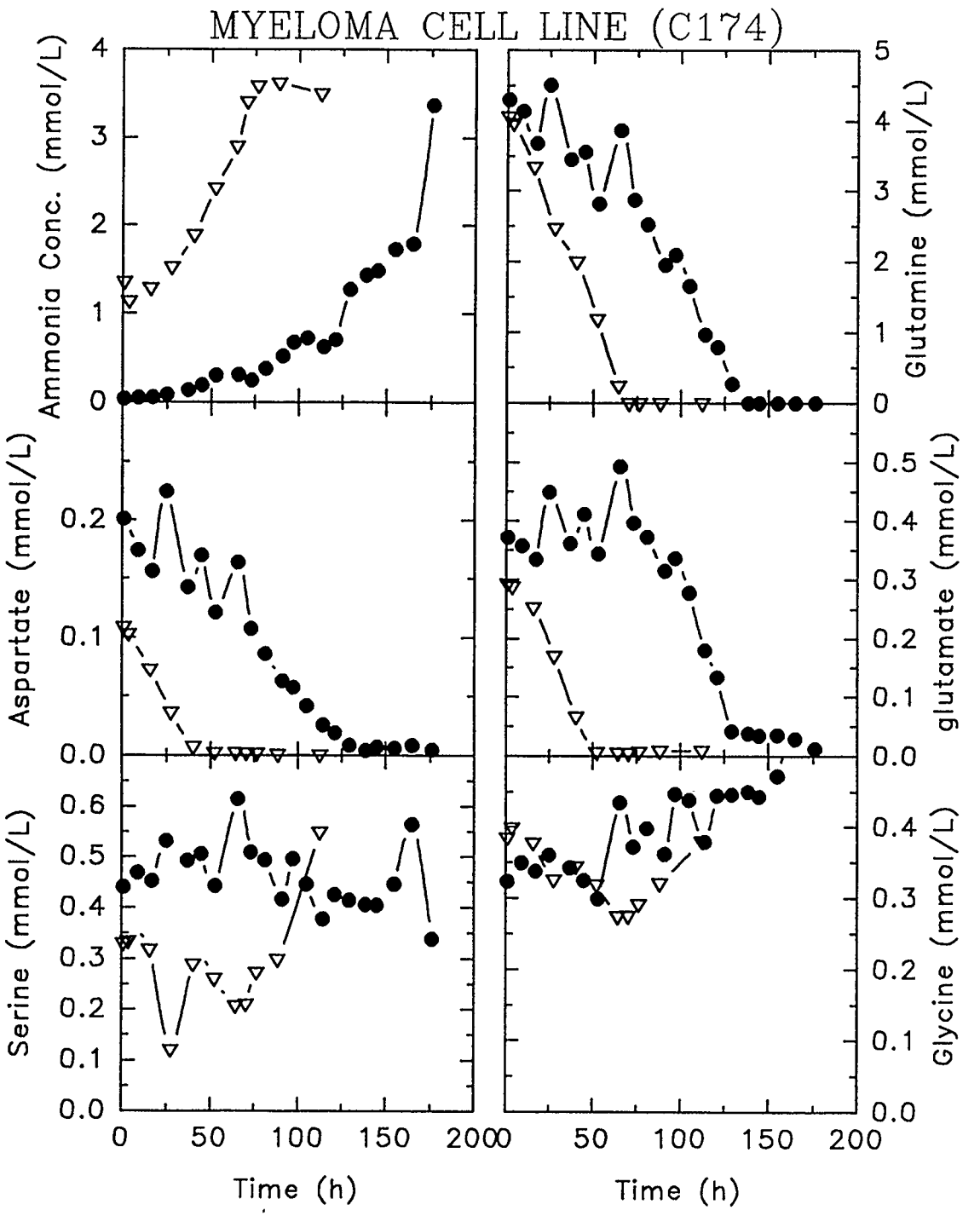


Figure 9.6: Graphs showing various recombinant myeloma (C174) cell characteristics. The symbols indicate a batch Celligen bioreactor run (1.5 L) in DIF medium supplemented with BITES and Pluronic F68 (●), and a batch run in a 150 mL Corning spinner flask in DIF medium supplemented with 10 % FBS (▽). Experiments in the Celligen bioreactor were performed with controlled pH (7.2) and temperature (37°C). Experiments in the spinner flask were performed in an incubator with a water saturated atmosphere at 37°C and 5 % CO_2.

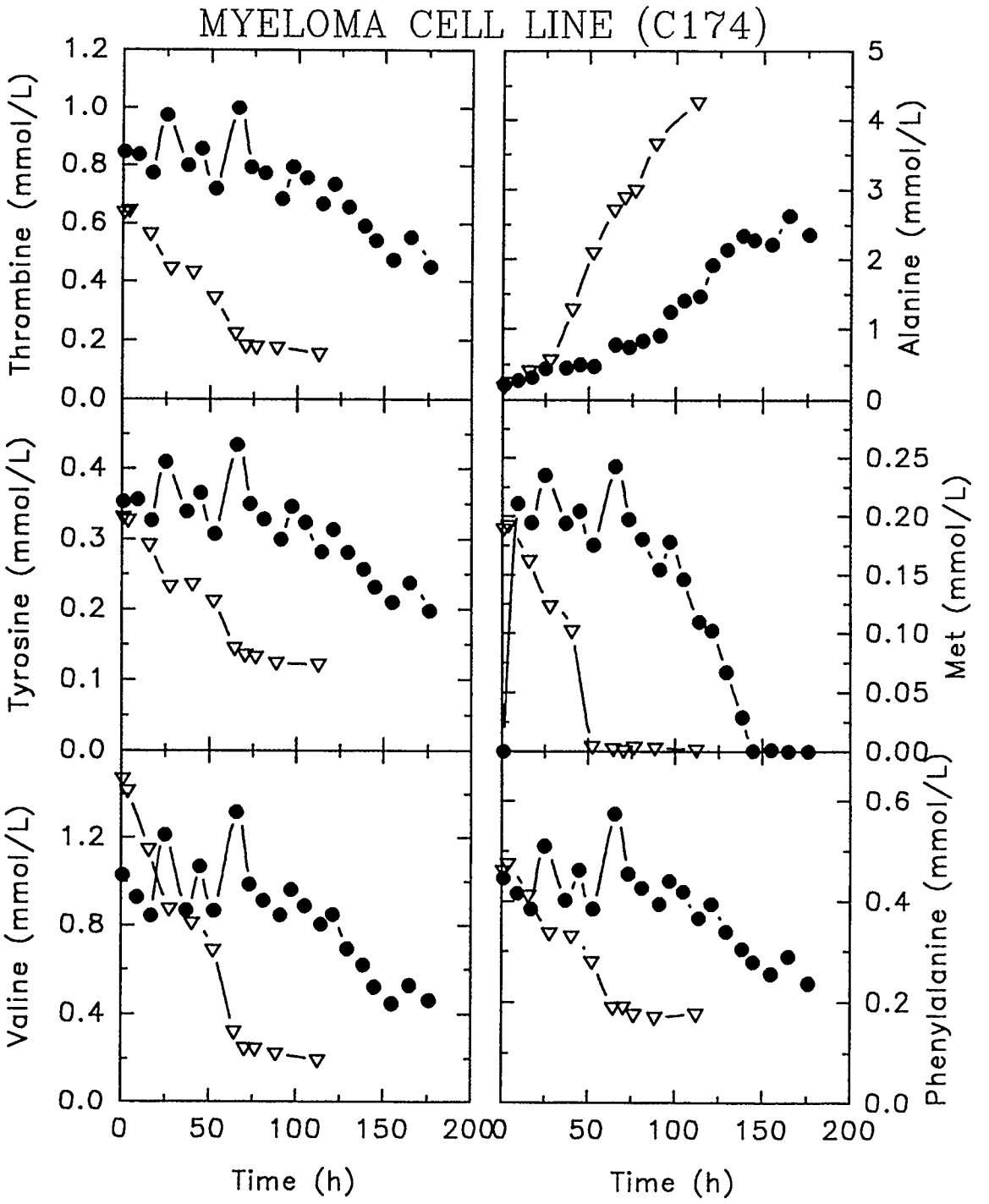


Figure 9.7: Graphs showing various recombinant myeloma (C174) cell characteristics. The symbols indicate a batch Celligen bioreactor (1.5 L) run in DIF medium supplemented with BITES and Pluronic F68 (●), and a batch run in a 150 mL Corning spinner flask in DIF medium supplemented with 10 % FBS (▽). Experiments in the Celligen bioreactor were performed with controlled pH (7.2) and temperature (37°C). Experiments in the spinner flask were performed in an incubator with a water saturated atmosphere at 37°C and 5 % CO_2.

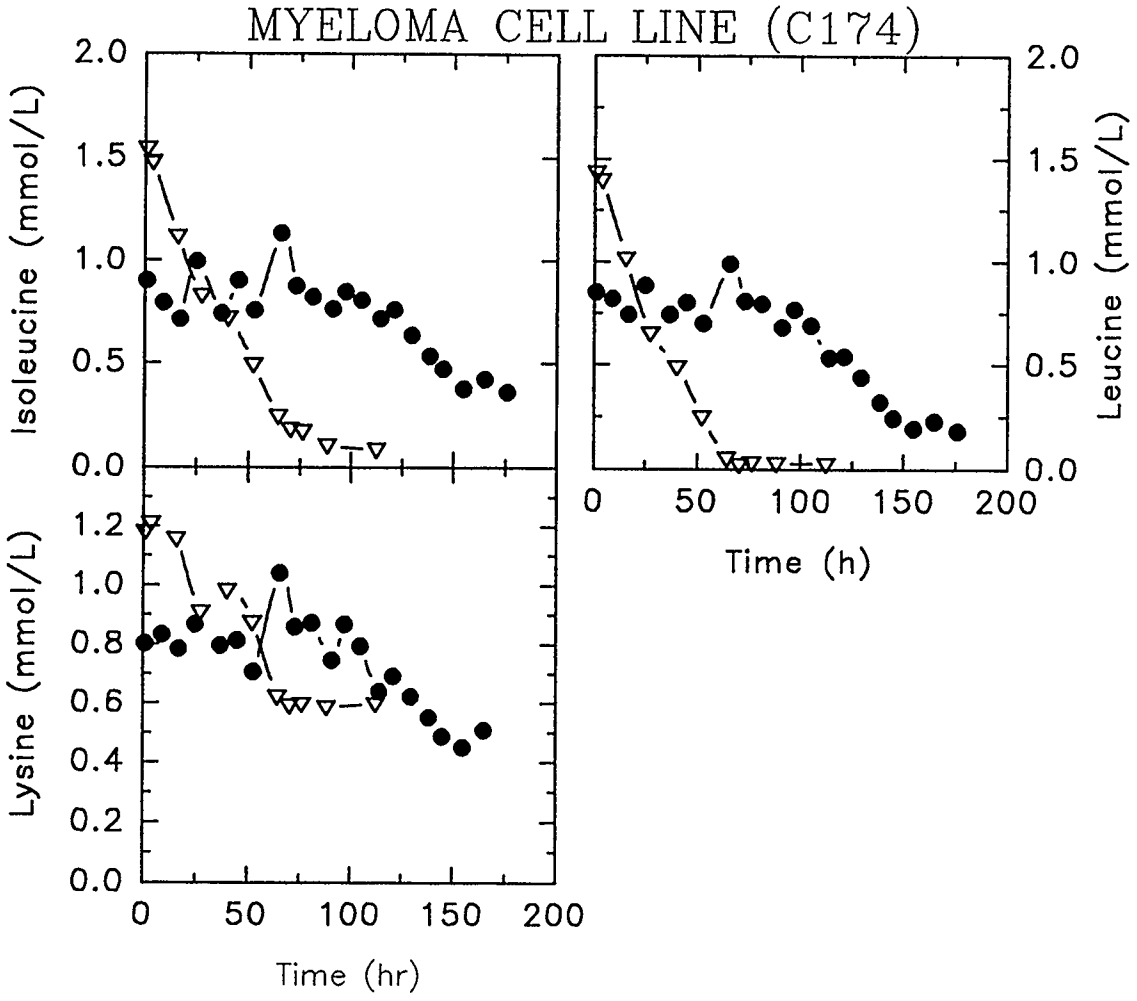


Figure 9.8: Graphs showing various recombinant myeloma (C174) cell characteristics. The symbols indicate a batch Celligen (1.5 L) bioreactor run in DIF medium supplemented with BITES and Pluronic F68 (●), and a batch run in a 150 mL Corning spinner flask in DIF medium supplemented with 10 % FBS (▽). Experiments in the Celligen bioreactor were performed with controlled pH (7.2) and temperature (37°C). Experiments in the spinner flask were performed in an incubator with a water saturated atmosphere at 37°C and 5 % CO_2.

9.4 Results - Recombinant Myeloma Cell Line (C174)

The results for the myeloma cell line are shown in Figures 9.5 to 9.8. Figure 9.5 shows the antibody production, viability, viable cell density, as well as the glucose concentration, lactate concentration and LDH activity. The maximum cell density reached was about 3 x 10^6 cells/mL and 1 x 10^6 cells/mL for the serum supplemented and serum free media respectively. The corresponding antibody produced was about 80 mg/L for both media. Again, note that the protein content of the serum free medium was 220 mg/L. As can be seen on all graphs the myeloma cells exhibit a significant lag when inoculated directly from 10 % FBS into the serum free medium (almost two days). The lag phase may be shortened by using Nutridoma NS to grow an inoculum for larger bioreactors. Again further experiments are required. The curves for glucose and lactate are very similar except that the cells growing on serum free medium grow slower in part to a longer lag phase. In addition, there is significant LDH activity for the serum free medium culture, due to lysis of dead cells. Death of live cells may be occurring due to excessive shear forces in the Celligen bioreactor. The solution is to increase the Pluronic F68 concentration (0.2 % Pluronic was used in the experiment).

Figure 9.6 to 9.8 show various amino acid concentrations for the myeloma cell culture. The following conclusions can be made concerning these figures:

- All amino acids show the same trends, except there is a longer lag phase in the serum supplemented media.
- Glutamine, Glutamate, Aspartate, and Methionine are completely consumed by the myeloma cells.
- The ammonia concentration reaches approximately 3 mmol/L for both media.
- Serine remains relatively constant in both media.
- Alanine and Glycine are produced for both media.

9.5 Conclusions

The following conclusions can be made concerning the scale-up of the hybridoma and

myeloma cell line into 1.5 L Celligen bioreactors:

Hybridoma Cell Line

- Glutamine was found to be the limiting substrate and amino acids such as leucine, isoleucine, and phenylalanine were completely consumed by the cells.
- LDH activity in the serum free medium increased dramatically as the dead cells were lysed in the Celligen bioreactor.
- No antibody was produced in the serum free medium due to the age of the cells used (70 generations old).

Myeloma Cell Line

- Again, glutamine was found to be the limiting substrate, and amino acids such as glutamate, aspartate, and methionine were completely consumed by the cells.
- The antibody produced in the serum free medium (Celligen bioreactor) was the same as the antibody produced in medium supplemented with 10 % FBS (Spinner flasks). It should be noted that the batch growth of hybridoma cells in a Celligen bioreactor is very similar to the growth in large spinner flasks.
- LDH activity was higher in the serum free medium.

Chapter 10: Economic Evaluation

10.1 Introduction

One potential advantage in using a serum free medium is the potential cost savings compared to serum supplemented media or commercially available serum free medium (i.e. Nutridoma NS). However, care must be taken in any SFM development program to keep the costs under control. This section will compare the cost of the serum free medium we have developed (DIF+BITES) to the cost of serum supplemented medium and to the cost of Nutridoma NS (a commercially available serum free medium). The cost comparison will be based on the cost per litre of medium. This approach is chosen since the antibody produced is almost identical when comparing the different media studied for the myeloma cell line and may also be true for the hybridoma cell line. However, it should be noted that medium supplemented with serum and medium supplemented with Nutridoma NS can support growth of the cell lines studied for long periods of time whereas our developed medium would be used in the final stages of the production process.

10.2 Cost Analysis

Table 10.1 shows the cost of the supplements used in our developed serum free medium. The highest priced items used are insulin, transferrin, trace elements, and lipoprotein. One possible cost saving measure would be to remove the trace element mix which would reduce the cost of the medium by 33 %. Removing this component would likely have no effect on the growth of the cells and the antibody produced.

Table 10.2 shows the cost comparison of different media for the myeloma cell line. The lowest cost (25.10 $/L) occurs for the medium supplemented with BITES and 1 % serum without any lipoprotein added. Although no results are shown, the myeloma cell line grew fairly well in this medium. Further experiments would have to be performed in order to determine the performance in a larger bioreactor. Our serum free medium has the second lowest cost at 37.80 $/L of the media listed in the table. The only drawback

Table 10.1 - Cost of Supplements from Sigma Chemical Co. for 1 L of our Developed Serum Free Production Medium (DIF + BITES).

Substance	Concentration in Media	Cost/L medium for desired conc.
Insulin	5 μg/mL	1.186 $/L
Transferrin	10 μg/mL	4.06 $/L
BSA	130 μg/mL	0.227 $/L
Ethanolamine	30 μmol/L	0.0000365 $/L
Trace Elements (100X)	10 mL/L	11.13 $/L
Selenium	10 nmol/L	0.00044 $/L
2-Mercaptoethanol	30 μmol/L	0.0009 $/L
Cholesterol	1 μg/mL	0.004 $/L
Basal Medium	----------	4.00 $/L
Total Cost of Supplements (hybridoma cell line)	----------	**20.60 $/L**
Excite Lipoprotein* (25g/L)	**125 μg/mL**	**17.20 $/L**
Total Cost of Supplements (myeloma cell line)	----------	**37.80 $/L**

***Note:** The Excite was only used for the myeloma cell line, it is not required for the hybridoma cell line. ICN LDL was not used because it is more expensive than the Excite lipoprotein.

Table 10.2 - Cost Comparison between our Developed Serum Free Medium for the Myeloma Cell Line and the Media Supplemented with Nutridoma NS and Serum.

Medium	Protein Content	Cost per L of Medium
1 % Nutridoma NS	150 mg/L	54.40 $/L
2 % Nutridoma NS	300 mg/L	102.80 $/L
3 % Nutridoma NS	450 mg/L	152.20 $/L
10 % Fetal Bovine Serum	3600 mg/L	49.00 $/L
1 % serum + BITES (no Lipoprotein)	510 mg/L	25.10 $/L
Developed Serum Free Medium	220 mg/L	37.80 $/L

to using this medium is that it can only be used for production purposes since the myeloma cells can only be grown in this medium for a short period of time (approximately seven subcultures). Three different concentrations of Nutridoma NS are given in the table because the Nutridoma supplement had to be used at 2 % and 3 % for the myeloma and hybridoma cells respectively to achieve good growth. Although the supplier recommends Nutridoma be used at a level of 1 %, we had no success with the supplement at this concentration. The result of having to use higher concentrations of Nutridoma NS is dramatic making the cost of using Nutridoma at 2 % 102.80 $/L and at 3 % 152.20 $/L. To put this in perspective lets consider the cost of the medium required to operate a 500 L batch bioreactor 20 times a year. If we use 2 % Nutridoma the cost of the medium will be $ 10280000.00. By using our developed medium the cost will be $ 3780000.00, a savings of 6.5 million dollars. Of course this depends heavily on the development of a simple production process in which our medium can be implemented.

Finally, Table 10.3 shows the relative costs of the media available for use in the hybridoma cell line. The medium we have developed can yield significant savings since it is much less expensive than the other media costing only $ 20.60. Nutridoma NS had to be used at 3 % for the hybridoma cell line leading to a much higher relative price. Again it should be noted that the hybridoma cells grow better in the long term on Nutridoma NS (3 %) than on our serum free formulation. However, this is not an issue when doing a batch bioreactor run since the cells will perform as well in the short term and significant cost savings can be achieved in medium costs.

10.3 Conclusions

The following conclusions can be made concerning the economic feasibility of the serum free media developed:

- The cost of using the serum free medium as a production medium costs $37.80 and $20.60 for the myeloma and hybridoma cell line respectively. This is cheaper than using 10 % serum or Nutridoma NS for both cell

Table 10.3 - Cost Comparison between our Developed Serum Free Medium (DIF+BITES) for the Hybridoma Cell Line and the Media Supplemented with Nutridoma NS and Serum.

Medium	Protein Content	Cost per L of Medium
1 % Nutridoma NS	150 mg/L	54.40 $/L
2 % Nutridoma NS	300 mg/L	102.80 $/L
3 % Nutridoma NS	450 mg/L	152.20 $/L
10 % Fetal Bovine Serum	3600 mg/L	49.00 $/L
Developed Serum Free Medium	145 mg/L	20.60 $/L

lines.

- Supplementation of the basal medium with Nutridoma NS is very expensive since it cannot be used at 1 % concentration as recommended by the supplier.

Chapter 11: Conclusions and Recommendations

11.1 Conclusions

A serum-free production medium for a hybridoma and a myeloma cell line has been developed. The medium consists of BITES (BAA, insulin, transferrin, ethanolamine, and selenium) in DIF (DMEM:IMDM:F12 mixed in a 1:1:1 ratio) basal medium. The following sections summarize the experiments performed and the resulting conclusions.

11.2 Cell Adaptation

The adaptation of the cells to serum-free medium was attempted using three different adaptation protocols (balanced, gradual, and shock) for each of three different media (450, 150, and 15 mg/L total protein content). Unfortunately, the myeloma cell line could not be adapted to the serum-free medium. After the experiments ended we found this cell line required a source of low density lipoprotein (LDL) in order to grow under serum free conditions. By adding the LDL the cell line could be subcultured for approximately 1 week under serum-free conditions. During this period the growth and viability would get progressively worse until the cells eventually perished. The best explanation seems to be that a certain component (growth factor, chemical, etc.) is missing. The hybridoma cell line was able to grow in the serum-free medium formulation, but at significantly reduced growth rates and viabilities than might be accepted. We also found that the hybridoma cells lose their antibody production capability after about 60-70 generations.

11.2 Testing of Supplements

Factorial experiments were designed to test 14 chemicals reported to stimulate the growth or antibody production of hybridoma and myeloma cells. Based on the factorial experiments performed no supplements were found to increase the antibody production of either cell line. In fact in the case of the myeloma cell line the antibody production was decreased by the chemicals tested. We also found that the growth of the myeloma

cell line was improved by practically all the supplements tested. This indicates that this cell line may require these components to support its growth. The fact that the antibody production was significantly reduced when these chemicals were added, was the reason for not adding them to the serum-free formulation. It is obvious that further testing with the myeloma cell line (with other supplements) should be done to try to obtain long term growth.

11.3 Media Optimization

Experiments for the hybridoma and myeloma cell line were performed to determine the optimal concentrations (in terms of growth and antibody production) for BAA-oleic, transferrin, insulin, and egg yolk LDL. The optimal values for the hybridoma cell line are 430 mg/L, 5 mg/L, and 5 mg/L for BAA-oleic, insulin, and transferrin respectively. The optimal values for the myeloma cell line are 130 mg/L, 5 mg/L, and 10 mg/L for BAA-oleic, insulin, and transferrin respectively.

11.4 Recommendations

1. Repeat adaptation experiments for the myeloma cell line. Use the same media formulations (450 mg/L, 150 mg/L, and 15 mg/L) supplemented with 150 mg/L egg yolk low density lipoprotein.

2. Test various other supplements for the myeloma cell line in order to improve growth, since currently the cell line can only be subcultured for approximately one week.

3. Repeat clonal selection process for the hybridoma cell line, because due to instability it loses its antibody production capacity after 70 generations.

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