DEVELOPMENT OF MATHEMATICAL DESCRIPTIONS OF MAMMALIAN CELL CULTURE KINETICS FOR THE OPTIMIZATION OF FED-BATCH BIOREACTORS

by

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ABSTRACT

The lack of mathematical descriptions of mammalian cell culture kinetics limits the ability to optimally design and control animal cell bioreactors. This thesis addresses this limitation by presenting a successful strategy for developing mathematical equations relating both the growth rate and the specific antibody productivity of the hybridoma cell line, CRL-1606, to its environmental state. Three steps were important in this development: 1) reduction of the state variable set via statistical experimental design; 2) functional description of the initial metabolic rates with respect to the reduced state variable set; and 3) mechanistic and experimental characterization of the deviations from the initial rate equations that occur at states other than the initial state. An equation was subsequently developed relating the initial growth rate to serum, ammonium, lactate, glutamine and cell concentrations, with serum, and ammonium exerting the greatest influence. The equation is a superposition of a non-competitive inhibition relation in ammonium and lactate, with Monod equations in serum and glutamine. The inhibition constants were inversely proportional to the ammonium and lactate levels, while the Monod constant for the serum level was proportional to the -0.2 power of the cell concentration.

The instability of the growth-promoting activity of serum was also demonstrated. This instability may be responsible for the rapid decline in the
growth rate that was observed in low serum cultures. The addition of thiols, or the elimination of cystine, a disulfide, from the media was shown to stabilize the growth-promoting activity of serum, thus suggesting an important role for the oxidation/reduction state of the culture. Thiols were also shown to stimulate growth in low serum medium at low initial cell levels. This stimulatory effect of thiols was reduced as the initial cell concentration was increased. These results suggest that the spontaneous oxidation of a active thiols in serum is responsible for the both the instability of serum activity, as well as the declining growth rates in low serum cultures. A hypothesis is presented suggesting that these thiols may actually be dithiols, such as lipoic acid.

Additionally, the results of statistically designed experiments demonstrated that lactate significantly inhibits both monoclonal antibody and ammonium production by the CRL-1606 hybridomas. A expression was formed relating the specific antibody productivity to the lactate level. Glutamine catabolism was shown not to be related to growth.

The utility of the mathematical relations developed was demonstrated by using Kelley's Transformation to determine the optimal control policy of a fed-batch culture of CRL-1606 hybridomas. The instability of serum activity was ignored for this determination. Experimental implementation of an optimal control policy thusly determined, resulted in cell and antibody levels twice that which is typically obtained in batch cultures. The resulting growth rate, cell, glutamine, ammonium and antibody profiles were reasonably predicted from a numerical fed-batch simulation only if serum instability was included. The results strongly indicate that both ammonium accumulation and serum instability limit the productivity of fed-batch mammalian cultures.
ACKNOWLEDGEMENTS

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I. INTRODUCTION

The significant developments in recombinant DNA techniques employing bacterial systems for producing protein products such as vaccines, hormones, and other therapeutic proteins, has not diminished the need for the commercial exploitation of mammalian cell culture technology. On the contrary, there will be a continuing need for advances in large-scale mammalian cell culture for a variety of reasons. First of all, many useful protein products that require posttranslational modification and precise folding are not correctly processed in bacteria. Also, for certain protein products that require unique glycosylation, mammalian cell systems offer ways to prepare such molecules. In addition, monoclonal antibody technology is going to result in the need for more cost-effective procedures for growing hybridomas. At present, genetic engineering companies are using mammalian cells to produce important commercial products such as tissue-plasminogen activator, factor-VIII, monoclonal antibodies, colony stimulating factor, and Hepatitis B vaccine (Klausner, 1985).

Since the advantages and continuing needs for a variety of products can be met by mammalian cell culture, the challenge then becomes developing engineering and biological principles that can be applied to reduce production costs. Costs are likely to be reduced by either developing more productive processes, or by developing control strategies which increase the efficiency of a particular process. Major technical bottlenecks that will need to be overcome for reducing production costs will be reducing or eliminating the requirement for serum, overcoming oxygen transfer limitations and shear effects at large scales, and reducing the rate of production of inhibitory waste products.

At this juncture in the technology, mammalian cell production processes can not be rigorously designed and optimized. What is lacking for this to occur are accurate and representative mathematical descriptions of culture kinetics which can be coupled to given bioreactor design equations. Numerical simulations of these coupled equations could then be used to predict mammalian culture dynamics, which, in turn, could serve as a basis for the comparison of alternative production methods. Such simulations would be essential for designing and controlling production units optimally. In the absence of such quantitative analysis, mammalian cell culture
technologists desiring to increase productivity were forced to resort to the purely qualitative descriptions that were currently available. The plethora of "novel" processes that resulted from such superficial analysis did little to advance a fundamental understanding of the limitations that confront large scale production of animal cell products, and were often solutions looking for problems (Feder and Tolbert, 1985).

The goal of this thesis research, then, is to provide the quantitative descriptions necessary to serve as a basis for the rational design and control of mammalian cell production processes. The model cell line used for this study was a mouse - mouse hybridoma cell line producing anti-fibronectin (CRL - 1606). Statistically designed experiments were first initiated to determine which nutrients and cellular waste products typically found in cell culture medium were the most important in determining the initial metabolic rates of the cells, such as the growth rate and the specific antibody productivity. The initial metabolic rates were than functionally related to the concentrations of these components from the rates calculated from cultures grown in various medium formulations.

The failure of these mathematical descriptions to hold at some process states was then investigated and characterized mathematically. A spontaneously loss of the growth - promoting activity of serum with time was the primary cause of the discrepancies observed. The mathematical description of the culture kinetics were then used to optimize a fed - batch culture of CRL - 1606 hybridomas. Techniques of optimal control theory were employed (Bryson and Ho, 1975).

It is hoped that the work described herein can serve as both a database with which to compare, and a methodology with which to develop, efficient production processes of any animal cell line and any animal cell product.
II. LITERATURE REVIEW

II.A. Large Scale Mammalian Cell Culture

There are two types of mammalian cells, anchorage-dependent cells (cells that require a surface for growth, such as human fibroblast cells WI-38 and FS-4), and suspension cells (non-anchorage dependent, such as HeLa cells and hybridomas). Mammalian cells that multiply in suspension can usually be cultivated by techniques similar to those used in microbial fermentations. The large-scale production of anchorage-dependent cells, however, presents novel problems.

Mammalian cells must be grown to high cell concentrations if their products are to be obtained at a reasonable cost. The greater the cell level, the more "biological catalyst" is present, resulting in increased product titers and reduced purification costs. Serum components added to the culture media catalyze cellular metabolic functions, and are assumed not to be consumed to a great extent. Thus, cultures at high cell concentrations should require no more serum than cultures at lower cell levels. As serum is the predominant cost of culture media, high cell concentrations greatly reduce the cost of producing mammalian biologicals. Using conventional techniques, it is usually possible to obtain cell levels of approximately $1 \times 10^6$ suspension cells/ml.

II.A.1.1 Anchorage-Dependent Cells

Anchorage-dependent cells have traditionally been grown on a large scale on the inside of rotating bottles. As the bottles roll, the cells are alternately exposed to oxygen in the air space and to the growth medium. The surface area for growth is only a small percentage of the total bottle volume, and thus many roller bottles are required to produce even small quantities of cells. For example, only $3 \times 10^7$ cells can be obtained from a bottle with a surface area of 500 cm$^2$. Roller bottle systems are cumbersome and expensive in labor and materials. Furthermore, the variation in the cultures obtained from a series of individual bottles makes it practically impossible to monitor cellular kinetics and to change the growth environment.
In an attempt to solve the problem of low ratios between surface area available for cell growth and the total culture volume, Van Wezel (1967) grew cells on charged dextran microcarrier beads suspended in liquid culture media. The attempt was successful, but the inhibitory effect of the microcarriers on the cells prevented the use of bead concentrations greater than 1 g beads/l. Levine et. al. (1977) eliminated this inhibitory effect by modifying the surface charge of the beads, thereby allowing some cell types to proliferate up to of $5 \times 10^6$ cells/ml at microcarrier concentrations up to 5 g/l.

Other schemes for growing mammalian cells are shown in Figure 1. In all of these methods, the surface-to-volume ratio is greater than that of roller bottles. However, the multiple propagator(Weiss and Schleicher, 1968), the spiral film(House et. al., 1972), plastic bags(Munder et. al., 1971), and the Gyrogen with tubes(Girard et. al., 1980) are of limited use for growing cells on a large scale, since their surface-to-volume ratios are much too low. The glass bead propagator(Wohler et. al., 1972) is not practical on a large scale, since the bead bed would shift due to the large rate of medium circulation required at larger scales, thereby damaging the cells on the glass bead surface. For the IL410 tubular spiral film propagator (Jensen, 1981), medium flows through a tube made of gas-permeable film. Cells grow on the inside of the tube and receive oxygen through the film. The surface-to-volume ratio, however, is low (Figure 1), and the scalability of the process is questionable.

The best methods for growing mammalian cells on a large scale are hollow-fiber membrane (Knazek et. al., 1972), and microcarrier suspension bioreactors. A hollow-fiber membrane bioreactor consists of a bundle of dialysis tubes packed inside a hollow cylinder. Typically, the medium flows through the inside of the fibers and the cells grow on the outside surface. Nutrients diffuse through the capillary wall to the cells.
Figure 1. Surface-to-volume ratios (cm⁻¹) of various methods used to culture anchorage-dependent cells.

II.A.1.2 Suspension Cell Systems

HeLa cells, hybridomas, lymphoblastoids and many other tumor cell lines do not require a surface on which to grow and can be cultured in equipment similar to that used for bacteria. However, mammalian cells lack a cell wall and are easily damaged
by shear forces. Thus, the development of novel techniques to cultivate suspension
cells has focused on the need to avoid creating high shear stresses.

Two types of agitators have been developed. The vibromixer consists of a
horizontal disk with conical apertures attached to a vertical shaft which rapidly
oscillates up and down (Girard, 1977). The vertical rather than a horizontal circulation
of the medium disperses the cells adequately with reduced shear forces. The other
design consists of four flexible sheets which span the depth of the culture fluid (Feder
and Tolbert, 1983). These sheets need only to be turned slowly to disperse the cells
adequately. Feder and Tolbert (1983) have shown that both types of agitators can yield
high cell concentrations.

Cells can be protected from shearing by the creation of an artificial cell wall, for
example, by encapsulating the cells inside a polymer - reinforced calcium alginate
membrane (Lim and Moss, 1981). Up to 10,000 cells can be trapped in a capsule 500
µm in diameter. Such processes produce high cell densities, increased cell viability,
and increased product yields. Encapsulation is now used commercially to produce

II.A.2. Production Modes: Batch, Fed-Batch and Perfusion

Three types of cultures can be used for microbial or mammalian cells: batch, fed-
batch or perfusion (Fig. 2). In a batch system, nutrients are not replenished and the
only addition to the medium is oxygen. The only parameters that can be controlled are
pH, temperature and aeration. Thus, the environment of cells in a batch culture is
constantly changing as nutrients are depleted and waste products accumulate; this may
cause cell growth and/or product formation to be inhibited prematurely. This problem
can be alleviated by replacing the "spent" medium with "fresh" medium, but the only
way of providing the cells with a constant environment is to replace the medium
frequently, and this can be very expensive. In addition, when the medium is replaced,
some expensive serum components, which may not be exhausted, would be
needlessly discarded.
Fed-batch systems feed vital components into the culture only as needed and thereby maintain constant nutrient concentrations. These processes require the ability to monitor and control the nutrients. Since more environmental parameters may be controlled, it is easier for operators to optimize cell growth and product generation. Although cellular waste products are not removed, it may be possible to limit their accumulation via controlling the nutrient flow (Glacken et. al., 1986, see Appendix B).

With perfusion and continuous processes, medium is constantly flowing into and out of the bioreactor at the same rate. In a perfusion process, the cells are retained inside of the bioreactor, while with a continuous process, the cells escape with the effluent. The best way to retain cells inside of the bioreactor is with a spin-filter device. The spinning action of the rotating filter keeps the cells from clogging the pores. Tolbert et. al. (1981) reported growing carcinosarcoma cells to cell levels up to 10⁷ cells/ml.

With these systems, both nutrient and waste product concentrations may be controlled by varying the dilution rate (i.e., the flow rate of medium divided by the bioreactor volume). Increasing the dilution rate results in increased nutrient concentrations and decreased waste product concentrations. The environment can be highly controlled, but this system does have some of the disadvantages of the repeated-
batch method. That is, nutrients, including valuable serum components, are needlessly discarded in the effluent. Since this is wasteful, perfusion may only be economical in comparison to fed-batch systems if the lower concentrations of waste products achieved by perfusion systems results in much higher cell levels than that attainable from fed-batch cultures.

II.A.3. Barriers to Scale-Up

II.A.3.1 Hydrodynamic Considerations

One very important consideration when scaling up mammalian cell processes is the hydrodynamic environment of the cells. This is especially true with microcarrier systems (Sinskey et al., 1981, Coughan and Wang, 1986; Cherry and Papoutsakis, 1986). Sinskey et al., 1981, correlated the maximum cell concentration obtained by chicken embryo fibroblasts (CEF) grown on microcarriers with what was termed the "integrated shear factor". The integrated shear factor (ISF) is the ratio of the tip speed to the distance between the impeller tip and the wall, given by:

\[
\text{ISF} = \frac{2\pi ND_i}{D_T - D_i}
\]

(1)

where

- \(D_i\) = diameter of impeller
- \(D_T\) = diameter of tank
- \(N\) = impeller speed

Croughan and Wang (1986) re-correlated the data of Sinskey et al. (1981), as well as the data of Hirtenstein and Clark (1980), and Hu (1983) with the turbulent eddy size in the agitated fluid. For microcarriers with a diameter of 185 µm, cell growth was found to be greatly inhibited in systems with eddies less than 100 µM. Croughan and Wang interpreted this to mean that eddies that are too small to move the microcarriers, dissipate their energy to cells immobilized onto the relatively immobile microcarrier.
The effect of the eddies on the culture kinetics was modeled as follows:

\[ \frac{dX}{dt} = \mu X \quad \text{for} \ L_e > L_c \]  
\[ \frac{dX}{dt} = (\mu - q)X \quad \text{for} \ L_e < L_c \]  
\[ q = K \left( \frac{P}{V^3} \right)^{0.75} = \text{death rate} \]  
\[ L_e = \left( \frac{V^3}{P} \right)^{0.25} = \text{Kolmogorov eddy length} \]  
\[ P = \frac{CN^3D_i^5}{V} = \text{average power dissipation rate per unit mass} \]

where \( X \) is the cell concentration (cells/l), \( L_c \) is the critical eddy size below which the eddy is harmful to the cells (\( \mu m \)), \( \mu \) is the specific growth rate (h\(^{-1}\)), \( K \) is a constant that reflects the likelihood of cell damage from a given eddy, \( V \) is the liquid volume, \( C \) is a constant, and \( v \) is the kinematic viscosity. Croughan and Wang suggest that scaling up at constant power per unit volume should not lead to detrimental effects.

Cherry and Papoutsakis (1986) also considered the effects of microcarrier - microcarrier collisions and collisions between microcarriers and the impeller. The severity of microcarrier collisions may be related to the following:

\[ SC = \frac{\rho \pi v^3k^{7/3}}{6d^{5/3}} \]  
\[ SC = \frac{9\pi^4 \rho_p \rho_b ND_i d}{512V} \]

where \( \rho \) is the fluid density, \( k \) is the surface to volume ratio of the microcarrier, and \( d \) is the microcarrier diameter. The severity of the collisions between the impeller and the microcarriers was related to the following:
where \( p_b \) is the microcarrier concentration. Cherry and Papoutsakis suggest using the smallest impeller diameter that still provides adequate mixing to reduce the effect of collisions.

II.A.3.2 Oxygen Supply

The provision of an adequate oxygen supply to large volumes of mammalian cells is the most critical barrier to scale-up, especially suspension systems. Oxygen is only sparingly soluble in cell culture medium (0.2 mmol O\(_2\)/l, Fleischaker, 1982). Oxygen demand ranges between 0.0531 mmol O\(_2\)/10\(^9\) cells - h (Katinger et al., 1978) to 0.59 mmol O\(_2\)/10\(^9\) cells - h (Green et al., 1958), depending on the type of cell.

Oxygen can be supplied from the head space of a small culture vessel. For example, HeLa cells at 1 x 10\(^7\) cells/ml respire at a rate of 5.0 mmol O\(_2\)/l-h (Danes et al., 1963). Cell concentrations of this magnitude are not typical of suspension cultures, but can sometimes be achieved under certain circumstances (Keay and Burton, 1979). The oxygen transfer rate of any vessel may be expressed as (Wang et al., 1977):

\[
N_0 = k_L a (C^* - C_L)
\]  

(9)

where:

\[
N_0 \quad \text{oxygen transfer rate, mmol O}_2/\text{l-h}
\]

\[
k_L a \quad \text{mass transfer coefficient, mmol O}_2/\text{atm-l-h}
\]

\[
C^* \quad \text{concentration of oxygen in the gas phase in equilibrium with the saturated concentration of oxygen in the liquid phase (atm)}
\]

\[
C_L \quad \text{concentration of oxygen in the gas phase in equilibrium with the actual concentration of oxygen in the liquid phase (atm)}
\]
The "a" term in the oxygen transfer coefficient for unaerated vessels is effectively a function of the ratio of the surface area available for aeration to the total volume of the culture. Using this assumption and some values of $k_L$ calculated from unaerated vessels of mammalian cells (Fleischaker, 1982), it can be calculated that oxygen limitation for $10^7$ HeLa cells/ml in a vessel with air in the headspace ($C^* = 0.21$ atm) occurs at a volume of less than 1.0 liters. If pure oxygen is used in the headspace instead of air, then the maximum bioreactor volume allowed before oxygen limitation occurs increases only to 3.5 liters. Thus, additional oxygen must be introduced directly to the medium if the culture volume exceeds 3.5 liters.

Hu et al. (1986) reported a four-fold enhancement of oxygen transport after installing an impeller at the liquid-gas interface. With the surface impeller, the scale of the culture using the previous example could be increased to 14 liters if pure oxygen is used in the headspace.

Air sparging, which is commonly employed in microbial fermentations, is generally unsuitable for mammalian cells since it causes lysis of the cell membrane and foaming of the medium (Kilburn and Webb, 1968). Foaming is of particular concern for microcarrier cultures, since the beads become entrained in the foam. One alternative is to aerate the medium using oxygen-permeable silicone tubing (Sinskey et al., 1981). Aeration via silicone tubing provided oxygen to a 7.5 liter microcarrier culture of FS-4 cells at a concentration of $2 \times 10^6$ cells/ml (Fleischaker, 1982).

To what scale may cultures be practically aerated using silicone tubing? Suppose we wish to oxygenate 1000 liters of HeLa cells at $10^7$ cells/ml. Since the $k_La$ for oxygen transfer through the silicone tubing is 1 mmol O$_2$/atm-m$^2$-s it would take about 30 m of 2.5 cm diameter silicone tubing to provide enough oxygen to the culture - a feasible, but somewhat expensive and quite unwieldy solution to the problem.

Another strategy is to circulate the medium through the culture vessel, while simultaneously oxygenating it in an external loop. The advantage of this method is that the circulation rate of the medium would dictate the amount of oxygen that is supplied to the cells. This can be described mathematically by:
\[ F(C_i - C_0) = \text{OUR} \]  \hspace{1cm} (10)

where:

- \( F \) = Flow rate of medium, l/h
- \( C_i \) = Dissolved oxygen concentration inlet, mM
- \( C_0 \) = Dissolved oxygen concentration, outlet, mM
- \( \text{OUR} \) = Oxygen uptake rate of the cells, mmol O\(_2\) / h

Clark and Hirtenstein (1981) externally oxygenated the medium by allowing it to equilibrate with the oxygen in the headspace of another vessel, but there is no reason why the medium could not be oxygenated externally via silicone tubing. How feasible is this strategy for large scale cultures? For a 1000 liter volume of HeLa cells at \(10^7\) cells/ml, a flow rate of 30,000 l/h would be required to ensure a dissolved oxygen concentration of 10\% of saturation in medium. This high rate might likely damage the cells and cause excessive foaming, and thus this method may be impractical.

Hollow-fiber membrane bioreactors have enhanced capability to provide oxygen to greater numbers of mammalian cells than conventional systems due to the relatively large surface area for transport. Oxygen diffuses from the medium flowing inside the fibers to the cell mass growing in the interstitial space. Knazek et al. (1972) has shown that cell levels of \(1 \times 10^8\) cells/ml could be obtained, however, it is not clear how many of these cells were viable. This is because gradients of oxygen exist, proceeding from the fiber radially outward. Thus, some of the cells in the interstitial spaces between the fibers may be anoxic, depending on how far the cells are from the nearest fiber (Knazek, 1974; Fike et al., 1977; Inoees et al. 1983). This problem may be overcome by packing many fibers as close together as possible, so that every cell is close enough to a fiber to receive adequate oxygen. Adema and Sinskey (1986) compared this solution with an alternative in which the cells were assumed to reside inside the fibers, concluding that under certain conditions, the alternate solution was more satisfactory.

Air-lift bioreactors have been used to directly introduce oxygen into the culture
medium (Katinger and Shreier, 1979; Birch et al., 1985). Katinger and Shreier determined that high viabilities could be maintained in an air-lift bioreactor if the power input per unit volume was kept below 10 W/m³.

The development of serum-free media may contribute to the solution to the problem of oxygen transfer. The high protein content of serum is responsible for the excessive foaming upon sparging. In the meantime, if one wants to grow cells on a large scale at high cell concentrations, stirred-tank bioreactors should be eschewed in favor of processes that have higher oxygen transfer capabilities (e.g., hollow fiber membrane or air-lift bioreactors).

II.A.3.3 Accumulation of Waste Products

Lactic acid can overcome the buffering capacity of the medium, resulting in a pH lower than optimal. Prolonged exposure to more than 4 mmol per liter of ammonia has been shown to inhibit cell growth (Glacken et al., 1986, Appendix B). A culture of MDCK cells growing from a cell level of 1 x 10⁶ cells/ml to a cell level of 5 x 10⁶ cells/ml could result in ammonium concentrations in excess of 18 mM, and lactate levels in excess of 90, assuming specific ammonium and lactate productivities of 7.0 and 35.0 mmol/10¹¹ cell-h, respectively (Glacken et al., 1986).

To overcome the problem of growth inhibition by ammonia and lactate, the culture medium may be modified so that the cell's metabolism is encouraged to generate less waste products. In 1958, Eagle et al. substituted galactose for glucose as the carbohydrate source in the medium, resulting in a 67-fold decrease in lactic acid generation. In 1982, Fleischaker reported the same effect by feeding glucose to FS-4 cells in a fed-batch fashion, ensuring that the glucose concentration in the medium remained relatively low (0.5 mM vs. 20 mM). Ammonia production has been shown to be reduced by 40% via implementation of a glutamine feeding strategy which maintained low glutamine concentrations (0.2 mM vs. 4 mM, Glacken et al., 1986).

Perfusion culture can also be used to limit the accumulation of metabolic end products. As noted earlier, perfusion techniques waste serum nutrients and may only be economical if very high cell levels may be achieved or if the expensive
macromolecular serum components are recovered via dialysis. Thus, whether a fed-batch system with a controlled environment designed to limit end product accumulation, or a perfusion system should be the method of choice, depends on: (1) the optimal turnover rate of the medium in the perfusion system which balances low consumption of the serum with a high final cell level; (2) the extent to which waste product accumulation can be reduced in a controlled fed-batch system; (3) the number of times the medium must be changed to keep the concentration of waste products below the maximum level in the fed-batch system (if the generation of waste products cannot be sufficiently reduced by adjusting only the environmental parameters); (4) the cost of medium dialysis; and (5) the cost of equipment peculiar to perfusion systems, i.e., pumps, spin filters, etc.

II.A.3.4 Instrumentation and Control

When cells are cultivated on a large scale, it is highly desirable that every production run should produce cells and cell products at the same maximal rate and level. Computer monitoring and control should be introduced to ensure consistent performance at optimal conditions. Most mammalian cell production units described in the literature to date have not utilized process control. One practical reason for this is that many production facilities are located in third world countries (where the great majority of human and animal vaccines are used) where the resources needed to maintain complex computer hardware are unavailable (Girard, 1977). Even in developed nations, process control has usually only been applied to monitoring the culture temperature and pH.

The lack of sophisticated methods for process control of mammalian cell cultures is not a problem of scale. The problem is that except for a few limited cases, process control strategies which will optimize cellular metabolism have not been developed, and that this deficiency becomes more serious as the scale of production increases.

In 1982, Fleischacker employed computer monitoring and control to optimize the production of interferon by FS-4 cells in a 7.5 liter culture. Carbon dioxide formation, oxygen uptake rate, glucose consumption and lactic acid production, were monitored
via a microcomputer to determine the ATP production rate (APR) with time (Fleischaker, 1982; Glacken et. al., 1986, see Appendix B). The concentration of cells in the culture could be continuously estimated from the calculated APR. The rate at which glucose was fed into the vessel was varied in response to the difference between the medium glucose level (measured after derivatization via an on-line spectrophotometer) and the glucose set point determined from the rate of lactate production. Two goals were realized: (1) the rate of lactic acid production was reduced; and (2) the optimal culture time for the induction of interferon production was determined. Thus, maximum interferon titers could consistently be obtained through the use of computer monitoring and control. This strategy could also be used to determine the best time to induce cells for the production of viruses or other products. In this way the run-to-run variability of virus titers that often affects biological manufacture may be reduced.

The concept of using the ATP flux to estimate the cell level on-line is important, since the use of material balances, which are often used to estimate either cell mass or product levels in microbial fermentations (Cooney et. al., 1977), would rarely be practical for animal cell cultures. The large number of components both consumed and produced by mammalian cells, in the absence of instrumentation to measure all of these components, would always result in the failure to close the material balance.

II.A.4 Comparison of Promising Mammalian Cell Culture Techniques

II.A.4.1 Strategies for Growing Anchorage-Dependent Cells

There are many advantages in using a microcarrier suspension system (Table 1) to produce anchorage-dependent cells on a large scale. First, a suspension system of microcarriers has the highest surface-to-volume ratio of any technique yet developed (Figure 1). Thus, with all other factors being equal, less labor, materials and medium are required to produce a given quantity of cells in microcarrier cultures as compared to other systems. For example, Fleischaker (1982) has demonstrated that one 7.5 liter microcarrier culture using a microcarrier density of 5 g beads/liter can produce as many cells as 500 roller bottles, using only 15% as much medium and serum. Microcarrier
cultures can typically support cell densities of 1-2×10^6 cells/ml in batch cultures (Levine, 1977), and 1×10^7 cells/ml in perfusion cultures (Butler et. al., 1983). It is not clear whether the higher cell levels obtained in perfusion cultures is due to a reduction of waste product accumulation or an increase in the rate of delivery of nutrients, especially oxygen.

Table 1. Comparison of two processes for growing anchorage - dependent cells on a large scale.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Hollow Fiber Bioreactor</th>
<th>Microcarrier Suspension</th>
</tr>
</thead>
<tbody>
<tr>
<td>S/V ratio</td>
<td>30.7</td>
<td>31 - 153</td>
</tr>
<tr>
<td>Oxygen transfer at high cell levels, and large volumes</td>
<td>Good, but some cells may be oxygen limited due to gradients through cells</td>
<td>Difficult, requires much silicone tubing</td>
</tr>
<tr>
<td>Uniform Environment</td>
<td>No - gradients will exist</td>
<td>Yes</td>
</tr>
<tr>
<td>Ability to control environment</td>
<td>Moderate with perfusion only gradients will exist</td>
<td>Excellent, fed-batch or continuous</td>
</tr>
<tr>
<td>Ability to optimize</td>
<td>Difficult because of gradients</td>
<td>Excellent</td>
</tr>
<tr>
<td>Ability to monitor growth</td>
<td>Difficult</td>
<td>Excellent, direct sampling</td>
</tr>
<tr>
<td>Max. cell levels</td>
<td>10^8 cells/ml (Knazek, 1972)</td>
<td>1 - 2×10^6 cells/ml, batch 10^7 cells/ml, perfusion</td>
</tr>
<tr>
<td>Ability to reduce serum usage</td>
<td>Excellent, via high cell levels</td>
<td>Excellent, with fed-batch and optimal control</td>
</tr>
<tr>
<td>Scale - up potential</td>
<td>Good, limited by difficulty in monitoring and control; also gradients may limit scale</td>
<td>Good, limited by oxygen transfer and bead cost and handling</td>
</tr>
</tbody>
</table>

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Another advantage of microcarrier suspension systems is that the cell population is homogeneous, and thus the system can be easily monitored and the process controlled. The culture environment can be easily adjusted to maintain maximum cell growth and product generation, while minimizing waste product formation. Since the environmental parameters can be controlled, greater consistency from culture to culture can also be expected. The average titers of interferon produced per production run of FS-4 cells in computer-controlled microcarrier cultures were shown to be higher than those produced in cultures without computer control (Fleischaker, 1982).

Microcarrier suspension cultures have a few undesirable characteristics. First, not all cell lines can be easily removed from the microcarriers while still maintaining high cell viability. Small microcarrier cultures cannot easily serve as inocula to larger microcarrier cultures. Hence, a large-scale production facility utilizing microcarriers must still depend on inocula from roller bottles. For example, approximately 200 roller bottles would be needed to seed a 10 liter microcarrier culture at 4 x 10^5 cell/ml. This may restrict the scale of a microcarrier production process. Hu et. al. (1985) developed a trypsinization technique using controlled pH to detach the cells from the microcarriers as a viable inocula. Using this technique, the authors demonstrated the ability to serially propagate both Vero and FS-4 cells on microcarriers continuously.

The cost of microcarriers must also be considered; a culture containing 5 g/l of microcarriers costs $50/l if the beads are not reprocessed and reused. Although reprocessing is less expensive than simply discarding the used beads, it still adds to the costs of producing the cells. There is evidence that recycled microcarriers do not support growth as well as unused microcarriers (Hu et. al., 1985).

Microcarrier suspension systems suffer from the same poor oxygen transfer characteristics as observed for any suspension culture of mammalian cells. Methods described previously can improve the transfer of oxygen within suspension cultures, but each of these methods has an inherent disadvantage. The main advantage of the two cell production methods yet to be described is that they have excellent oxygen transfer potential. With the hollow-fiber membrane propagator, cells may be attached to an oxygen-permeable membrane. High cell concentrations have been achieved because
of the high oxygen transfer characteristic of the bioreactor (Knazek, 1972, Table 1).

The hollow-fiber membrane bioreactor does not provide a uniform cellular environment. Gradients in concentrations of nutrients and waste products exist. If the gradients are large, cell growth and metabolism may be adversely affected in some sections. In addition, these gradients would complicate process control. Since cellular metabolism is a function of nutrient and waste product concentrations, many different populations of cells would exist in these systems. Thus, monitoring the overall concentrations would provide only an indication of average cellular activity, masking cell populations that were operating less than optimally. Actions taken by the control system based on the average values may, therefore, only optimize certain cell populations. Of course, gradients could be kept to a minimum by increasing the flow rate, but this would increase the pressure drop across the propagator, which would eventually become prohibitive at increasing flow rates. In addition, if the gradients were too small, the accuracy of subsequent calculations estimating cellular kinetics (e.g., oxygen uptake rate) would suffer.

Additionally, it is quite difficult to take representative cell samples with which to estimate cell growth in hollow-fiber systems since the cells cannot be removed conveniently during operation. In contrast, a given volume of the suspension medium is simply withdrawn for evaluation from a microcarrier suspension system. Cells on the beads can be viewed under a microscope or removed from the microcarriers and counted (Van Wezel, 1973). Cells can not be viewed under a microscope in hollow-fiber membrane bioreactors. Indirect, non-invasive measurements, such as the volumetric nutrient and/or waste product production rates or ATP production rates (Fleischaker, 1982; Glacken et. al., 1986), are required for estimating the cell level and metabolic state on-line.

In summary, because it is very amenable to process control, microcarrier suspension culture has the best scale-up potential of the systems mentioned, provided the problem of oxygen transfer limitation is solved. If the oxygen transfer problem cannot be easily solved, then the hollow-fiber membrane bioreactor becomes an attractive alternative.
II.A.4.2 Strategies for Growing Suspension Cells

The only really novel procedure for cultivating suspension cells has been developed by Lim and Moss (1981) and applied commercially by the Damon Corporation. Cells are encapsulated in a semipermeable membrane and cultivated in standard suspension culture vessels. The advantages of this system are: (1) greater cell levels compared to standard suspension systems; (2) increased product generation per cell; (3) simpler separation of cells via gravity settling, as opposed to centrifugation; and (4) controlled product partitioning into either the microcapsule or the medium, separate from the cells, whichever is desired (Table 2).

A disadvantage of this system is, of course, the costs of encapsulation and removing cells and products from the capsule. Also, oxygen transfer is a more serious problem. In addition to the limitations of delivering oxygen to the bulk medium, an additional obstacle is the limited access of oxygen to cells in the center of the capsule. This could adversely affect cell metabolism and product formation, and complicate process control. The magnitude of the oxygen transfer resistance of the cell mass in the capsule is a function of the capsule radius according to the following relation (Bailey and Ollis, 1977):

$$\Phi = \frac{r_s r^2}{9D_s S_0}$$  \hspace{1cm} (11)

where:

- $\Phi$ = modified form of Thiele modulus, dimensionless
- $r_s$ = respiration rate of cells, $\frac{\text{mmol O}_2}{\text{l - h}}$
- $r$ = radius of cell floc, cm
- $D_s$ = diffusivity of oxygen through cell mass, $\frac{\text{cm}^2}{\text{h}}$
- $S_0$ = dissolved oxygen concentration in the liquid media, mM
The Thiele modulus is functionally related to the effectiveness factor, \( \eta \), which is the ratio of the observed oxygen uptake rate to the oxygen uptake rate that could be obtained in the absence of oxygen transfer resistance in the cell floc. For example, an

Table 2. Comparison of two processes for growing suspension cells on a large scale.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Microencapsulated Suspension</th>
<th>Free Suspension</th>
</tr>
</thead>
<tbody>
<tr>
<td>Max. cell level</td>
<td>( 10^7 ) cells/ml</td>
<td>( 2 \times 10^6 ) cells/ml</td>
</tr>
<tr>
<td>Oxygen transfer at high cell levels, and large volumes</td>
<td>Limited by oxygen diffusion through cell mass</td>
<td>Limited by ( k_L a )</td>
</tr>
<tr>
<td>Productivity of cells</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Ability to control environment</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Cell separation</td>
<td>Gravity settling</td>
<td>Centrifugal (expensive)</td>
</tr>
<tr>
<td>Cell recovery</td>
<td>Capsule lysis</td>
<td>No extra step</td>
</tr>
<tr>
<td>Protection of product from shear</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Ability to reduce serum usage</td>
<td>Excellent, via high cell levels</td>
<td>No advantage</td>
</tr>
<tr>
<td>Product Separation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Very high mol. wt.</td>
<td>Easier, trapped in capsule. Separation in situ possible</td>
<td>No advantage</td>
</tr>
<tr>
<td>Lower mol. wt.</td>
<td>Easier, separated from cells - important is using transformed cells</td>
<td>No advantage</td>
</tr>
<tr>
<td>Scale - up potential</td>
<td>Oxygen transfer problem; cost of encapsulation vs. reduced serum usage and easier separation</td>
<td>In use for large scale production of vaccines and hybridomas</td>
</tr>
</tbody>
</table>
effectiveness factor of 1.0 implies that no transfer resistance exists, while an effectiveness factor of 0.5 implies the oxygen transfer resistance of the cell floc decreases the observed oxygen utilization rate of the capsule by one half. For illustrative purposes, assume that: 1) 10,000 cells completely fill a 500 μm microcapsule (Damon Corporation press release, 1981); 2) the volumetric oxygen uptake rate of the cells is a zero-order function of the oxygen concentration (Fleischaker, 1982), equal to 0.59 mmol O2/l-h (for 10^7 cells/ml); 3) the diffusivity of oxygen through the cell floc is 2.1x10^-5 cm^2/s (Bailey and Ollis, 1977), and; 4) the concentration of dissolved oxygen in the bulk phase can be kept at saturation. With these assumptions, the Thiele modulus may be calculated to be 1.84. This corresponds to an effectiveness factor of approximately 0.6 and implies that 40% of the cells in the capsule are oxygen limited. The Thiele modulus must be less than 0.3 if all oxygen limitation is to be avoided. This would occur at a capsule diameter of 170 μm. Thus, development of new technologies designed to increase the rate of oxygen transfer from the gas phase to the liquid phase will be futile unless strategies for producing smaller microcapsules are concomitantly developed.

II.B. IN VITRO MAMMALIAN METABOLISM

II.B.1 Glutamine, Glucose, and Energy Metabolism

The nutrients required for mammalian cells to grow in vitro are complex and difficult to define. Amino acids, vitamins, salts, serum components and carbohydrates are all normally required for growth. Glucose and glutamine have traditionally been the most abundant essential nutrients in cell culture medium (Eagle, 1955). However, glucose has been replaced with other carbohydrate sources, such as galactose and fructose (Eagle, 1958; Butler and Thilly, 1982). The requirement for a carbohydrate source has been replaced entirely with the combined addition of hypoxanthine, glycine, thymidine, and uridine (Zielke et al., 1976). The absolute requirement for glutamine
determined by Eagle has not been circumvented. Except for a few mutant cell lines, growth cannot be maintained without glutamine for more than 48 hours. These observations, combined with the high rate of glutamine utilization by cultured cells (Eagle, 1959), led researchers to suspect that glutamine may be a major source of metabolic energy.

The pathways of glucose and glutamine energy metabolism in cultured cells are depicted in Figure 3. The experimental proof for the existence of these pathways are as follows. First, it was determined, via labeling studies, that CO₂ was the major product of glutamine metabolism for many cell types (Lavetres, 1974; Stoner and Merchant, 1972; and Kovacevic and Mooris, 1972). This implied that glutamine may be oxidized in the citric acid cycle. Supporting this hypothesis, Zielke et al. (1980) showed that labeled glutamine resulted in labeled intercellular lactate, glutamate, pyruvate, citrate, malate and aspartate in human diploid fibroblasts. In addition, [6-C̄]glucose resulted in much less labeled CO₂ (indicative of citric cycle oxidation) and lactic acid than would be expected if glucose was the sole energy source, while 50% of labeled glutamine was recovered as labeled CO₂ and labeled lactate in HeLa cells (Reitzer et al., 1979).

Figure 3 presents the versatility with which glutamine may be catabolized. Glutamine may be completely oxidized to CO₂ if the pyruvate produced via the malic enzyme is re-introduced into the Kreb's cycle as acetyl-CoA. Glutamine may also be incompletely oxidized to either lactate, which is excreted, or aspartate, which may then be used to synthesize cellular material. These three pathways may be represented as follows:

a) \([\text{GLN}] + 5\text{NAD} + 2\text{FADH} + 2\text{GDP} \Rightarrow 5\text{CO}_2 + 5\text{NADH} + 2\text{FADH}_2 + 2\text{GTP} + 2\text{NH}_4\)

b) \([\text{GLN}] + 3\text{NAD} + \text{FADH} + \text{GDP} \Rightarrow \text{CO}_2 + 3\text{NADH} + \text{FADH}_2 + \text{GTP} + \text{aspartate} + \text{NH}_4\)

c) \([\text{GLN}] + \text{NAD} + \text{FADH} + \text{GDP} \Rightarrow 2\text{CO}_2 + \text{NADH} + \text{FADH}_2 + \text{GTP} + \text{lactate} + 2\text{NH}_4\)
Figure 3. Pathways of glutamine carbodilym and ammonium excrction.

Glutamine → Glutamate → NADH → α-Ketoglutarate → Oxaloacetate → Pyruvate → Acetyl-CoA → α-Ketoglutarate → NADH → FAD → GTP → Ribose units → Pathway of ribose phosphate pathway of pentose phosphate pathway of glycolysis

Lactate → Aspartate → NADH → Glutamate → Glutamine
Respectively, these pathways yield 21, 12 and 6 moles of ATP per mole of glutamine utilized, suggesting the cell has significant flexibility for utilizing glutamine to satisfy its energy requirements. Depending on the ratios of the carbon fluxes through each pathway, one to two moles of ammonia may be excreted per mole of glutamine catabolized.

The relative regulation of glucose and glutamine metabolism, as well as the energy contribution from each, is of importance. In almost all cell types examined, very little glucose is oxidized to CO₂ via the Kreb’s cycle. The possibility exists that the enzyme that takes acetyl-CoA and oxaloacetate to citric acid is either inactive or barely active (Reitzer et al., 1979). The bulk of the carbon flux from glucose goes to either lactate via glycolysis or CO₂ via the pentose - phosphate pathway (Reitzer et al., 1979; Tildon, 1973; Cristofalo, 1970; Morell and Foresch, 1973; Zielke et al., 1978).

The relative distribution of carbon flux through each of these two pathways depends upon the relative concentration of glucose and glutamine. Some examples are as follows:

- Reitzer et al. (1979) has observed that a 50-fold increase in glucose concentration reduced the glucose carbon flux through the pentose phosphate pathway of HeLa cells by 50%. Recall that increasing glucose levels has been shown to depress glucose utilization (Zielke et al., 1978; Fleischaker, 1982).

- More slowly utilized carbohydrates, such as fructose and galactose, have been shown to shunt a higher percentage of their carbon chains through the pentose phosphate pathway, and less through glycolysis, than glucose (Reitzer et al., 1979).

- Zielke has shown et al. (1978) showed that the addition of glutamine to a culture of human fibroblasts increased the flux of glucose carbon through the pentose - phosphate pathway by 70%, while simultaneously reducing the glucose carbon flux through the glycolytic pathway to 12% of its initial rate.

A general principle may be formulated: increased rates of carbohydrate uptake
may shunt carbohydrate carbon chains preferentially through glycolysis and away from the pentose-phosphate pathway. Reiterz has shown that the intercellular energy charge decreases as the carbohydrate uptake rate increases, presumably because the transport of carbohydrate into the cell utilizes ATP. Since phosphofructokinase is stimulated by a low energy charge, carbon flux through the glycolytic pathway may be stimulated by high carbohydrate uptake, thus explaining the increased flux of carbon through the glycolytic pathway relative to the pentose-phosphate pathway.

In a reciprocal fashion, the glucose level may regulate glutamine metabolism. It has been shown that increased concentrations of glucose inhibit glutamine uptake (Zielke et al., 1978), while the substitution of fructose or galactose as the carbohydrate source stimulates glutamine uptake. Thus, a high rate of carbohydrate uptake inhibits glutamine uptake. The low energy charge resulting from increased carbohydrate transport may limit the rate of glutamine transport into the cell, since the transport of glutamine is energy dependent.

The volumetric ATP production rate (APR) can be estimated by monitoring both the volumetric oxygen uptake (OUR) and volumetric lactic acid production (LPR) rates of the culture. It is known that 1/2 mole of oxygen is required to oxidize 1 mole of either NADH or FADH. The oxidation of 1 mole of NADH provides energy for the creation of 3 moles of ATP, while the oxidation of 1 mole of FADH, provides energy for the production of 2 moles of ATP. Fortunately, FADH generation always occurs simultaneously with GTP generation in the Kreb's cycle. So, for ATP accounting purposes, the utilization of 1/2 mole of oxygen can be said to produce 3 moles of ATP, regardless of whether NADH or FADH is oxidized.

If the carbohydrate moiety is the only source of lactic acid production, 1 mole of ATP is formed for each mole of lactate produced. However, it can be seen from Figure 3 that lactic acid produced from glutamine is simply a waste product of glutamine oxidation, and does not represent any additional production of ATP over that produced via respiration. Therefore, to estimate the APR of mammalian cells properly, the percentage of total lactic acid produced from the carbohydrate must be known. The APR may then be calculated as follows:
\[ \text{APR} = 6\text{OUR} + (\text{LPR})(L_c) \]  \hspace{1cm} (12)

where

\[ \begin{align*}
\text{APR} & = \text{volumetric rate of ATP production, } \frac{\text{mmole}}{\text{l - hr}} \\
\text{OUR} & = \text{volumetric oxygen uptake rate, } \frac{\text{mmole}}{\text{l - hr}} \\
\text{LPR} & = \text{volumetric lactic acid production rate, } \frac{\text{mmole}}{\text{l - hr}} \\
L_c & = \text{the fraction of lactic acid produced from the carbohydrate/total lactic acid produced}
\end{align*} \]

As will be presented subsequently, \( L_c \) for cells grown on fructose approaches 0, whereas \( L_c \) is significantly non-zero for cells grown on glucose.

The percentage of the total cellular energy derived from glutamine varies with the cell type and environmental conditions. For HeLa cells grown on glucose, 80% of the glucose carbon goes through glycolysis to lactate, while 35 and 13 percent of the glutamine is converted, via the Kreb's cycle, to CO\(_2\) and lactate, respectively (Rietzer, 1979). Consequently, 70% of the ATP produced results from glutamine metabolism. HeLa cells grown on fructose instead of glucose shunt almost all of the fructose carbon to the pentose-phosphate pathway, while the same proportion of glutamine is converted to CO\(_2\) and lactate. Thus, glutamine provides nearly all of the total cellular energy. The increased rate of glutamine uptake on fructose with respect to glucose may make up for the ATP that was provided by glucose via glycolysis. Since no lactate is formed from fructose, \( L_c \) in equation (4) is zero. In contrast, glutamine provides only a maximum of 40% of the total cellular energy in fibroblasts grown on glucose (Zielke, 1980; Donnelly and Schaffler, 1976). Glutamine accounts for only one third of the lactic acid produced by these cells (i.e., \( L_c = 0.67 \)).

In light of the preceding discussion concerning glutamine energy metabolism, the
data presented by Fleischaker (1982) may be expanded upon. Fleischaker observed a decrease in lactic acid production and an increase in respiration of human fibroblast cells, both when glucose was reduced to low concentrations (0.2 - 1 mM) and when glucose was replaced by galactose. These observations were interpreted as the "Crabtree effect", that is, readily utilizable carbohydrates at increasing concentrations are shunted through glycolysis toward lactate at increasing rates, while proportionally less are oxidized in the Kreb's cycle. Kuchka et al. (1981), however, demonstrated that human fibroblast cells grown on galactose oxidized 4.5 to 6.5 times more glutamine than cells grown on glucose. In addition, Kuchka et al. (1981) observed an increase in glutamine oxidation as the glucose concentration was decreased below 1.8 mM. Therefore, the stimulation of respiration observed by Fleischaker (1982) in galactose or in low glucose medium was probably the result of increased glutamine oxidation at these conditions, and not the result of the "Crabtree" effect.

Other medium components may be involved in the energy metabolism of mammalian cells. Sumbilla et al. (1980) compared the rates of oxidation of glutamine with those of glucose and fatty acids, and determined that the oxidation rates were insignificant in comparison to glutamine. Amino acids other than glutamine are depleted from the medium by mammalian cells (Butler and Thilly, 1982; Glacken and Sinskey, unpublished). However, the rates of utilization are much lower than that of glutamine. Because these amino acids are required in significant quantities for protein synthesis, it is doubtful that they are catabolized to produce energy. As support for this hypothesis, it has been observed that cultures temporarily depleted of glutamine no longer excrete ammonia, indicating that amino acid catabolism has ceased (personal observation).

II.B.2 Ammonia Metabolism and Toxicity

Mammalian cells and tissues produce ammonia (Lund, 1970). Stone and Pitts (1967) have shown that the primary source of ammoniagenesis in mammalian tissue is glutamine. In addition, ammonia is generated in cell culture medium during storage (Ryan and Cardin, 1966; McLimans et al., 1981) via the spontaneous first order
deamidation of glutamine (Tritsh and Moore, 1962).

Since intracellular glutamine is responsible for cellular ammonia production, it would be instructive to review glutamine transport and deamination/deamidation pathways in mammalian cells. First, glutamine transport across the cell membrane is mediated by a protein carrier. In hepatocytes, this carrier is specific for glutamine and is Na⁺-dependent (Joseph et al., 1978), while with rat intestinal cells, the glutamine carrier also transports alanine and is both Na⁺ and energy-dependent (Bradford and McGivan, 1982). The Km for these carriers range from 0.25 mM in the rat brain (Benjamin et al., 1981) to 2.1 mM in rat intestinal cells (Bradford and McGivan, 1982).

Since the mitochondria is responsible for much of the glutaminase activity in the cell, transport of glutamine across the mitochondrial membrane is important. Welbourne and Bazer (1980) have shown that, while glutamine does not traverse the mitochondrial membrane of normal rat kidneys easily, renal mitochondrial membranes of acidic rats demonstrate increased permeability to glutamine, thereby allowing increased glutamine transfer and consequently, increased glutamine deamidation/deamination and ammoniagenesis. In addition, the ratio of ammonia produced per glutamine utilized increases from 1.22 for normal rats to 1.85 in acidic rats. This altered permeability may be a buffering response by the mammalian system to acidic conditions. When glutamine is hydrolyzed in the mitochondria, ammonia is produced. Since ammonia is basic, it attracts a proton from the surrounding fluid to form an ammonium ion. Thus, an increase in glutamine permeability across the mitochondrial membrane may help buffer the cell against acidic conditions. Cultured mammalian cells probably have glutamine permeable mitochondria, since glutamine is taken up and deamidated/deaminated at a rapid rate. Mammalian cells grown in vitro are analogous to acidic cells, in this respect.

Glutamine can be deamidated/deaminated by one of two mechanisms; via glutaminase, which hydrolyzes glutamine to glutamate and ammonia; and/or via L-glutamine-oxoacid aminotransferase, which transfers the α-amino group of glutamine to an α-oxoacid to yield α-oxoglutaramate and an amino acid (Lund, 1970). The α-
oxoglutarate irreversibly deamidates to α-ketoglutarate and ammonia. Stone and Pitts (1967) have shown that the latter pathway predominates in dog kidney, while Goldstein (1967) has shown glutaminase is predominant in the rat kidney. However, both mechanisms are often lumped together and referred to as "glutaminase activity." The $K_m$ of the glutaminase activity ranges from 1.6 mM in the rat brain (Benjamin, 1981) to 5 mM for the purified rat renal glutaminase (Klingman and Handler, 1958).

The glutaminase activity is regulated by ammonia in many systems. Ammonia inhibits glutaminase activity in the brain of the rat (Benjamin, 1981) and in the rat kidney (Klingman and Handler, 1958), while ammonia has no effect on the glutaminase of cultured astrocytes (Schousboe et al., 1980). In rat liver mitochondria, ammonia stimulates glutaminase activity. The $K_i$ of ammonia on glutaminase ranges from 0.7 mM in the rat brain (Benjamin, 1981) to over 5 mM in the rat kidney (Goldstein and Schooler, 1967). Inhibition of glutaminase by ammonia may account for its toxic effect in vivo, as well as in vitro, for some cell types.

Ammonia is toxic for most animals. In humans, 300 μM ammonia in the blood is lethal (McReynolds et al., 1978). Cultured mammalian cells can survive much higher ammonia concentrations, depending on the cell type. For example, while the growth of strain L mouse cells are inhibited in 0.5 mM of ammonia (Ryan and Cardin, 1966), 10 mM of ammonia is needed to inhibit the growth of chorioallantoic tissue (Eaton et al., 1962), and 40 mM of ammonia is needed to inhibit the growth of mouse ascites tumor cells (Furusawa and Cutting, 1962). The absolute value of these or other ammonia concentrations mentioned subsequently must be viewed with caution, since these values usually reflect only the concentration of ammonia added to the culture. Since there is usually a background of at least 1 mM ammonia, even at the best conditions, the value of 0.5 mM ammonia reported toxic for mouse L cells must be viewed with some skepticism. In our laboratory, we have shown that prolonged exposure (over 36 hours) to actual medium concentrations of 5 to 8 mM inhibits growth of canine kidney cells (MDCK) (Glacken et al., 1986, Appendix B).

From a commercial viewpoint, ammonia can affect the profitability of large-scale mammalian cell processes, not only by inhibiting cell growth, but by inhibiting cellular
metabolism and product production as well. For example, interferon production by human fibroblast cells is inhibited by 50% at ammonia concentrations of 2 mM (Ito and McLimans, 1981), while 50% inhibition of interferon production does not occur until 20 mM with mouse L cells (Commooy-Chevalier, 1978). Vaccine production can be deleteriously affected by ammonia. It has been shown that the propagation of influenza (Jensen and Liu, 1961), Newcastle Disease (Eaton and Scala, 1961), and Columbia SK (Furusawa and Cutting, 1962) viruses are inhibited by ammonia. Influenza virus production can be completely halted at ammonia concentrations of 1 mM, while 10 mM ammonia is necessary to completely inhibit NDV production.

How does ammonia affect cellular metabolism, and what is the basis of its toxic action? It has been shown that ammonia inhibits glutaminase in many mammalian tissues (Bejamin, 1981; Goldstein and Schooler, 1967; Klingman and Hardler, 1967; Sayre and Roberts, 1958). The brain is particularly sensitive to ammonia inhibition of glutaminase since glutamate, the product of glutaminase activity, is involved in neuron transmitter function. This may explain why very low concentrations of ammonia (300 µM) are toxic to humans. The inhibitory effect of ammonia on glutaminase may also explain its toxic effect on some cultured cells. Since glutamine is a major source of energy in cultured cells, any inhibition of glutaminase may be expected to have an inhibitory effect on growth. However, as mentioned previously, not all glutaminase activity of all cell types are inhibited by ammonium, so this can not be a universal explanation.

Hypotheses explaining the toxic effect of ammonia on cultured cells and tissues are varied. One explanation is based on the premise that ammonia may stimulate glutamate formation from α-ketoglutarate via an increase in activity of glutamate dehydrogenase (McFarlane-Anderson and Alleyne, 1981; Lund, 1970). This action is thought to deplete the pool of α-ketoglutarate and consequently inhibit the operation of the Kreb's cycle. This explanation does not seem plausible for cultured cells since glutamine is deamidated/deaminated so rapidly in culture, that the flux of glutamate should be heavily oriented towards α-ketoglutarate.
It has been demonstrated that ammonia stimulates glycolysis, while inhibiting respiration in Ehrlich ascites tumor cells (Olavarria et al., 1981) and in rat adipocytes (Stelling et al., 1981). Olavarria showed that this effect was a result of phosphofructokinase inhibition, a decrease in the ATP/ADP ratio, and the oxidation of cytochromes c and a. Related to these findings is the observation that ammonia metabolism is intimately related to the NADH/NAD ratio in the mitochondria (Goldstein et al., 1981; Lund, 1970). This suggests that ammonium formation may act to decouple proton gradients in the mitochondria, thereby reducing the ATP/ADP ratio and slowing growth. As stated previously, formation of un-ionized ammonia from glutaminase activity in the mitochondria would act as a base by sequestering hydrogen ions away from the mitochondrial environment.

That ammonium may affect intracompartmental pH is substantiated by the results of Karin et al. (1981), who determined that ammonium inhibited transferrin-meditated iron uptake. The ammonium did not affect the binding of transferrin to its receptor. Rather, the ammonium was believed to raise the intralysosomal pH, thereby interfering with the release of iron from the transferrin transported there. The lysosome is maintained at a pH of 5 to shift the equilibrium of iron-transferrin binding to the free iron state (Dautry-Varsat and Lodish, 1984).

II.C Optimal Control Theory Applied to Microbial Bioreactors

II.C.1 Background:

There has been strong interest in applying modern control theory to fed-batch cultures of microbial fermentations in recent years. Methods of optimal control theory have been implemented to determine the best control policy for feeding nutrient to microbial fermentations. Fed-batch operation is more suitable than batch or continuous fermentations for achieving high yields and productivities of desired products at the culture terminus, especially when undesired components are produced in parallel with the desired product (Fishman and Biryukov, 1974; Ohno et al., 1976; Staniskis and Levisauskas, 1984). Fed-batch fermentations have also been found to be most efficient in overcoming the effects of substrate inhibition and
catabolic repression (Parulekar and Lim, 1985). In general, whenever the specific rates of growth and/or product formation are non-monotonic functions of the substrate concentration, fed-batch operation may be superior. Constantinides (1979) has written an extensive review of this area.

The following is a brief overview of the theoretical basis for the determination of an optimal control policy for problems in variational calculus. The derivation that is to follow combines the elements of the derivations of both Bryson and Ho (1976) and Beveridge and Schechter (1970). Consider the problem of determining a set of functions \( u(t) \) that maximize (or minimize) an objective function, \( J \), defined by:

\[
J = \phi(x(t_f), t_f) + \int_{t_0}^{t_f} L[x(t), u(t), t]dt
\]

(13)

where the state variables, \( x(t) \), must satisfy the following differential equations:

\[
\dot{x} = f[x(t), u(t), t] \text{ with } x(t_0) \text{ given, } t_0 \leq t \leq t_f
\]

(14)

This is a constrained problem. That is, \( u(t) \) must be chosen such that equation (14) is at all times satisfied. That is,

\[
F = f - \dot{x} = 0
\]

(15)

Now consider the variation in \( J \), \( \delta J \), due to variations in the control vector \( u \), \( \delta u \), for fixed times \( t_0 \) and \( t_f \). A variation differs from a differential, \( dJ \), in that the variation computes the change in the function \( J \) for small arbitrary changes in \( u(t) \) at each time \( t \) between \( t_0 \) and \( t_f \), whereas a differential evaluates the change in \( J \) for corresponding changes in \( u( t_f) \) at a fixed time, \( t_f \).

To clarify, suppose \( u^*(t) \) is a vector function of \( t \) which minimizes \( J \), and \( u(t) \) is a second vector function which is at most infinitesimally different from \( u^*(t) \) at every
point \( t \). Then the variation of \( u(t) \) is defined as:

\[
\delta u = u^*(t) - u(t)
\]  

(16)

The variation of a function represents an infinitesimal change in both the function and its derivatives at each time \( t \), the change being arbitrary. Even though \( u(t) \) may be changed by changing \( t \), the variation symbol implies that \( t \) is fixed and the function itself is altered. This is different from differentiation, which represents the measure of the change of a function resulting from a specified change in the independent variable. If \( u^*(t) \) is the control vector that minimizes \( J \), then any small change away from \( u^*(t) \) results in non-improving \( J \) or:

\[
\delta J = 0
\]  

(17)

\[
\delta J = \delta x J + \delta u J = 0 \quad \text{for every} \quad t_0 \leq t \leq t_f
\]  

(18)

Note that \( u(t) \) may only be varied in a manner that does not violate the local constraints in (14). That is, \( F \) must equal zero at all times, implying that \( \delta F = 0 \), such that:

\[
\delta F = \delta x F + \delta u F = 0 \quad \text{for every} \quad t_0 \leq t \leq t_f
\]  

(19)

For equations (18) and (19) to both hold and yield a non-trivial solution the equations must be linearly consistent, for all \( t \). In other words, a set of vector functions \( \lambda(t) \) may be found, such that

\[
\frac{\delta J}{\delta Y} + \lambda^T \frac{\delta F}{\delta Y} = 0
\]  

(20)

where \( Y^T = (x_1, \ldots, x_n, u_1, \ldots, u_n) \).
Solving equation (20) is equivalent to finding the stationary point of a new objective function $J^*$:

$$J^* = \phi[x(t_f), t_f] + \int_{t_0}^{t_f} \left\{ L[x(t), u(t), t] + \lambda^T(t) f[x(t), u(t), t] \right\} dt \tag{21}$$

$$J^* = \phi[x(t_f), t_f] + \int_{t_0}^{t_f} \left\{ L[x(t), u(t), t] + \lambda^T(t) f[x(t), u(t), t] - \dot{x} \right\} dt \tag{22}$$

The functions, $\lambda(t)$, are called adjoint functions. Integration of the last term in the integral by parts gives:

$$J^* = \phi[x(t_f), t_f] - \lambda^T(t_f) x(t_f) + \lambda^T(t_0) x(t_0) + \int_{t_0}^{t_f} \left\{ H[x(t), u(t), t] + \lambda^T(t) x(t) \right\} dt \tag{23}$$

where $H[x(t), u(t), t] = L[x(t), u(t), t] + \lambda^T(t) f[x(t), u(t), t]$

$H$ is called the Hamiltonian.

The variation in $J$ due to variations in the control vector $u(t)$ for fixed times $t_0$ and $t_f$ may be computed:

$$\delta J = \left[ \frac{\partial \phi}{\partial x} - \lambda^T \right] (\delta x)|_{t_f} + \left[ \lambda^T (\delta x) \right]_{t_f} + \int_{t_0}^{t_f} \left\{ \frac{\partial H}{\partial x} + \dot{\lambda}^T \right\} \delta x + \frac{\partial H}{\partial u} \delta u \right\} dt = 0 \tag{24}$$

Since $\delta J$ must be zero for any arbitrary changes in vectors $\delta x$ and $\delta u$, the coefficients of the differentials must all be zero. Thus, the necessary conditions for the
extrema are:

\[
\frac{\partial H}{\partial u} = 0
\]  
(25)

\[
\lambda^T(t) = -\frac{\partial H}{\partial x}
\]  
(26)

\[
\lambda^T(t_f) = \left[\frac{\partial \phi}{\partial x}\right]_f
\]  
(27)

Since the initial values of \( x \) are specified at \( t = t_0 \) then \( \delta x|_{t=t_0} = 0 \), which implies that \( \lambda^T(t_0) \) are not necessary zero. If the final time is not specified, an additional necessary condition is that \( H = 0 \) (Bryson and Ho, 1976).

Thus, by solving the system of differential equations in (14), (25), and (26) the vector function \( u^* \) that minimizes the function \( J \) may be found by satisfying equation (16). This is a split two-point boundary-value problem.

The solution of this two-point boundary value problem is, in general, difficult to solve. Analytical solutions rarely can be obtained, so one must usually resort to numerical solutions. A description of such general numerical procedures may be found in Chapter 7 of Bryson and Ho (1976). In general, these solutions involve making an initial guess for \( u^*(t) \), followed by integration of the differential equations, which is then used to upgrade the initial estimate of \( u^*(t) \), from where the process is repeated until the boundary conditions are satisfied.

Returning to the subject at hand, many methods have been developed to solve optimal control problems for fed-batch microbial bioreactors. The state equations of these bioreactors are linear with respect to the control variable, \( F \), the nutrient feed rate. This implies that \( \partial H/\partial F \) is independent of \( F \), and consequently cannot be used to determine the feed rate. This type of problem is a singular problem. For these problems, Pontryagin's Maximum Principle may be invoked (Pontryagin et al., 1962).
This principle states that $F$ should be chosen according to the following (for $J$ maximizing):

$$\frac{\partial H}{\partial F} > 0, \quad F = F_{\text{max}} \quad (28)$$

$$\frac{\partial H}{\partial F} < 0, \quad F = F_{\text{min}} \quad (29)$$

$$\frac{\partial H}{\partial F} = H_F = 0, \quad F = F_{\text{singular}} \quad (30)$$

$F_{\text{singular}}$ is determined from repeated time differentiations of the $H_F$, until $F$ appears in the derivative (Bryson and Ho, 1976).

Determination of $F_{\text{singular}}$ may be quite complex. Ohno et al. (1976) used Green's Theory to develop an analytical solution for $F_{\text{singular}}$. However, this method only applies to state variable spaces of dimension two. A number of methods have been developed that convert the singular problem into a non-singular one. Via Kelley's Transformation of the state variables (Kelley, 1965), Staniskis and Levisaukas (1984) and Hong (1986) transformed singular systems of dimension 3 to non-singular systems of dimension 2. Both authors used this transformation to determine when singular control was initiated (termed the conjunction point), as well as the functional form of $F_{\text{singular}}$. In both cases, the control variable of the transformed system was the liquid volume, $V$, and not the medium flow rate, $F$.

Yamane et al. (1977) converted the singular problem into a non-singular one by specifying, $\mu$, the growth rate, as the control variable. Similarly, Guthke and Knorre (1981) used the substrate concentration as the control variable. A practical problem with the latter two methods was that certain states of the system could not be achieved due to physical constraints.

When the dimension is low and when the stated problem is of a fortuitous form, the adjoint variables may be ignored and the control profile may be determined.
directly and analytically. Weigand et al. (1979) used this method to optimize a repeated fed-batch microbial reactor, while San and Stephanopoulos used this method to optimize microbial fed-batch bioreactors (1984, 1986) as well as enzyme bioreactors (1983). The common feature of all of these systems is that the dimensionality was three or less. Generalized computational algorithms based on this procedure have been developed by Modak, Lim and co-workers (1986a,b).
III. MATERIALS AND METHODS

III.A. Cell Lines and Stock Culture Maintenance

CRL-1606, a mouse-mouse hybridoma producing anti-fibronectin IgG monoclonal antibody (Schoen et al., 1982), was obtained frozen from the American Type Culture Collection (ATCC, Rockville, MD) at an unknown passage number. The culture was thawed and diluted 10-fold with Dulbecco’s Modification of Eagle’s Medium (DMEM, Flow Laboratories, Inc., McLean, VA) supplemented to 8% newborn calf serum (NCS, Hazleton Dutchland, Inc., Denver, PA) 2% fetal calf serum (FCS, Hazleton), 64 μg/ml potassium penicillin G (Sigma Chemical, St. Louis, MO), and 100 μg/ml streptomycin sulfate (Sigma Chemical). The cells were maintained at 37°C in a 10% CO₂ environment and were diluted 10-fold every 2 to 3 days with DMEM (8% NCS, 2% FCS) until the viability was determined to be 99+% via trypan blue vital staining (Phillips, 1973). An aliquot from the culture was then centrifuged at 1500 RPM for 10 minutes and concentrated to 3x10⁶ cells/ml in DMEM supplemented to 8% NCS, 2% FCS, and 5% dimethyl sulfoxide (DMSO). One ml aliquots were frozen in stages at successively lower temperatures and stored permanently in liquid nitrogen. For each experiment, a one ml vial was rapidly thawed, and diluted 10-fold with DMEM (8% NCS, 2% FCS). The cultures were passaged every 2 to 3 days so that viability was maintained at 99+%. Only cultures that had been maintained in exponential growth for less than one month were utilized in experiments, to ensure that the cell passage did not affect the results.

III.B. Growth Rate and Specific Antibody Productivity Modeling Experiments

Hybridoma cultures were grown in T-75 flasks (Corning Glass Products, Corning NY) at various glutamine, glucose, serum, sodium lactate, and ammonia chloride concentrations, as well as at various b-DMEM dilutions; i.e., dilutions of basal 1X DMEM (without NaCl, NaHCO₃, glutamine, glucose, or serum) adjusted to 3.7 g/l NaHCO₃, and 325 mosmol with NaCl. The b-DMEM dilutions used in the
experiments were 1X (no dilution), 0.6X (diluted to 60% of 1X), and 0.2X (diluted to 20%). Concentrated isotonic (325 mosmol) solutions of glutamine, glucose, serum (FCS), sodium lactate, and ammonium chloride were made in 1X, 0.6X and 0.2X b-DMEM-diluted medium. Also, isotonic solutions of 1X, 0.6X, and 0.2X diluted b-DMEM were prepared. All solutions were filter-sterilized (0.2 micron, Millipore, Milford, MA). Solutions at a given b-DMEM dilution were mixed aseptically such that isotonic solutions at the desired component concentrations and b-DMEM dilutions were obtained. Sodium lactate stock solutions were prepared by neutralizing the free lactic acid (Sigma Chemical) with an equal molar concentration of NaOH. Glutamine and glucose were obtained from Sigma Chemical, while ammonium chloride was obtained from Merck (Rahway, NJ). The b-DMEM solution was prepared from the individual components as described by Dulbecco (Morton, 1970). The amino acids and the vitamins were supplied by Sigma Chemical.

After the various media formulations were prepared and added to the T-75 flasks, an aliquot of exponentially growing hybridoma cells were centrifuged at 1500 RPM for 10 minutes. The spent medium was drawn off and replaced with b-DMEM medium of the lowest b-DMEM dilution and the lowest serum level to be used in a given experiment. The cultures were inoculated from this mixture (usually 1 ml into 50 or 100 ml) to achieve cell levels of 5000 to 7000 cells/ml. These low cell concentrations were necessary so that the cells would not appreciably alter the nutrient and waste product concentrations in the medium over a period of 30 to 40 hours. Therefore, the results would not be dependent on either the extent of growth or the culture time. Occasionally, experiments were initiated at high initial cell levels (i.e., 50,000 cells/ml). The cell concentration of the inoculum in such cases was adjusted so that only 1 ml of cell suspension was added to the 50 or 100 mls of experimental medium. The cultures were grown at 37 °C in a 10% CO₂ environment.

Samples were taken from the flasks every 9 to 12 hours. Total cell levels were measured via a Coulter Counter model ZF with a 100-micron aperture tube (Coulter Electronics, Inc., Hialeah, FL). The samples were diluted with Isotone (Coulter Electronics) to 2,000-20,000 cells/ml. For subsequent antibody assay, samples were
diluted 1:1 with a 15 mg/ml BSA (Sigma Chemical) solution in PBS (0.05% tween-20) after removal of the cells via centrifugation. The samples were stored at -70°C. For subsequent nutrient and waste product assay, supernatant was stored frozen at -15°C.

III.C Experiments To Determine Specific Ammonium Productivity (q_A), and Specific Glutamine Utilization (q_G)

The experimental procedures followed to determine q_A and q_G as functions of glucose, glutamine, serum, lactate, ammonium, and b-DMEM dilution levels was essentially identical to the procedures used to determine μ and q_A, as described previously, with the following exceptions:

- b-DMEM dilutions of 1X and 0.4X were used in the statistical design grid.
- The initial cell concentrations were 120,000 cells/ml instead of 5000 cells/ml. Samples were taken every 6-7 hours for 24 hours (12-36 hrs.). The higher cell levels were used so that perceptible changes in the ammonium and glutamine profiles could be accurately measured.
- Two medium samples were taken at every time point so that replicate ammonium assays could be obtained.

III.D. Experiments to Determine the Rate-Limiting Components in Serum and to Characterize Cell-Dependant Growth Rate Stimulation:

All medium used to grow cells in these experiments was prepared from powdered medium. DMEM powder (KC Biologicals, catalog no. DM-326) was added 13.43 g per liter of double distilled water. Sodium bicarbonate was added to 3.7 g/l. Fifty percent mixtures of DMEM and F-12 (DMEM/F-12) were formulated by adding 6.72 g of DMEM powder, 5.31 g of F-12 powder (Gibco, catalog no. 430-1700), and 3.7 g of NaHCO_3 per liter of water. All medium was filtered sterilized prior to the addition of any supplements. Hazleton fetal calf serum (FCS) from the same lot was used in all experiments.
For all experiments, cultures were inoculated at two different initial cell levels: 50,000-90,000 cells/ml and 5,000-9,000 cells/ml. Inocula was prepared from late exponential or stationary phase stock cultures. The cells were spun down and the spent medium was replaced with DMEM supplemented to the lowest serum level to be used in a given experiment. The inocula was counted with a hemocytometer (Phillips, 1973), and medium was added to achieve a cell level 50 to 100 times higher than that to be used in the cultures to be initiated at high cell concentrations. This cell suspension was used to inoculate these cultures. A fraction of the cell suspension was diluted 10-fold and was used to inoculate cultures to be started at the low initial cell levels. Inocula was added as either 1% or 2% of the culture volume.

Growth factors were added from either 50X or 200X stock solutions. Addition of these supplemental growth factors would slightly dilute the serum level in the medium. To account for this, control cultures (cultures in which no additional growth factors were added) were diluted with equivalent volumes of PBS. Insulin (Sigma, catalog no. I5500) and human transferrin (Sigma, catalog no. T2252) were prepared as described by Iscove (1984). BSA - linoleic acid, ethanolamine cysteine, cystine, thiolglycerol, Na₂SeO₃, SnCl₂, MnSO₄·H₂O, (NH₄)₆ Mo₇O₂₄·4H₂O, NiCl, Na₃VO₄·3H₂O, and 3CdSO₄·H₂O were all supplied by Sigma. All solutions were filter-sterilized with 0.2 micron low - protein binding syringe filters (Milllex-GV, Millipore) prior to supplementation. Cysteine solutions were prepared immediately before usage to avoid oxidation to cystine.

Experimental cell concentrations were determined via a Coulter Counter, as described previously.

III.E Fed-Batch Cultivation with Medium Addition Determined via Optimal Control Theory

III.E.1 Experimental Methods - Medium:

The medium used was standard Dulbecco's Modification of Eagle's Medium(DMEM) with four times the standard concentration of all amino acids except
glutamine and cystine (0.4 mM, 2X concentration). Higher cystine levels could not be used due to its low solubility. The initial glutamine and glucose levels in the bioreactor were 1.3 mM and 12.5 mM respectively, while the levels in the medium feed were 33.6 mM and 165 mM, respectively. The sodium chloride level in both the start-up medium and the feed medium was adjusted to maintain an osmolality of 325 mosmol. The initial serum level was 2.6 %FCS. There was no serum in the feed medium.

III.E.2 Experimental Methods - Bioreactor Operation:

A 20-l spinner vessel with a magnetic agitator was used. The initial and final liquid volumes were 932 and 2000 mls, respectively. The paddles on the agitators at all times extended through the liquid surface. A humidified gas purge of air, pure oxygen, and carbon dioxide, metered through flowmeters, was constantly supplied. Readings from a polarographic dissolved oxygen probe (Ingold) were used to adjust the flowmeter settings such that the DO level was maintained between 20 and 70% of air saturation. The rate of CO₂ addition was adjusted based on the pH of the medium, estimated from the color of the phenol red indicator in the medium. The vessel was maintained in the dark in a 37° C room.

The flow rate of medium into the vessel was determined based on the volume profile determined from optimal control theory and given in Figure 35. The RPM of a variable speed peristaltic pump (Cole Palmer, Chicago, IL) was adjusted every six hours. The weight of the medium feed vessel was constantly monitored. At no time did the actual volume in the 20-l vessel vary from the desired volume by more than 10 mls (0.5%). The medium feed vessel was at all times maintained at 4° C.

Prior to inoculation, cells were concentrated in fresh DMEM at 5% FCS to 1.6 x 10⁶ cells/ml and allowed to grow for 24 hours to 3.2 x 10⁶ cells/ml. This represents an average growth rate of 0.03 h⁻¹. This cell suspension was used to inoculate the 20-l spinner vessel to 2.05 x 10⁵ cells/ml.
III.E.3 Experimental Methods - Assays:

Every eight hours, 5 mls of culture medium was collected. The total cell concentration was measured via a Coulter Counter, while the culture viability was determined via microscopic observation on a hemocytometer after trypan-blue staining. After removing the cells via centrifugation, the samples were stored at 15°C for subsequent analysis.

III.F. Analytical Methods

Anti-fibronectin monoclonal antibody titers were measured by means of an enzyme-linked immuno-sorption assay (ELISA). The samples were measured against G1-kappa mouse anti-human fibronectin.2 monoclonal antibody obtained from BRL (Bethesda, MD, cat. no. 95236A, lot no. 20617) as a standard. First, 50 μl of 10 μg/ml fibronectin in PBS was added to each well of a 96-well microtiter plate (Flow Laboratories, Inc.), and incubated overnight at 4°C. The fibronectin was removed and was replaced with 105 μl/well of 10 mg/ml bovine serum albumin (BSA, Sigma Chemical) in 0.05% Tween-20 in PBS. After the plates had incubated at room temperature for 3 hours, they were rinsed four times with 150 mM NaCl in 0.05% Tween-20 in distilled water (Tween-saline, pH 7). Then, 90 μl of diluted sample was placed in each well, and was incubated at room temperature for 4 hours. Each plate contained standard samples at 0, 25, 40, 60, 80, 100 and 130 ng/ml anti-fibronectin antibody(Sigma). Each sample was repeated in triplicate. All samples, if diluted, were diluted with 7.5 mg/ml BSA in Tween-PBS. After each plate was rinsed 8x with Tween-saline, 55 μl of anti-mouse IgG antibody linked to horseradish peroxidase (Sigma Chemical, cat. no. A2028) diluted 167-fold in 7.5 mg/ml BSA in Tween-PBS was added to each well. After incubation at room temperature for 3 hours, each plate was rinsed 8x with Tween-PBS. A substrate solution of ABTS (Mannheim Boehringer Biochemicals, Indianapolis, IN) was added at 200 μl/well to each plate. After 12 minutes, the reaction was stopped by adding 50
μl of a 5% hydrogen peroxide solution to each well. The color was read for each well at 420 nm via a microtiter plate reader (Dynatech Instruments, Inc., Santa Monica, CA). The ABTS solution was prepared by adding 160 mg of ABTS and 400 μl of 5% hydrogen peroxide to 100 ml of a phosphate-citrate buffer (5.16 g citric acid 8.27 g Na₂HPO₄·7H₂O, 400 ml H₂O, pH 4).

Ammonia was assayed enzymatically with glutamate dehydrogenase (Sigma Chemical) as described by Lund (1977). Glutamine was determined via high pressure liquid chromatography (Waters Associates, Milford, MA) after derivatization with o-phthalaldehyde (Hill et al., 1979). Lactate was determined enzymatically with lactate dehydrogenase (Gutmann and Nahlefeld, 1974).

III.F Calculations

Specific growth rates (μ) and standard deviations of calculation for each culture were determined from the slope of the linear regression of the log of the cell concentrations vs. time (Bevington, 1962). Four to six time points were used for each calculation. Standard deviations of μ for each experimental condition were calculated using the standard deviations from replicate cultures (Bevington, 1962).

Specific antibody productivities (qₚ) were calculated as follows (see Appendix for legend of symbols):

i) \[
\frac{dP}{dt} = q_p X(t) = q_p X_0 e^{\mu t}
\]

\[
P(t) = \left[ P_0 + \frac{q_p X_0}{\mu} \right] + \left[ \frac{q_p}{\mu} \right] X(t)
\]

\[
P(t) = A + B X(t) \quad \text{where} \quad B = \frac{q_p}{\mu}
\]

ii) A linear regression between the antibody titer and cell concentration for each culture was initiated to obtain the slope B, and the standard deviation of B. The specific antibody productivity was obtained by the multiplication of B by the specific
growth rate. The standard deviation of $q_p$ can be calculated from the standard deviation of $B$ and $\mu$ (Bevington, 1969).

The specific glutamine uptake rate, $q_G$, and the specific ammonium uptake rate, $q_A$ (mmol/cell-hr), can be estimated by solving the following differential equations (assuming $\mu$, $q_G$, and $q_A$ are approximately constant over the interval):

$$\frac{dX}{dt} = \mu X$$

$$\frac{d[GLN]}{dt} = -k[GLN] - q_G X$$

$$\frac{d[NH_4]}{dt} = k[GLN] + q_A X$$

The solution of these differential equations is (see Appendix A for legend):

$$X = X(0)e^{\mu t}$$

$$[GLN] = [GLN]_0 e^{-kt} + \frac{q_G \left[ e^{-kT} - e^{\mu t} \right] X_0}{\mu + k}$$

$$[NH_4] = [NH_4]_0 + \left[ [GLN]_0 + \frac{q_G X_0}{\mu + k} \right] \left[ 1 - e^{-kt} \right] + \frac{X_0 \left[ q_A - \frac{q_G k}{\mu + k} \right] \left[ e^{\mu t} - 1 \right]}{\mu}$$

The parameters $\mu$, $q_G$, and $q_A$ were estimated simultaneously from these equations by minimizing the weighted sum of square of the residuals:

$$(\bar{X} - X)^2 + 16(\bar{G} - G)^2 + 25(\bar{A} - A)^2$$
Since the change in concentration of both ammonium and glutamine was relatively less than the change in the cell concentration, scaling was used to ensure equitable contribution to the objective function from all three terms. The method of Powell was used to perform the multivariable non-linear regression (Kuester and Mize, 1973).

Methods to calculate linear and co-linear coefficients from factorial experiments is described by Box et al. (1978). Gaussian elimination was used to solve the system of linear equations resulting from minimizing of the mean square error of the resolution experiment in Table 5 (Strang, 1980).
IV. RESULTS

IV.A.1 Variable Reduction: Growth Rate.

A priori, it must be assumed that all possible nutrients, growth factors, and waste products may, either singularly or in concert with other medium components, affect the growth rate. The goal of the following set of experiments was to reduce this set of possible relevant environmental variables and interactions of variables to a smaller, more manageable set. This was achieved using the concept of fractional factorial experimental design (Box et al., 1978). This technique assumes that the dependent variable, in this case, the specific growth rate, \( \mu \), is linear and/or co-linear with respect to the independent variables. That is

\[
\mu = \sum_{i=1}^{n} \mu_i Z_i + \sum_{i=1}^{n} \sum_{k=1+1}^{n} \mu_{i,k} Z_i Z_k
\]  

(41)

where

\[
Z_i Z_k = \text{scaled parameters proportional to the component concentrations, usually 1 or -1}
\]

\[
\mu_i = \text{linear coefficients representing the relative effect on } \mu \text{ of scaled component } Z_i
\]

\[
\mu_{i,k} = \text{co-linear coefficient representing the interaction between scaled components } Z_i \text{ and } Z_k \text{ on } \mu.
\]

The independent variables were chosen to be glucose, glutamine, serum, sodium lactate, ammonium chloride, and b-DMEM concentration (i.e., the concentration of DMEM salts, amino acids (excluding glutamine), vitamins, and pyruvate relative to the standard DMEM levels, maintaining an osmolarity of 325 mosmol). The coefficients, \( \mu_i \) and \( \mu_{i,k} \) were directly evaluated from the results of cultures grown at the experimental conditions specified by the factorial design, and were consequently used to rigorously
determine which components, $Z_i$, and which interactions, $Z_iZ_k$, were significant.

The concentrations of the medium constituents used to grow CRL-1606 hybridomas are presented in Table 3, and represents a $2^6-2$ fractional factorial design in two blocks (two sets of experiments performed at different times). Any block effects may be accounted for, and separated from, the calculation of any of the coefficients. Since it was assumed that not every interaction would be significant, the number of experiments in the design was reduced from $2^7$ to $2^7-3$ (Box et al., 1978). Not all co-linear coefficients may be uniquely determined in this case, however. All growth medium shown in Table 3 was isotonic (325 mosmol). The growth rates were calculated from five cell concentrations measured at equal intervals over 40 hours, beginning several hours after the end of lag phase and are shown in column 9 of Table 3. Since the initial cell concentrations were low ($\sim 5000$ cells/ml) and since the period of measurement was relatively brief, the growth rates shown in Table 3 represent initial or intrinsic rates. These rates do not reflect any cell-cell interactions or influences of the cells on the culture medium. Duplicate cultures were grown for each condition, so that standard deviations could be calculated from the standard deviations from replicate cultures (column 10 in Table 3). The average standard deviation for this experiment was 0.002 h$^{-1}$.

An examination of Table 3 indicates that decreased serum, and b-DMEM concentrations, and increased ammonium levels reduce the growth rate. This observation is reflected in Table 4 by the calculation of the values of the linear and co-linear coefficients defined in equation (41) based on the results from Table 3. Any coefficient whose value is less than one half the standard deviation is considered insignificant (Box et al., 1978). Table 4 clearly shows that serum, ammonium and b-DMEM levels had the greatest affect on $\mu$ over the ranges examined. There was a small difference in the average growth rate between the two blocks of experiments (0.0044 h$^{-1}$). Note that lactate levels as high as 40 mM do not significantly affect the growth rate. The growth rate was also independent of the glucose and glutamine levels at concentrations greater than 1 mM. Additionally, the results indicate that four two-factor
Table 3. CRL - 1606 hybridomas were grown in DMEM at the nutrient and waste product levels shown in the table. The initial cell level was 5000 cells/ml, and replicate cultures were inoculated for each formulation shown in the table. Growth rates were calculated from five cell measurements taken every 10 hours for 40 hours, and the rates shown are an average of replicate cultures. The constituent concentrations were selected via the statistical design formulation of Box et al. (1978).

<table>
<thead>
<tr>
<th>#</th>
<th>Glutamine (mM)</th>
<th>Glucose (mM)</th>
<th>Serum (%)</th>
<th>Sodium Lactate (mM)</th>
<th>Ammonium Chloride (mM)</th>
<th>b-DMEM</th>
<th>Block</th>
<th>Growth Rate (hr⁻¹)</th>
<th>Standard Deviation (h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>16.7</td>
<td>9</td>
<td>40</td>
<td>1</td>
<td>0.2</td>
<td>1</td>
<td>0.0287</td>
<td>0.0016</td>
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<td>2</td>
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<td>9</td>
<td>40</td>
<td>6.7</td>
<td>0.2</td>
<td>-1</td>
<td>0.0159</td>
<td>0.0011</td>
</tr>
<tr>
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<td>9</td>
<td>40</td>
<td>6.7</td>
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<td>0.0238</td>
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<tr>
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<td>9</td>
<td>40</td>
<td>1</td>
<td>1</td>
<td>-1</td>
<td>0.0338</td>
<td>0.0016</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
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<td>1</td>
<td>40</td>
<td>6.7</td>
<td>1</td>
<td>-1</td>
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<tr>
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<td>1</td>
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</tr>
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<td>-1</td>
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<td>1</td>
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<td>12</td>
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<td>13</td>
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<tr>
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<td>5</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0.0229</td>
<td>0.0010</td>
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<tr>
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<td>5</td>
<td>6.7</td>
<td>1</td>
<td>-1</td>
<td>0.0024</td>
<td>0.0038</td>
</tr>
</tbody>
</table>

Interactions may be significant. As stated earlier, each co-linear coefficient could not be uniquely determined, since each must be lumped with two other coefficients.

Additional experiments were implemented to uniquely determine the co-linear coefficients in question. The results of such experiments are shown in Table 5. Culture
conditions were identical to that of the previous experiment, except that all cultures were
grown at 9% FCS, thus ensuring relatively high growth rates, and an increased
capability of resolving the interactions. The four equations in rows 5 - 8 of Table 4 were
combined with the results of Table 5 to determine the linear and co-linear coefficients
via a least-mean squares analysis (Beveridge and Schechter, 1970). The results are
presented in Table 6.

Again, ammonium and b-DMEM levels affected the growth rate to a greater extent
than any two-factor interaction and are consistent with the values calculated from the
previous experiment (Table 4). Thus, the results are repeatable. The two-factor
interactions that may be significant are interactions between serum and lactate,
glutamine and block, serum and ammonium, and ammonium and b-DMEM. The
glutamine-block interaction suggests that the stock solution of glutamine may have
degraded slightly on storage at 4 °C between the time the first block and the second
block of experiments were initiated. This may indicate that the growth rate may begin to
become a function of the glutamine level at concentrations only slightly lower than 1
mM. This will subsequently be shown to be the case. This clearly illustrates the
sensitivity of the technique. The interaction between serum and ammonium deserves
special attention since these two variables exhibit the two largest single effects on \( \mu \).
The potential of this interaction was kept in mind in developing a mathematical
relationship for the growth rate.

IV.A.2 Mathematical Description of the Growth Rate, \( \mu \)

The previous results suggested that the growth rate was most sensitive to changes
in the ammonium and serum levels, and that both components must be examined
together in developing a functional form for \( \mu \), since there was determined to exist a
significant interaction between these two variables in their effect on \( \mu \). As a preliminary
step in developing a functional form for \( \mu \), cultures were grown in standard DMEM (25
mM glucose, 4 mM glutamine, 0 mM lactate) at various concentrations of serum and
ammonium. The cultures were inoculated at approximately 8500 cells/ml. The growth
rates were determined from four cell concentrations measured between 16 and 52
hours after inoculation. The results are shown in Figure 4. The growth rate exhibits a
Table 4. Linear and co-linear coefficients defined in equation (41) are calculated from the results in Table 3. The average standard deviation of all of the growth rates in Table 3 was 0.002. Any coefficient that is less than half of the standard deviation is considered insignificant. The subscripts correspond to the following medium constituents: 1 = glutamine; 2 = glucose; 3 = serum; 4 = lactate; 5 = ammonium; 6 = b-DMEM; 7 = block effect.

<table>
<thead>
<tr>
<th>Coefficients</th>
<th>Value(hr(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\mu_3)</td>
<td>0.0098</td>
</tr>
<tr>
<td>(\mu_5)</td>
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</tr>
<tr>
<td>(\mu_6)</td>
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<td>(\mu_7)</td>
<td>0.0022</td>
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<td>(\mu_{1,4} + \mu_{5,6} + \mu_{3,7})</td>
<td>-0.0013</td>
</tr>
<tr>
<td>(\mu_{1,2} + \mu_{3,5} + \mu_{6,7})</td>
<td>0.0012</td>
</tr>
<tr>
<td>(\mu_{2,6} + \mu_{3,4} + \mu_{1,7})</td>
<td>0.0012</td>
</tr>
<tr>
<td>(\mu_{1,6} + \mu_{4,5} + \mu_{2,7})</td>
<td>0.0010</td>
</tr>
<tr>
<td>(standard deviation)/2</td>
<td>0.0010</td>
</tr>
<tr>
<td>(\mu_{1,5} + \mu_{2,3} + \mu_{4,6})</td>
<td>0.0009</td>
</tr>
<tr>
<td>(\sum \mu_{i,k,l})</td>
<td>-0.0009</td>
</tr>
<tr>
<td>(\mu_{2,5} + \mu_{1,3} + \mu_{4,7})</td>
<td>-0.0008</td>
</tr>
<tr>
<td>(\mu_1)</td>
<td>0.0006</td>
</tr>
<tr>
<td>(\mu_4)</td>
<td>-0.0005</td>
</tr>
<tr>
<td>(\mu_2)</td>
<td>-0.0004</td>
</tr>
<tr>
<td>(\mu_{3,6} + \mu_{5,7} + \mu_{2,4})</td>
<td>0.0003</td>
</tr>
</tbody>
</table>
Table 5. Experiments initiated to resolve the two factor interactions in rows 5 - 8 of Table 4 are presented. The experimental methods were as described in Table 3. The serum level in all of the cultures was 9% FCS.

<table>
<thead>
<tr>
<th>#</th>
<th>Glutamine (mM)</th>
<th>Glucose (mM)</th>
<th>Sodium Lactate (mM)</th>
<th>Ammonium Chloride (mM)</th>
<th>b-DMEM</th>
<th>Growth Rate (hr⁻¹)</th>
<th>Standard Deviation (h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>5</td>
<td>16.7</td>
<td>40</td>
<td>1</td>
<td>1</td>
<td>0.0361</td>
<td>0.0016</td>
</tr>
<tr>
<td>20</td>
<td>1</td>
<td>16.7</td>
<td>40</td>
<td>1</td>
<td>1</td>
<td>0.0345</td>
<td>0.0014</td>
</tr>
<tr>
<td>21</td>
<td>5</td>
<td>1.7</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>0.0372</td>
<td>0.0019</td>
</tr>
<tr>
<td>22</td>
<td>1</td>
<td>1.7</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>0.0412</td>
<td>0.0011</td>
</tr>
<tr>
<td>23</td>
<td>5</td>
<td>1.7</td>
<td>40</td>
<td>1</td>
<td>0.2</td>
<td>0.0309</td>
<td>0.0014</td>
</tr>
<tr>
<td>24</td>
<td>1</td>
<td>1.7</td>
<td>40</td>
<td>1</td>
<td>0.2</td>
<td>0.0316</td>
<td>0.0011</td>
</tr>
<tr>
<td>25</td>
<td>5</td>
<td>16.7</td>
<td>5</td>
<td>1</td>
<td>0.2</td>
<td>0.0323</td>
<td>0.0014</td>
</tr>
<tr>
<td>26</td>
<td>1</td>
<td>16.7</td>
<td>5</td>
<td>1</td>
<td>0.2</td>
<td>0.0332</td>
<td>0.0018</td>
</tr>
<tr>
<td>27</td>
<td>5</td>
<td>1.7</td>
<td>5</td>
<td>6.7</td>
<td>1</td>
<td>0.0223</td>
<td>0.0024</td>
</tr>
<tr>
<td>28</td>
<td>5</td>
<td>16.7</td>
<td>5</td>
<td>6.7</td>
<td>1</td>
<td>0.0234</td>
<td>0.0024</td>
</tr>
<tr>
<td>29</td>
<td>1</td>
<td>16.7</td>
<td>40</td>
<td>1</td>
<td>0.2</td>
<td>0.0291</td>
<td>0.0017</td>
</tr>
<tr>
<td>12</td>
<td>1</td>
<td>1.7</td>
<td>5</td>
<td>1</td>
<td>0.2</td>
<td>0.0333</td>
<td>0.0011</td>
</tr>
</tbody>
</table>
Table 6. The linear and co-linear coefficients in equation (41) calculated from rows 5 - 8 in Table 4 and the results of Table 5 are presented. The subscripts are defined as in Table 4.

<table>
<thead>
<tr>
<th>Coefficients</th>
<th>Value (hr(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>average</td>
<td>0.0274</td>
</tr>
<tr>
<td>(\mu_5)</td>
<td>-0.0065</td>
</tr>
<tr>
<td>(\mu_6)</td>
<td>0.0035</td>
</tr>
<tr>
<td>(\mu_{3,4})</td>
<td>-0.0017</td>
</tr>
<tr>
<td>(\mu_{1,7})</td>
<td>0.0012</td>
</tr>
<tr>
<td>(\mu_{3,5})</td>
<td>0.0011</td>
</tr>
<tr>
<td>(\mu_{5,6})</td>
<td>-0.0011</td>
</tr>
<tr>
<td>(\mu_{4,5})</td>
<td>0.0009</td>
</tr>
<tr>
<td>(\mu_{2,6})</td>
<td>0.0008</td>
</tr>
<tr>
<td>(\mu_{1,6})</td>
<td>0.0006</td>
</tr>
<tr>
<td>(\mu_{3,7})</td>
<td>0.0005</td>
</tr>
<tr>
<td>(\mu_{1,4})</td>
<td>0.0006</td>
</tr>
<tr>
<td>(\mu_{1,2})</td>
<td>0.0001</td>
</tr>
<tr>
<td>(\mu_{6,7})</td>
<td>-0.0001</td>
</tr>
<tr>
<td>(\mu_{2,6})</td>
<td>0.0001</td>
</tr>
</tbody>
</table>
cultures. Each point represents the average of two cultures. From equation (42) in the text. The solid line represents results predicted to the growth rates were measured from four cell concentrations taken every 12 hours between 16 and 52 hours. The cultures were inoculated at 8500 cells/ml of serum and ammonium levels. The cultures were shown as a function of growth rate of CRL-1666 hybridomas as a function of growth rate of CRL-1666 hybridomas as a function of growth rate of CRL-1666 hybridomas.
Monod-like dependency with respect to the serum level, while ammonium appears to inhibit growth non-competitively. A double reciprocal plot of $1/\mu$ vs. $1/$serum confirms this observation (Figure 5). The lines in Figure 5 all intersect the x-axis at approximately the same point, indicating non-competitive inhibition (Stryer, 1981) at ammonium levels less than 7.5 mM. The inhibition constant ($K_i$) calculated from these plots was found to be inversely proportional to the ammonium level (Table 7). That is, $K_i = K_A/A$.

Table 7. The Monod constants and the ammonium inhibition constants are calculated from the data presented in Figure 5 for each ammonium level. The values were calculated from a linear regression of the double-reciprocal plots.

<table>
<thead>
<tr>
<th>Ammonium Concentration (mM)</th>
<th>Monod Constant for Serum ($K_s$) (% FCS)</th>
<th>Inhibition Constant for Ammonium ($K_i$) (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.2</td>
<td>$(\mu_{\text{max}} = 0.056 \text{ hr}^{-1})$</td>
</tr>
<tr>
<td>2.5</td>
<td>1.4</td>
<td>14.1</td>
</tr>
<tr>
<td>5.0</td>
<td>1.3</td>
<td>5.5</td>
</tr>
<tr>
<td>7.5</td>
<td>1.1</td>
<td>3.7</td>
</tr>
</tbody>
</table>

Thus, the following mathematical form is proposed:

$$\mu = \frac{\mu_{\text{max}} S}{[S + K_s]\left[1 + \frac{A}{K_i}\right]} = \frac{\mu_{\text{max}} S}{[S + K_s]\left[1 + \frac{A^2}{K_A}\right]}$$ (42)

This functional form is similar to that which describes the kinetics of allosteric
Figure 5. A double reciprocal plot of the inverse growth rate versus the inverse serum level is shown for various ammonium levels. The data is shown in Figure 4.
enzymes. The constants computed from a non-linear regression analysis (Kuester and Mize, 1973) using equation (42) were: $\mu_{max} = 0.55 \pm 0.002$ h$^{-1}$; $K_S = 0.93 \pm 0.12$ % FCS $K_A = 26 \pm 2.3$ mM$. The standard deviation for this regression was 0.002 h$^{-1}$. This equation represents the data well, as shown by the solid lines in Figure 4.

Since ammonium greatly inhibits growth, it would be desirable to control the glutamine concentration at a low level, since spontaneous glutamine decomposition and cellular ammonium excretion have both been shown to be reduced at low glutamine levels (Glacken et al., 1986). The question then is, to what extent is the growth rate is reduced at low glutamine levels. Although the growth rate was shown not to be a function of glutamine concentrations in excess of 1.0 mM, there was some indication (the glutamine block effect in Table 6) that slightly lower glutamine levels might result in lower rates. To address this, cells were inoculated at 8500 cells/ml into DMEM medium (25 mM glucose, w/o glutamine) supplemented to 1.75% FCS and to various glutamine concentrations. The growth rates were calculated from four cell concentrations measured between 16 and 52 hours after inoculation and are shown in Figure 6 as a function of the average adjusted glutamine concentration. The average adjusted glutamine concentration is the amount of glutamine added to the medium from a stock solution, plus the glutamine added from the serum, and minus the average amount of glutamine that is expected to be spontaneously degraded during the length of the experiment, which is:

$$[\text{GLN}] = \frac{[\text{GLN}]_0 e^{k(t_b - t_i)}}{2}$$  \hspace{1cm} (43)

$[\text{GLN}]_0$ is the initial concentration of glutamine. The first order decomposition rate constant, $k = 0.0048$ h$^{-1}$, has been determined previously (Tritsch and Moore, 1966; Glacken et al., 1986). The glutamine level in serum was measured to be 2 mM. Note that the cells are estimated to utilize very little glutamine at such low cell levels:
$$\Delta[\text{GLN}] = q_G X_{\text{avg}} \Delta t = \left[ \frac{7 \times 10^{-11} \text{mmol}}{\text{cell} \cdot \text{h}} \right] \left[ 1.5 \times 10^7 \text{cells/l} \right] [50 \text{h}] = 0.05 \text{mM}$$

The value for \( q_G \), the specific glutamine uptake rate, was taken from Table 17. Thus, the above expression for the average adjusted glutamine levels should suffice, since the actual adjustment to the initial added concentration of glutamine is minor. Not surprisingly, Figure 6 demonstrates typical Monod kinetics. The Monod constant, \( K_G \) calculated from a non-linear regression is 0.15 mM. Thus, not until very low glutamine levels are reached is the growth rate considerably reduced.

At this point, it is tempting to superimpose equation (42) with Monod's equation in glutamine to formulate:

$$\mu = \frac{\mu_{\text{max}} S G}{[S + K_s] \left[ 1 + \left( \frac{A}{K_a} \right)^2 \right] \left[ G + K_G \right]}$$ (44)

Is this superposition valid? Hybridoma cultures were inoculated at 8500 cells/ml in medium at low glutamine levels with varying concentrations of serum and ammonium. The growth rates were calculated from four cell concentrations measured between 16 and 52 hours after inoculation and are shown in Table 8. The growth rates predicted by the superposition equation, although somewhat high in two of the cases, is seen to predict the actual growth rates reasonably well.

Table 4 indicates that serum and lactate levels may interact in determining the growth rate. Indeed, note in Table 3, that the addition of 40 mM to cultures grown in 9% FCS at identical ammonium and b-DMEM levels inhibited the growth rate slightly (0.003 h\(^{-1}\)). However, much higher lactate levels may be reached in cell culture medium, especially if high cell levels (1 x 10^7 cells/ml) can be obtained. Therefore, cultures were inoculated at 8500 cells/ml into DMEM at various serum, ammonium, and lactate levels, shown in Table 9, and the growth rates were computed from 4 cell concentrations.
at 8500 cells/ml. The growth rate of CRL-1606 cells is shown as a function of the glutamine concentration in DMEM medium supplemented to 1.75 μg/ml. Each point represents the average of two cultures. The solid line represents the equation with a monod constant equal to 0.15 mg/l. The cultures were inoculated
measured between 16 and 52 hours after inoculation.

Table 8. Growth rates of cultures initiated at 8500 cells/ml and grown at low glutamine levels and at various serum and ammonium levels, are compared with the rates predicted from equation (44). The constants in equation (44) were determined previously and were taken to be: $\mu_{\text{max}} = 0.55$ h$^{-1}$; $K_S = 0.93$ % FCS; $K_A = 26$ mM$^2$. Each experimental growth rate shown is the average of two replicate cultures.

<table>
<thead>
<tr>
<th>Serum (mM)</th>
<th>Ammonium (mM)</th>
<th>Glutamine (mM)</th>
<th>Experimental Growth Rate hr$^{-1}$</th>
<th>Predicted Growth Rate hr$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0</td>
<td>0.31</td>
<td>0.0396</td>
<td>0.0341</td>
</tr>
<tr>
<td>10</td>
<td>5.0</td>
<td>0.31</td>
<td>0.0227</td>
<td>0.0174</td>
</tr>
<tr>
<td>3</td>
<td>2.5</td>
<td>0.20</td>
<td>0.0191</td>
<td>0.0195</td>
</tr>
</tbody>
</table>

It is clear from Table 9 that lactate inhibits growth only slightly, if at all, at concentrations less than 40 mM. However, significant inhibition did occur in medium with 70 mM lactate. Thus, it appears that extent of growth inhibition may be related to the square of the lactate concentration, analogous to equation (42) developed to describe inhibition from ammonium, that is:

$$\mu = \frac{\mu_{\text{max}}SG}{[S + K_S][G + K_G]\left[1 + \frac{A^2}{K_A}\right]^{1/2}\left[1 + \frac{L^2}{K_L}\right]}$$

The parameters in equation (45) determined from a non-linear regression using the data from Table 9 are as follows: $\mu_{\text{max}} = 0.061 \pm 0.004$ h$^{-1}$, $K_S = 1.6 \pm 0.3$ %FCS, $K_A = 45 \pm 8$ mM$^2$, and $K_L = 12,000$ mM$^2$. The standard deviation for the regression was 0.002 h$^{-1}$,
indicating that equation (45) represents the data very well. If the exponent of the lactate concentration is changed from 2 to 1, the resulting fit was not as good ($\sigma_b = 0.0055 \text{ h}^{-1}$).

Table 9  Cultures were grown at various serum, ammonium, and lactate levels to determine the extent to which lactate affects the growth. Each growth rate shown is the average from replicate cultures. Cultures were initiated at 8500 cells/ml. The glutamine concentration of each culture was 1 mM.

<table>
<thead>
<tr>
<th>Serum (mM)</th>
<th>Ammonium (mM)</th>
<th>Sodium Lactate (mM)</th>
<th>Growth Rate hr$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.25</td>
<td>3.75</td>
<td>0.0</td>
<td>0.0286</td>
</tr>
<tr>
<td>4.25</td>
<td>3.75</td>
<td>22.5</td>
<td>0.0293</td>
</tr>
<tr>
<td>4.25</td>
<td>3.75</td>
<td>70</td>
<td>0.0202</td>
</tr>
<tr>
<td>7.5</td>
<td>1.5</td>
<td>5.0</td>
<td>0.0389</td>
</tr>
<tr>
<td>7.5</td>
<td>1.5</td>
<td>40</td>
<td>0.0355</td>
</tr>
<tr>
<td>1.0</td>
<td>1.5</td>
<td>5.0</td>
<td>0.0192</td>
</tr>
<tr>
<td>1.0</td>
<td>1.5</td>
<td>40</td>
<td>0.0194</td>
</tr>
<tr>
<td>7.5</td>
<td>6.0</td>
<td>5.0</td>
<td>0.0258</td>
</tr>
<tr>
<td>10.0</td>
<td>3.75</td>
<td>22.5</td>
<td>0.0360</td>
</tr>
<tr>
<td>0.6</td>
<td>3.75</td>
<td>22.5</td>
<td>0.0071</td>
</tr>
<tr>
<td>4.25</td>
<td>0.0</td>
<td>22.5</td>
<td>0.0388</td>
</tr>
<tr>
<td>4.25</td>
<td>9.0</td>
<td>22.5</td>
<td>0.0106</td>
</tr>
</tbody>
</table>

The large value of $K_L$ is a clear indication that lactate is a weak inhibitor of the growth rate. Note that the parametric values determined from the set of experiments shown in
Table 9 differ somewhat from the values determined from Figure 4. The difference may be due the different lots of serum used for each experiment. It will be shown in section IV.D that the growth stimulatory activity of serum degrades slowly with time, and thus, may contribute to the discrepancy.

The growth rate has been mathematically related to the four most important components in cell culture medium: 1) serum, the most expensive raw material, the usage of which should be kept to a minimum while still maintaining culture productivity; 2) ammonium and lactate, waste products whose accumulation may be reduced via medium manipulation (Glacken et al., 1986) but never eliminated; and 3) glutamine, the nutrient from which ammonium, the most potent growth rate inhibiting waste product, is derived. All other nutrients could conceivably be constantly maintained at levels above their respective Monod values through simple medium manipulation. For example, Table 10 shows that the growth rate of hybridomas was not affected at glucose levels as low as 0.2 mM or b-DMEM levels above 0.6 X at typical culture conditions (3.75 mM ammonium, 22.5 mM lactate, 1.0 mM glutamine, and 4.3% FCS). Thus, if the concentration of glucose and amino acids are sufficiently elevated, they need not be included in equation (45). Cells cultivated at such elevated levels of DMEM amino acids (4 X, including glutamine except cystine, which was only 2 X for solubility reasons) and glucose (70 mM, osmolarity adjusted by NaCl level) grow normally, as shown in Table 11.

All of the results shown up to this point were taken from cultures initiated at low cell levels (5000 - 8500 cells/ml). To determine if the cell level might have any affect on the growth rate, cells were grown in DMEM at various serum levels and at initial cell levels of 8500 cells/ml and 85,000 cells/ml. Cells were counted every 12 hours, between 16 to 52 hours after inoculation and growth rates were determined from the four points. The results are shown in Figure 7. The figure implies that the Monod constant, Ks, is a function of the cell level, while \( \mu_{\text{max}} \) remains essentially the same, as seen in Table 12.
Table 10. The growth rate of CRL-1606 hybridomas are shown for various medium formulations. All medium contained 1.0 mM glutamine, 1.5 mM glucose, 4.3% FCS, 3.8 mM NH₄ and 22.5 mM lactate in 0.6X b-DMEM unless otherwise specified. Each value is the average of replicate cultures.

<table>
<thead>
<tr>
<th>Medium Composition</th>
<th>Growth Rate (hr⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 mM glucose</td>
<td>0.029</td>
</tr>
<tr>
<td>0.2 mM glucose</td>
<td>0.030</td>
</tr>
<tr>
<td>1.25x b-DMEM</td>
<td>0.031</td>
</tr>
<tr>
<td>1.0x b-DMEM</td>
<td>0.030</td>
</tr>
<tr>
<td>0.6x b-DMEM</td>
<td>0.029</td>
</tr>
</tbody>
</table>

Table 11. The growth rate of CRL-1606 hybridomas are shown for various elevated levels. Unless otherwise noted, the cells were cultured in DMEM at 2.5% FCS, 25 mM glucose, and 4 mM glutamine. Each value is the average of two cultures.

<table>
<thead>
<tr>
<th>Medium Composition</th>
<th>Growth Rate (hr⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard DMEM</td>
<td>0.047</td>
</tr>
<tr>
<td>70 mM glucose</td>
<td>0.046</td>
</tr>
<tr>
<td>4x amino acids</td>
<td>0.049</td>
</tr>
</tbody>
</table>
in the text. The solid lines represent an average of two cultures. Each point on the graph corresponds to two different initial cell levels. The growth rate of CRL-1690 hybridomas are shown as a function of serum (%).
Table 12. The Monod constant, $K_s$ and the maximum growth rate $\mu_{\text{max}}$ are calculated for the two curves shown in Figure 7 via a non-linear regression of equation (42).

<table>
<thead>
<tr>
<th>Cell Level (cells/ml)</th>
<th>(% FCS)</th>
<th>$\mu_{\text{max}}$ (hr$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8,500</td>
<td>0.84</td>
<td>0.054</td>
</tr>
<tr>
<td>85,000</td>
<td>0.54</td>
<td>0.055</td>
</tr>
</tbody>
</table>

Thus, $K_s$ may be related to the cell level, $X$, by the relation:

$$K_s = (K_s)_0 X^\beta.$$  \hfill (46)

A non-linear regression of the data in Figure 7 using equation (46) results in the following parametric values: $(K_s)_0 = 6.5 \pm 2.9$, and $\beta = -0.21 \pm 0.002$. The standard deviation of the regression was 0.002 h$^{-1}$. In microbial systems, the Monod constant has also been found to be related to the cell level (Contois, 1959), except that the exponent of the cell level in those cases was positive.

Thus, the initial growth rate of CRL-1606 hybridoma cells is a complex function of serum, glutamine, ammonium, lactate and cell levels, and is summarized by the following equation:

$$\mu = \frac{\mu_{\text{maxSG}}}{\left[ S + (K_s)_0 X^\beta \left[ G + K_G \right] \left[ 1 + \frac{A^2}{K_A} \right] \left[ 1 + \frac{L^2}{K_L} \right] \right]}.$$  \hfill (47)

For most cases, the lactate term may be ignored.
IV.B.1 Variable Reduction: Specific Antibody Productivity, $q_p$

The anti-fibronectin antibody concentration was measured as a function of time for all of the cultures represented in Tables 3 and 5. The specific antibody productivities (pg/cell-hr) and standard deviations were computed for each condition represented and are shown in Table 13. The productivities shown in Table 13, however, must be interpreted with caution. Since the method for measuring cell concentration (i.e., Coulter Counter) does not exclude dead, (i.e., trypan blue stained) and presumably non-producing cells, the productivities shown in Table 13 may be indicative of culture viability, as well as the rate of cellular antibody synthesis. Note from Table 13 that the cultures with the lowest growth rates (see Table 3) appear to have the lowest productivities, indicating that not all of the cells may be viable. This hypothesis is supported by Birch (1984), who noted that as the growth rate of hybridoma cells were decreased below a given threshold value (0.002 h$^{-1}$), the cells started to die.

Unfortunately, because the initial cell concentration was very low (<5000 cells/ml), the viability could not be measured directly via microscopic examination following trypan blue staining. Thus, to determine if low culture viability might be responsible for the decreased antibody productivities exhibited at low growth rates, additional hybridoma cultures were grown at the medium conditions in Table 3 that resulted in growth rates below 0.025 h$^{-1}$, with the exception that the cultures were initiated at 100,000 cells/ml instead of 5000 cells/ml. The culture viability could then be monitored via trypan blue staining as a function of time for each culture. Figure 8 plots the culture viability with time for cultures growing at four different rates. It may be seen that at growth rates (based on total cells) below 0.01 to 0.02 h$^{-1}$, the culture viability decreases as a function of time. For the culture exhibiting a growth rate of 0.032 h$^{-1}$, the cells remain viable throughout. Thus, there appears to exist a minimum growth rate, below which the viability of the culture suffers.

Note that the six cultures in Table 3 with growth rates below 0.014 h$^{-1}$ were grown in low serum medium (1 %FCS). Therefore, to ensure that low culture viability does not skew the results of the experimental design, all cultures grown at low serum levels were excluded from the analysis. Table 14 represents the restructured data. All entries in
Viability was determined via trypan blue staining at 100,000 cells/ml. Viability was determined via trypan blue staining at 100,000 cells/ml. All cultures were incubated adjusting the serum, ammonium, and d-DMEM levels. All cultures were incubated adjusting the serum, ammonium, and d-DMEM levels. The growth rates were varied by function of time and growth rate in [1/hr]. The growth rates were varied by function of time and growth rate in [1/hr]. Figure 8: The viability of cultures of CR-1606 hybrids was shown as growth rates.

Growth Rates:

- 0.021 I/l
- 0.010 I/l
- 0.017 I/l
- 0.0319 I/l
Table 13 represent cultures grown in high serum (9 % FCS) media. Note that the first eight rows in Table 14 represent a 2⁵-² fractional factorial design in two blocks. Thus, despite the elimination of eight experiments, the structure of the experimental design was not destroyed, and the techniques of Box et al. (1978) used to determine $\mu_i$ and $\mu_{i,k}$ may still be used to calculate $(q_p)_i$ and $(q_p)_{i,k}$. The results of such calculations are shown in Table 15.

The results indicate that the lactate level is the only variable that has any significant impact on $q_p$. As was the case with the growth rate (Table 3), there was a significant difference between values of $q_p$ for the two blocks of experiments presented in Table 13. Again, this was probably due to the temperature control problems mentioned previously. Since it has been determined that glucose, glutamine and ammonium levels have no effect on $q_p$, the effect of serum may now be examined from the results of Table 13. The specific antibody productivity of the only two low-serum cultures having growth rates above 0.02 h⁻¹ in Table 3 may be compared with cultures grown in high serum medium at the same time (same block) and at identical lactate levels (Table 16). Table 16 represents a 2² factorial design. The linear and co-linear coefficient calculated from Table 16 are: $(q_p)_5$ (lactate) = -0.4, $(q_p)_3$ (serum) = -0.10, and $(q_p)_{3,5} = 0.05$. Again, the lactate level is shown to be the only variable significant in determining the specific antibody productivity, and agrees with the value calculated and presented in Table 15.

The effect of low culture viability at low growth rates on the apparent specific antibody productivity is shown in Figure 9, which plots all of the data in Table 14 versus the growth rate, based on total cells. The apparent specific antibody productivity is seen to be linearly dependent on $\mu$ below a minimum growth rate of approximately 0.02 h⁻¹, and independent at higher growth rates. This is consistent with the observations made from Figure 8. At growth rates above 0.020 h⁻¹, the antibody productivity is a function of the lactate level only.
Table 13. The specific antibody productivities were calculated for each culture grown at the conditions specified in Tables 3 and 5. Each value is the average of replicate cultures.

<table>
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<tr>
<th>#</th>
<th>Glutamine</th>
<th>Glucose</th>
<th>Serum</th>
<th>Sodium Lactate</th>
<th>Ammonium Chloride</th>
<th>b-DMEM</th>
<th>Block</th>
<th>q&lt;br&gt; p</th>
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86
is due to cell death (Figure 8). The low productivities at the low growth rates and DFM addition levels. The growth rates of the cultures were varied by adjusting the serum, ammonium, and growth rates of the growth rate and the lactate concentration. Figure 9: The specific rate of antibody production by CRL-1666 hybridomas.
Table 14. Cultures grown at high serum levels from Table 13 are re-presented.

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<th>Ammonium Chloride (mM)</th>
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<th>Block</th>
<th>( q_p ) cell - hr</th>
<th>Standard Deviation cell - hr</th>
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Table 15. The linear and co-linear coefficients defined in equation (41) are calculated from the results in Table 14. The subscripts correspond to the medium constituents, as presented in Table 4.

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<th>Coefficients</th>
<th>Value (pg/cell-h)</th>
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<tr>
<td>( q_p ) sub_7</td>
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<td>(standard deviation)/2</td>
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<tr>
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Table 16. The effect of serum on $q_p$ is shown to be insignificant by comparing the cultures in Table 13 that had growth rates less than 0.02 h$^{-1}$ and had different serum levels, but identical lactate levels and in the same block group.

<table>
<thead>
<tr>
<th>#</th>
<th>Serum (% FCS)</th>
<th>Sodium Lactate (mM)</th>
<th>Block</th>
<th>$q_p$ (pg cell$^{-1}$ hr$^{-1}$)</th>
<th>Standard Deviation (pg cell$^{-1}$ hr$^{-1}$)</th>
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IV.B.2 Mathematical Description of $q_p$

It has been shown that $q_p$ is a function only of the lactate level. To determine this functional form, especially at high lactate levels, the antibody titers were measured for the cultures represented in the first five rows of Table 9. The results are shown in Figure 10. The growth rates of all of the cultures were above 0.02 h$^{-1}$, so the viability is assumed to be 98%+. The data may be approximated by the following relationship:

$$q_p = \frac{64 + 0.25[Lactate]}{37 + [Lactate]} \left[ \frac{pg}{cell \cdot hr} \right]$$  \hspace{1cm} (48)

Recall that at growth rates below 0.02 h$^{-1}$, the culture viability is less than 100%, and consequently results in a decrease in the apparent specific antibody productivity. The relationship between the growth rate, the culture viability and the subsequent effect on $q_p$ may be determined in two ways: 1) the growth rate, $\mu$, and the death rate, $\mu_d$, may be determined from chemostats in which the growth rate is controlled by the concentrations of a variety of limiting substrates or inhibiting waste products, or 2) the
The serum level was 4 & FCS. The antibody productivity was calculated from four points taken every 12 hours. The antibody level was 4 & FCS. The antibody productivity was calculated from four levels in cultures of hybrido cells grown in DMEM and incubated at 8500 cells/ml. The specific antibody productivity is shown as a function of the lactate.
culture viability may be approximated from Figure 9 by assuming that the low specific antibody productivities attained for cultures with growth rates less than 0.02 h\(^{-1}\) are due solely to cell death; that is, the viability, \(\alpha\), may be related to the growth rate via the following relation:

\[
\alpha = \begin{cases} 
1 & \text{for } \mu \geq 0.02 \text{ hr}^{-1} \\
50\mu & \text{for } \mu \leq 0.02 \text{ hr}^{-1}
\end{cases}
\]  

(49)

The first suggestion would be rigorous, accurate, but very time-intensive, while the second idea could be used to provide quick, quantitative estimates.

IV.C.1 Variable Reduction - Glutamine Utilization and Ammonium Production

From previous experience with a different cell line (MDCK), it is known that the specific cellular glutamine utilization rate \(q_G\), mmol/cell-h) may be a function of the glutamine concentration, exhibiting saturation kinetics typical of substrate transport (Glacken et al., 1986, see Appendix B). Thus, it is clear that glutamine should be one of the variables to consider when developing mathematical descriptions for \(q_G\). To determine what other variables may have to be considered, a 2\(^5\)-2 factorial design was initiated as represented in Table 17. The cultures were initiated at high cell concentrations \((1.7 \times 10^5 \text{ cells/ml})\) so that perceptible changes in the glutamine and ammonium concentrations could be accurately measured. The initial glutamine level in all cultures was 1 mM. Four equally spaced culture samples, taken during exponential growth over a period of 21 hours, were assayed for cell, glutamine, and ammonium concentrations.

Since it is known that cell viability decreases at growth rates below 0.02 h\(^{-1}\), cultures were also assayed for viability via microscopic observation following staining with trypan blue. Cultures were initiated in duplicate. The values of \(q_G\) and \(q_A\) shown in Table 17 were calculated using the concentration of viable cells. The viability of the last
Table 17. Cultures initiated at 170,000 cells/ml were grown in DMEM at constituent levels determined via a factorial statistical design, as shown below. Each value of $\mu$, $q_G$, or $q_A$ is an average of replicate cultures. The values of $q_G$ and $q_A$ are based on viable cell levels.

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<th>Ammonium Chloride (mM)</th>
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<th>$\mu$ (h$^{-1}$)</th>
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<td>100</td>
<td>0.036</td>
<td>5.4</td>
<td>3.2</td>
</tr>
<tr>
<td>A3</td>
<td>25</td>
<td>0.9</td>
<td>40</td>
<td>4</td>
<td>40</td>
<td>0.014</td>
<td>9.1</td>
<td>4.0</td>
</tr>
<tr>
<td>A4</td>
<td>2.5</td>
<td>0.9</td>
<td>40</td>
<td>4</td>
<td>100</td>
<td>0.017</td>
<td>7.0</td>
<td>3.9</td>
</tr>
<tr>
<td>A5</td>
<td>25</td>
<td>2.5</td>
<td>0</td>
<td>4</td>
<td>100</td>
<td>0.027</td>
<td>6.8</td>
<td>5.2</td>
</tr>
<tr>
<td>A6</td>
<td>2.5</td>
<td>2.5</td>
<td>0</td>
<td>4</td>
<td>40</td>
<td>0.023</td>
<td>8.1</td>
<td>7.3</td>
</tr>
<tr>
<td>A7</td>
<td>25</td>
<td>0.9</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0.036</td>
<td>6.9</td>
<td>6.5</td>
</tr>
<tr>
<td>A8</td>
<td>2.5</td>
<td>0.9</td>
<td>0</td>
<td>0</td>
<td>40</td>
<td>0.030</td>
<td>7.1</td>
<td>7.0</td>
</tr>
</tbody>
</table>

Samples taken of cultures A3, A4, and A6A was approximately 80%. The rest of the cultures had 98+ % viability throughout. The average standard deviation for the calculated values of $\mu$, $q_G$ and $q_A$, are 0.002 h$^{-1}$, 0.6 mmol/cell-h, and 0.4 mmol/cell-h, respectively. The results of Table 17 indicate that the glutamine utilization rate does not vary greatly over the range of concentrations of constituents considered (5.4 - 9.1 mmol/10$^{11}$ cell-h), whereas the ammonium production rate varied more considerably (3.2 - 7.3 mmol/10$^{11}$ cell - h).

Table 18 presents the values of the linear coefficients calculated from the data in Table 17. Any coefficient less than one-half the standard deviation are not considered significant. From Table 18 it is clear that lactate significantly inhibits cellular production of ammonium, while ammonium appears to stimulate glutamine utilization. Less significant is the inhibition of increasing b - DMEM levels (i.e., DMEM salts, amino acids, and vitamins) on both $q_G$ and $q_A$, and the inhibition of increasing serum levels on $q_G$. The inhibition of cellular ammonium excretion, as well as antibody production by lactate,
may indicate a fundamental physiological phenomenon, which may involve lactate's proficiency in chelating divalent cations (E. Adema, personal communication, Gosh and Nair, 1970). Divalent cations are critical for membrane exocytosis (Douglas and Nemeth, 1982).

Table 18  The linear and co-linear coefficients defined in equation (41) are calculated for the results in Table 17.

<table>
<thead>
<tr>
<th>Variable</th>
<th>$q_G$ $\text{mmol} \times 10^{11} \text{cells} \cdot \text{h}$</th>
<th>$q_A$ $\text{mmol} \times 10^{11} \text{cells} \cdot \text{h}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose</td>
<td>0.1</td>
<td>-0.2</td>
</tr>
<tr>
<td>serum</td>
<td>-0.5</td>
<td>-0.2</td>
</tr>
<tr>
<td>lactate</td>
<td>-0.2</td>
<td>-1.4</td>
</tr>
<tr>
<td>ammonium</td>
<td>0.7</td>
<td>-0.0</td>
</tr>
<tr>
<td>b-DMEM</td>
<td>-0.5</td>
<td>-0.4</td>
</tr>
<tr>
<td>(standard deviation)/2</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>average</td>
<td>7.0</td>
<td>5.1</td>
</tr>
</tbody>
</table>

IV.C.2 Mole Balance on Glutamine Nitrogen

It is desired to express glutamine utilization ($dG/dt$) in standard form (Wang et al., 1977); i.e., as a function of a growth related term with a constant yield coefficient, $Y$, a maintenance term, $m$, and a production term, $q_A$, which, in this case, represents ammonium production, as follows:

$$
\frac{-dG}{dt} = kG + q_GX = kG + \left[ \frac{\mu}{Y} + m + q_A \right] X
$$

(50)

where

- $kG$ = first order decomposition rate of glutamine, $\text{mmol/h}$
- $q_G$ = specific cellular glutamine utilization rate, $\text{mmol/cell-h}$

This equation may be related to a material balance on glutamine nitrogen (Figure 11).
Figure 11. Schematic diagram of a mole balance of glutamine nitrogen.

We wish to develop an equation that determines the amount of glutamine nitrogen \( A^* \) that is unaccounted for at any time. From Figure 11, this is easily seen to be:

\[
-2 \frac{dG}{dt} - \frac{dA}{dt} - \frac{d(PC)}{dt} = \frac{dA^*}{dt}
\]  

(51)

where 

- \( A \) = ammonium level 
- \( PC \) = pyrrolidone carboxylate level 
- \( A^* \) = residual ammonium level

Pyrrolidone carboxylate is a degradation product from the spontaneous decomposition of glutamine. A fraction of the residual glutamine ammonium may be
directly incorporated into cell mass, which, assuming a constant yield coefficient, $Y$, may be described by:

$$\frac{d[A^{*}]_{x}}{dt} = \frac{1}{Y} \frac{dX}{dt}$$

(52)

Residual glutamine ammonium may also be converted to other compounds, such as amino acids, and excreted into the medium in a form that is not accounted for by the balance. Assume that this rate is proportional to the total cell number:

$$\frac{d[A^{*}]_{m}}{dt} = mX$$

(53)

Now the balance takes the form

$$-2 \frac{dG}{dt} = \frac{dA}{dt} + \frac{d(PC)}{dt} + \frac{1}{Y} \frac{dX}{dt} + mX$$

(54)

Now, substitute the following into the above equation.

$$\frac{dX}{dt} = \mu X$$

(55)

$$\frac{dA}{dt} = kG + q_{A}X = kG + \frac{q_{A}}{\mu} \frac{dX}{dt}$$

(56)

$$\frac{d(PC)}{dt} = kG = \frac{dA}{dt} - \frac{q_{A}}{\mu} \frac{dX}{dt}$$

(57)

The new balance is then

$$\frac{dG}{dt} = kG + \frac{\mu}{Y + m + q_{A}} X$$

(58)

Now relating this to equation (50) we see that:
\[ q_G = \left[ \frac{\mu Y + m + q_A}{2} \right] \]  \hspace{1cm} (59)

\[ q_G - \frac{q_A}{2} = \frac{\mu}{2Y} + \frac{m}{2} \]  \hspace{1cm} (60)

Thus, if the left side of equation (60) is plotted versus the growth rate, a straight line with an slope of \(1/2Y\), and a intercept of \(m/2\) should be obtained.

In Table 18, \(q_A\) and \(q_G\) were calculated for replicate cultures grown at the given concentrations of serum, ammonium, lactate, glucose and amino acid levels. This data is presented again in Table 19, except the difference \(q_G - q_A/2\). Note that each entry in Table 18 represents the average of data taken from replicate cultures, whereas each entry in Table 19 represents data from a single replicate culture.

Figure 12 shows the plot of \(q_G - q_A/2\) vs. \(\mu\). It is obvious that there is no trend, so we may conclude that glutamine utilization is not growth associated (at glutamine levels above 1 mM). This is consistent with what was found in cultures of MDCK cells grown on microcarriers (Glacken et al., 1986, Appendix B). Note, however, that for growth rates below 0.02 h\(^{-1}\), the values of \(q_G - q_A/2\) are much higher than the values calculated for higher growth rates. Recall that previous data showed that cultures with growth rates less than 0.02 h\(^{-1}\) exhibited declining levels of cell viability (Figure 9). This suggests that at growth rates below 0.02 h\(^{-1}\), a drastic shift in metabolic activity may occur which results in high maintenance energy requirements and thus, concomitant decreases in cell viability.
Table 19. The specific glutamine utilization rate and the specific ammonium production rate were calculated from replicate cultures grown at various serum, ammonium, glucose, glutamine, lactate and b-DMEM levels, as presented in Table 18. The data is presented below for each replicate culture, so that the term $q_G - q_A/2$ may be calculated and related to the growth rate, as in equation (60).

<table>
<thead>
<tr>
<th>$\mu$ (h$^{-1}$)</th>
<th>$q_A$ (mmol/10$^{10}$ cells - h)</th>
<th>$q_G$ (mmol/10$^{10}$ cells - h)</th>
<th>$q_G - q_A/2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.043</td>
<td>3.80</td>
<td>6.10</td>
<td>4.20</td>
</tr>
<tr>
<td>0.040</td>
<td>4.00</td>
<td>5.40</td>
<td>3.40</td>
</tr>
<tr>
<td>0.037</td>
<td>2.80</td>
<td>5.80</td>
<td>4.40</td>
</tr>
<tr>
<td>0.036</td>
<td>6.30</td>
<td>6.80</td>
<td>3.60</td>
</tr>
<tr>
<td>0.034</td>
<td>6.70</td>
<td>7.00</td>
<td>3.60</td>
</tr>
<tr>
<td>0.033</td>
<td>3.60</td>
<td>5.10</td>
<td>3.30</td>
</tr>
<tr>
<td>0.031</td>
<td>6.10</td>
<td>7.20</td>
<td>4.20</td>
</tr>
<tr>
<td>0.030</td>
<td>6.90</td>
<td>7.40</td>
<td>4.00</td>
</tr>
<tr>
<td>0.029</td>
<td>6.00</td>
<td>7.30</td>
<td>4.80</td>
</tr>
<tr>
<td>0.028</td>
<td>7.00</td>
<td>6.70</td>
<td>3.30</td>
</tr>
<tr>
<td>0.026</td>
<td>5.60</td>
<td>6.30</td>
<td>3.50</td>
</tr>
<tr>
<td>0.026</td>
<td>3.80</td>
<td>6.80</td>
<td>5.90</td>
</tr>
<tr>
<td>0.025</td>
<td>6.30</td>
<td>8.00</td>
<td>4.90</td>
</tr>
<tr>
<td>0.024</td>
<td>8.20</td>
<td>8.20</td>
<td>4.10</td>
</tr>
<tr>
<td>0.020</td>
<td>4.90</td>
<td>9.40</td>
<td>7.00</td>
</tr>
<tr>
<td>0.015</td>
<td>3.10</td>
<td>8.80</td>
<td>7.20</td>
</tr>
</tbody>
</table>
Utilization is not growth associated and is dominated by the maintenance term.

Figure 12. The difference between the specific glutamine uptake rate and the specific ammonium production rate of CRL-1666 hybrids are plotted as a function of the growth rate. The growth rate of the cultures were varied by adjusting the serum and ammonium levels. This plot shows that glutamine utilization is not growth associated and is dominated by the maintenance term.
IV.D. Deviation From Initial Metabolic Rates

IV.D.1. Determination of Rate Limiting Components In Serum

It has been shown that as the serum level is reduced, the growth rate decreases. Also, it was observed that as the cell level is increased, the growth rate increases in low serum medium. This was crudely modeled with a cell level - dependent Monod constant. If a more molecular mechanistic model is desired, it must be determined what component or components in serum are rate-limiting in low serum media. A set of experiments was initiated to determine what these components may be. Knowing the rate-limiting components at various serum levels would facilitate the development of molecular kinetic models, from which, more accurate mathematical descriptions of the metabolic rates could follow. These molecular kinetic models could be especially useful for extrapolating the initial rate data gathered in the previous section to states at higher cell levels and at later culture times.

As shown in Table 20, various growth factors often found in serum-free medium formulations were added to DMEM medium supplemented with 1% FCS to determine which, if any, of the growth factors may be limiting at this serum level. These growth factors were categorized into four separate groups: 1) peptides; 2) lipids; 3) trace metals; and 4) small molecular weight nutrients (F-12). Concentrated mixtures of the given growth factors were formulated for each category, and added to the medium at three different levels: 1x, 2x or 4x. The growth factors and their concentrations for each of the four groups corresponding to the 1x level were as follows:

- **Peptides** = Insulin (6 μg/ml), Epidermal growth factor (50 ng/ml), Transferrin (20 μg/ml)
- **Lipids** = Ethanolamine (1.2 μg/ml), BSA-linoleic acid (100 μg/ml)
- **Trace Metals** = SnCl₂ - H₂O (57 ng/l), MnSO₄ - H₂O (85 ng/l), NiCl (60 ng/l), [NH₄]₆Mo₇O₂₄ - 4H₂O (618 ng/l), NaSeO₃ (3.5 μg/l), Na₃VO₄ - 3H₂O (595 ng/l), 3CdSO₄ - 8H₂O (77 μg/l)
- **F - 12 DMEM:** = 50% DMEM + 50% F-12
Cultures were inoculated at approximately 9000 cells/ml and the cell concentrations were determined every 8 hours for 16 hours after the onset of exponential growth (approximately 12 hours after inoculation). Three cell concentrations were utilized to calculate the growth rates (\(\mu\)). Each value in Table 20 represents the average of 2 duplicate cultures. Cultures grown at 1% FCS and 10% FCS without any additives were used as controls. The average standard deviation of the calculated growth rates was 0.004 h\(^{-1}\). The standard deviations are relatively large since only three cell measurements were used to calculate the growth rates.

Table 20. The growth rate of CRL-1606 cells in DMEM at 1% FCS as a function of various concentrations of growth factors (1x, 2x, or 4x). The growth rates are expressed in h.

<table>
<thead>
<tr>
<th>Additions</th>
<th>1X</th>
<th>2X</th>
<th>4X</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide Factors</td>
<td>0.037±0.004</td>
<td>0.035±0.003</td>
<td>0.036±0.007</td>
</tr>
<tr>
<td>Trace Metals</td>
<td>0.032±0.003</td>
<td>0.032±0.005</td>
<td>0.034±0.003</td>
</tr>
<tr>
<td>Lipids</td>
<td>0.030±0.003</td>
<td>0.029±0.002</td>
<td>0.032±0.004</td>
</tr>
<tr>
<td>F-12/DMEM</td>
<td>0.029±0.005</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>None (control, 1% FCS)</td>
<td>0.034±0.005</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>FCS to 10%</td>
<td>0.046±0.002</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

The results from Table 20 indicate that none of the factors tested significantly increased the growth rate above that achieved in DMEM supplemented to 1% FCS. Based on a student - t test analysis, a difference of 0.006 h\(^{-1}\) is required before two growth rates could be considered to be significantly different with greater than 99.5% certainty (Larsen and Marx, 1981). Thus, it can be concluded, that none of these factors appear to be the rate-limiting components for cells grown in medium at serum levels greater than 1% FCS.

Since the specific rate-limiting growth factor in low serum medium was not
identified, a less restrictive question was asked: that is, are the rate limiting components small molecular weight compounds, or large polypeptides? To answer this question, 30 ml of FCS was dialyzed against an agitated 2-l solution of PBS for 6 hours at 37 °C. Prior to this, the membrane (Spectra/por 6, MWCO 1000) was soaked once in distilled water for 16 hours at 4 °C, and soaked twice in PBS for 16 hours each. Then a Spectra/por closure was applied to one end, placed in a beaker of PBS, and autoclaved for 20 minutes at 121 °C prior to dialysis.

As the FCS was dialyzing, 30 ml of FCS from the same batch was also incubated at 37°C. Both the non-dialyzed and the dialyzed FCS was added to separate flasks of DMEM medium supplemented to 0.7% with non-dialyzed FCS. The final serum concentration in each case was 1.4% FCS. Cells were also inoculated in DMEM at 0.7% FCS (not dialyzed) for comparison. The growth rate of each of the cultures was measured from four equally-spaced cell level measurements and are presented in Table 21. The table shows that the addition of dialyzed serum, while increasing the growth rate of the hybridomas somewhat, did not increase the rate as much as complete (un-dialyzed) serum did. From this, it may be concluded that small molecular weight components, as well as large molecules, may be rate limiting in low serum medium. Based on a student-t test analysis, a growth rate difference of 0.002 h⁻¹ is significantly different to a certainty of 99.9%.

Since it could not be determined which growth factors limit growth at 0.7-1.0% FCS, experiments were performed to determine which growth factors were limiting at 0% FCS. Murakami et al. (1982) showed that some hybridoma cells could be grown in a 50% mixture of DMEM and F-12, supplemented with 35 μg/ml transferrin, 5 μg/ml insulin, 20 μM ethanolamine, and 25 nM selenium without the presence of serum. The serum-free preparation with all of the growth factors (ITES) were prepared as in Murakami et al. (1982) except the following concentrations were used: insulin (10 μg/ml), transferrin (50 μg/ml), ethanolamine (30 μM), and sodium selenite (40 nM).
Table 21. The relative size of components in serum that may limit the growth rate was determined. The growth rate of CRL-1606 cells grown in DMEM supplemented with non-dialyzed and/or dialyzed serum is presented. Cultures were inoculated at 10,000 cells/ml. Replicate cultures were initiated in each case. FCS was dialyzed 30 ml against 2000 ml PBS with SPECTRA/POR 6 (MWCO = 1000) for 6 hours.

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Growth Rate, h⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.7 % FCS</td>
<td>0.026 ± 0.001</td>
</tr>
<tr>
<td>0.7 % FCS + 0.7 % dialyzed FCS</td>
<td>0.030 ± 0.001</td>
</tr>
<tr>
<td>1.4 % FCS</td>
<td>0.033 ± 0.001</td>
</tr>
</tbody>
</table>

These concentrations are approximately 50% higher than those used by Murakami et al. (1982) to ensure there were no limitations. Preparations of each individual growth factor in DMEM/F-12 medium at each of the concentrations shown in Table 22 were also prepared.

To reduce the effect of serum carry-over, cells in stationary phase were taken from a 10% FCS stock culture in DMEM (1 x 10⁶ cells/ml), and diluted 10-fold with DMEM/F-12 without serum, so that the resulting culture contained 1% FCS. After stationary phase was reached, the cells were spun down and resuspended in DMEM/F-12 without serum. The cells were then incubated without serum at 37°C at 10% CO₂ for 20 hours. These cells were then spun down and resuspended in DMEM/F-12 without serum again. Each culture was inoculated with 0.8 ml of cell suspension into 40 ml of medium to give 50,000 cells/ml. Upon inoculation, 85% of the cells were viable. Samples were taken every 12 hours, starting from 23.5 hours after inoculation, until 54.5 hours. The growth rates calculated from 5 samples points are given in Table 22. Each value in Table 22 is an average of replicate cultures.
Table 22. The growth rate of cells cultured in DMEM/F-12 medium, without serum, supplemented with various growth factors. The cultures were inoculated at 50,000 cells/ml.

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Growth Rate, h⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>35 µg/ml Transferrin</td>
<td>0.016</td>
</tr>
<tr>
<td>5 µg/ml Insulin</td>
<td>0.005</td>
</tr>
<tr>
<td>20 µM Ethanolamine</td>
<td>no growth</td>
</tr>
<tr>
<td>25 nM Sodium Selenite</td>
<td>no growth</td>
</tr>
<tr>
<td>ITES</td>
<td>0.022</td>
</tr>
<tr>
<td>1% FCS</td>
<td>0.026</td>
</tr>
</tbody>
</table>

It is obvious that only when all of the growth factors are added together is the growth rate comparable to that achieved in 1% FCS. From the data, it is not clear whether the addition of ethanolamine or selenite was beneficial, or whether insulin and transferrin alone were responsible for the growth observed in ITES medium. Thus, it may be concluded that transferrin, insulin, and possibly, ethanolamine and selenium may be rate limiting at serum levels below 1% FCS, but that other small and large molecular weight components are rate limiting at higher serum levels.

IV.D.2 Growth Rate Stimulation with Increasing Initial Cell Levels: Hypothesis Testing

Increasing the initial cell level from 8,500 to 85,000 cells/ml resulted in an increase in the growth rate for cells grown at all serum levels, although the stimulation was greater for cells cultured at serum levels less than 1.75% FCS (Figure 7). This phenomenon was characterized mathematically by including a cell level-dependent Monod constant (serum) in the relation for the growth rate (equation (46)). The purpose of the following research was to develop a physiological explanation for this
observation.

Five hypotheses were formulated (Figure 13). The first hypothesis was that additional growth factors introduced into the medium from cellular membrane receptors were responsible for the stimulation of the growth rate at high cell levels. Desorption of growth factors from cellular membranes may occur if the medium contains a low concentration of growth factors, since the free growth factor exists in dynamic equilibrium with the bound growth factor (Tolkovsky, 1983; Reseigho and Goren, 1982). Insulin and transferrin are examples of some of the growth factors that stimulate mammalian cell growth via membrane receptors (Jacobs and Cuatrecasas, 1976; Hamilton, 1983).

If the initial cell concentration is low, the relative amount of growth factor introduced from the cell surfaces is small, while at high initial cell levels, the amount of growth factors introduced may be significant in comparison to the amount added with the serum. At low cell levels, much of the growth factor initially bound to the receptor may be released to the medium, due to equilibrium considerations, while at higher cell levels, less would be released. If it is assumed that the growth rate is related to the amount of growth factor bound to receptors (Ariens, 1954; Tolkovsky, 1983), this hypothesis predicts that the growth rate would increase with increasing initial cell levels, especially at low serum concentrations, exactly as it must if it is to explain the experimental observations.

Of course other complexities may exist with growth factor-receptor interactions. It has been shown that bound growth factors may be internalized (Carpenter and Cohen, 1976), and that receptor-growth factor complexes may interact with membrane effector moieties, which then elicit a response (Jacobs and Cuatrecasas, 1976). For the present, these potential complexities will be neglected in preference to the simpler models.
HYPOTHESIS 1. GROWTH FACTOR - RECEPTOR EQUILIBRIUM

HYPOTHESIS 2. REACTION - DIFFUSION

HYPOTHESIS 3. GROWTH FACTOR SYNTHESIS
HYPOTHESIS 4. CELL-CELL INTERACTIONS

HYPOTHESIS 5. CELLULAR DETOXIFICATION OR REACTIVATION

Figure 13. Five hypothesis for the cell level stimulation of the growth rate.

The second hypothesis formulated to describe the stimulation of the growth rate with increasing initial cell concentrations is that small molecular weight metabolic intermediates or cofactors diffuse out of the cell at low cell concentrations. This
explanation is often given to explain why mammalian cells will not grow at very low cell concentrations (Paul, 1975). There are, however, problems with this hypothesis. First, the ratio of the cellular volume to the total liquid volume at 5000 cells/ml is approximately $10^{-5}$, while the ratio is approximately $10^{-4}$ at 50,000 cells/ml. Thus, while the final intracellular steady state concentration of a freely diffusible component could be as much as ten times higher at 50,000 cells/ml than at 5000 cells/ml, the initial intracellular concentration at steady state would be reduced by at least a factor of 10,000 in either case. This implies that at some point during the culture period, possibly during late exponential growth or stationary phase, when some other growth factor is rate limiting, the diffusible growth factor must be manufactured at a rate much faster than the rate of diffusion, so that it would become reconcentrated to the original level inside the cell. If the intermediate were not reconcentrated inside the cell to the same level, the cells would fail to grow upon subculturing, no matter what the serum level was. This 10,000-fold reconcentration of intermediates seems unlikely, since this would be a tremendous waste of cellular resources. While this is an unlikely hypothesis, it is nonetheless included for the sake of completion.

The third hypothesis is that the stimulation of the growth rate at high initial cell levels results from the manufacture and excretion of growth factors into the culture medium by the hybridoma cells. Although the cells may well secrete a growth factor into the medium, it is unlikely that this accounts for the observed stimulation of the growth rate at high initial cell levels, since 1) the disparity in the growth rates between cultures grown at high initial cell levels and low initial cell levels is seen immediately, within the first 20 to 30 hours; and 2) this hypothesis would imply that the growth rate of cultures grown in 1% FCS at low initial cell levels would increase as the cell levels increase with time. This is not the case. On the contrary, the growth rate decreases with time for cultures grown at 1% FCS at both high and low initial cell levels (Figure 14). While this validity of this hypothesis is questionable, it is nonetheless included for completeness.

A fourth hypothesis is that the cells stimulate growth via specific cell-cell interactions. This is not unreasonable, since it has been shown that for cells cultured
on microcarriers, a minimum number of cells per unit surface area is required for
growth (Fleischacker, 1982; Hu et al., 1985). Additionally, certain cell populations of
the immune system are stimulated to grow via intercellular contact with other cell
populations (Kimball, 1986). Since in the present case, the hybridoma cells are grown
in static culture on the bottom of flasks, it is possible that the stimulation of growth at
high initial cell levels may be due to the high number of cells per unit area and not per
unit volume.

Another hypothesis is that the cells may detoxify their environment, and that the
higher the cell concentration, the greater the level of detoxification. For example, it
has been shown that hydrogen peroxide is spontaneously formed in cell culture
medium when exposed to light, and that catalase activity alleviates the toxic effects of
peroxides in serum-free media (Darfler and Insel, 1983). It is known that the cells
contain glutathione peroxidase (Hoekstra, 1975), an enzyme which destroys
peroxides.

Finally, mammalian cells may help reverse the inactivation of essential nutrients.
For example, cysteine, an essential nutrient that is rapidly transported into the cell, is
converted to cystine in the medium spontaneously (Toohey, 1975). Cystine, however,
is transported much more slowly than cysteine into cells (Ohmori and Yamamoto,
1983). Mammalian cells may play a role in converting cystine to cysteine by
converting oxidized serum thiols to their reduced form (Ohmori and Yamamoto, 1983).
Thus, high cell concentrations may allow greater reversion of cystine to cysteine,
resulting in higher growth rates.

Five hypotheses have been developed to explain the stimulation of the growth
rate of CRL-1606 hybridoma cells with increasing initial cell level (Figure 13). The
first two hypotheses were tested by washing cells in 50/50 mixture of DMEM/F-12
without serum so that any growth factors on the cell surface are allowed to desorb
from surface receptors and/or diffuse out of the cells. If either of these hypothesis are
valid, washing the cell inoculum should significantly decrease the growth rate
observed in low serum cultures inoculated with high cell levels, while the growth rate
of cultures initiated at low cell levels would remain unchanged. It was determined
previously that CRL-1606 cells maintain high viability in a 50% mixture of DMEM and F-12 without serum for about 24 hours at high cell concentrations (>500,000 cells/ml, data not shown). The viability of cells maintained in DMEM without serum was observed to rapidly decline. Thus, the possible detrimental effects from washing cells without serum is alleviated by using a DMEM/F-12 mixture. Recall that the addition of F-12 medium to DMEM did not stimulate growth in low serum medium (Table 20).

A fraction of a culture grown in DMEM with 10% FCS was harvested in stationary phase at 1.2x10^6 cells/ml, and was spun down, and resuspended in DMEM/F-12 without serum. This cell suspension was incubated without serum for 23 hours at 5x10^5 cells/ml at 37°C and 10% CO_2 in DMEM/F-12 medium. These cells will be referred to as "washed" cells. The rest of the cells in the DMEM medium with 10% FCS was set aside in the incubator and will be referred to as "un-washed" cells.

Both washed and unwashed cells were spun down and resuspended in DMEM-F/12 without serum and were used to inoculate cultures to 5000 and 50,000 cells/ml in DMEM at 1% FCS. However, the supernatant from the washed cells was saved and filtered with a low-protein binding 0.2 micron syringe filter (Millipore, Millex-GV). This "conditioned" medium was combined with DMEM in a ratio of 25 ml to 75 ml, to which FCS was added to 1%. Unwashed cells were added to give 5000 cells/ml. In all cases, one ml of cell suspension was used to inoculate 50 mls of culture medium. The cell viability after the washing was 90%. The cell density was determined every 11 hours for 50 hours, and the growth rates were calculated from the five data points.

The results of this experiment are shown in Table 23. Washing had no effect on the growth rate of cultures. The addition of "conditioned" medium did not stimulate the growth rate. Thus, it appears that the enhancement of the growth rate at high initial cell levels cannot be fully explained by either growth factor desorption from cell surface receptors, or diffusion of growth factors from the intracellular environment into the growth medium. Based on a student - t test analysis, differences in growth rate of more than 0.003 h^-1 signify a significant difference with a certainty of greater than 99.5%.
Table 23. Presented are the growth rates of cultures grown in DMEM at 1% FCS at various initial cell levels with and without washed inocula. Cultures were grown for 24 hours in DMEM/F-12 without serum at 37°C. Culture B5 was grown in 75% DMEM and 25% "conditioned" DMEM/F-12 supplemented to 1% FCS.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Initial Cell Level (cells/ml)</th>
<th>Inocula Washed?</th>
<th>Growth Rate h⁻¹</th>
<th>Standard Deviation h⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>50,000</td>
<td>No</td>
<td>0.031</td>
<td>0.003</td>
</tr>
<tr>
<td>B2</td>
<td>50,000</td>
<td>Yes</td>
<td>0.029</td>
<td>0.002</td>
</tr>
<tr>
<td>B3</td>
<td>5,000</td>
<td>No</td>
<td>0.024</td>
<td>0.002</td>
</tr>
<tr>
<td>B4</td>
<td>5,000</td>
<td>Yes</td>
<td>0.024</td>
<td>0.004</td>
</tr>
<tr>
<td>B5</td>
<td>5,000</td>
<td>No/conditioned</td>
<td>0.025</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Hypothesis #4 was tested by independently varying both the number of cells per unit volume and the number of cells per unit area in flask-grown cultures. Cells were grown in flasks of varying surface area and with varying volumes of medium, as shown in Table 24. Inoculum was prepared as usual. One ml of cell suspension was added to each 50 ml of culture medium. Growth rates were calculated from four cell measurements taken between 13 and 45 hours after inoculation. If there were cell-cell interactions, one would expect higher growth rates for cells grown at low initial cell levels (5000 cells/ml), but at a high level of cells per unit surface area (40,000 cells/cm², C3 & C4) than for cells grown at low initial cell levels, and at a low level of cells per unit surface area (4000 cells/cm², C7 & C8). This was not the case; cultures C3 and C7 had almost identical growth rates, as did cultures C4 and C8. It can therefore be concluded that cell-cell growth stimulation does not describe the stimulation of growth at high initial cell levels. Based on a student - t test analysis,
differences in growth rate of more than 0.004 h\(^{-1}\) signify a significant difference with a certainty of greater than 99.9%.

Table 24. Growth rates are shown for cultures grown in DMEM at 10% and 1% FCS in flasks of varying surface areas, and with various liquid volumes such that the amount of cells per unit surface area could be varied independently of the cell concentration. Replicate cultures at each condition were initiated.

<table>
<thead>
<tr>
<th></th>
<th>Volume of Medium (ml)</th>
<th>Surface Area (cm(^2))</th>
<th>Serum (%)</th>
<th>Initial Cell Level (cells/ml)</th>
<th>Cells/Area (cells/cm(^2))</th>
<th>Growth Rate (h^{-1})</th>
<th>Standard Deviation (h^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>50</td>
<td>75</td>
<td>1.0</td>
<td>60,000</td>
<td>40,000</td>
<td>0.040</td>
<td>0.001</td>
</tr>
<tr>
<td>C2</td>
<td>50</td>
<td>75</td>
<td>10.0</td>
<td>60,000</td>
<td>40,000</td>
<td>0.051</td>
<td>0.001</td>
</tr>
<tr>
<td>C3</td>
<td>33.3</td>
<td>5</td>
<td>1.0</td>
<td>6,000</td>
<td>40,000</td>
<td>0.030</td>
<td>0.002</td>
</tr>
<tr>
<td>C4</td>
<td>33.3</td>
<td>5</td>
<td>10.0</td>
<td>6,000</td>
<td>40,000</td>
<td>0.046</td>
<td>0.002</td>
</tr>
<tr>
<td>C5</td>
<td>14</td>
<td>175</td>
<td>1.0</td>
<td>60,000</td>
<td>4,800</td>
<td>0.026</td>
<td>0.004</td>
</tr>
<tr>
<td>C6</td>
<td>14</td>
<td>175</td>
<td>10.0</td>
<td>60,000</td>
<td>4,800</td>
<td>0.047</td>
<td>0.004</td>
</tr>
<tr>
<td>C7</td>
<td>60</td>
<td>75</td>
<td>1.0</td>
<td>6,000</td>
<td>4,800</td>
<td>0.029</td>
<td>0.001</td>
</tr>
<tr>
<td>C8</td>
<td>60</td>
<td>75</td>
<td>10.0</td>
<td>6,000</td>
<td>4,800</td>
<td>0.043</td>
<td>0.002</td>
</tr>
</tbody>
</table>

It must be noted that oxygen limitation was possible in cultures C3 and C4, even at the low cell levels used, since the height of the liquid layer above the cells was very high. Solution of the diffusion equation for oxygen indicates that it would take at least 17 hours under static conditions to deplete the cells of oxygen for these cultures. Since samples were taken after considerable liquid agitation every 7 hours, oxygen limitation should not have occurred. As a rough verification of this theoretical analysis, the growth rate of culture C4 (10% FCS) did not decrease with time even though the cell concentration increased over 5-fold. It should be noted that during the latter
stages of the experiment, the cell level in culture C4 was about twice that of culture C3, so if a significant oxygen limitation did occur in culture C3, a decrease in the growth rate of culture C4 should have been observed.

Note that there is an inconsistency in Table 24. That is, the growth rate of culture C5 was similar to the growth rate of culture C7, even though the initial cell density of C5 was 60,000 cells/ml. This may be an experimental artifact. Since only 13 mls of medium was covering 175 cm² of area, the layer of liquid on covering the surface of the flask was extraordinary thin. Since the cells do adhere loosely to the surface of the flask, it was difficult to accurately remove all of the cells from the surface into the liquid phase via agitation with such a small quantity of fluid. The resulting cell counts were very erratic and thus, not very accurate. Therefore, the growth rates calculated for cultures C5 and C6 were not very accurate and may be artificially low.

The results thus far indicate that the first four physiological models proposed in Figure 13 do not adequately to explain the growth rate enhancement observed at high initial cell levels. Experiments were initiated to determine if the formation of peroxides in the medium might limit growth. The addition of catalase at 30 units/ml did not stimulate the growth rate for cultures grown in DMEM at 1% FCS at either low or high initial cell concentrations when compared to control cultures (1% FCS) (Table 25). Thus, it is unlikely that the cell-mediated removal of peroxides is responsible for cell-level dependent stimulation of the growth rate.

The final possibility to be tested, is that the cells may reactivate some spontaneously inactivated growth factor. Cysteine (a free thiol) is spontaneously oxidized to cystine (a disulfide) in the presence of oxygen (Toohey, 1975). Cystine, however, is transported very slowly into mammalian cells, whereas cysteine is transported rapidly (Ohmari and Yamamoto, 1983). Thiol compounds in serum can participate in a thiol-exchange reactions with cysteine, that result in a product that may be transported rapidly into the cell (Ohmari and Yamamoto, 1983). These thiol compounds, however, may spontaneously oxidize to disulfides, and therefore may be unable to easily participate in thiol exchange reactions (Ohmari and Yamamoto, 1983). There is some evidence that mammalian cells may have the capability to
Table 25. Growth rates are presented for cells cultured in DMEM at 1% FCS supplemented with or without 30 units/ml catalase. Each entry represents the average of replicate cultures.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Initial Cell Level (cells/ml)</th>
<th>Addition</th>
<th>Growth Rate ( h^{-1} )</th>
<th>Standard Deviation ( h^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>70,000</td>
<td>None</td>
<td>0.025</td>
<td>0.002</td>
</tr>
<tr>
<td>E2</td>
<td>7,000</td>
<td>None</td>
<td>0.016</td>
<td>0.001</td>
</tr>
<tr>
<td>E9</td>
<td>70,000</td>
<td>30 u/ml catalase</td>
<td>0.027</td>
<td>0.002</td>
</tr>
<tr>
<td>E10</td>
<td>7,000</td>
<td>30 u/ml catalase</td>
<td>0.016</td>
<td>0.002</td>
</tr>
</tbody>
</table>

reduce these components to the active form (Ohmari and Yamamoto, 1983). It is possible that the observed enhancement of the growth rate at high initial cell concentrations in low serum medium may be due to an increased rate of cell-mediated reduction of inactive serum disulfides that may occur at higher cell concentrations, especially if these thiols are rate-limiting in low serum medium.

To test this hypothesis, cultures were grown in DMEM at 1% FCS to which either nothing, 20 mM cystine, 20 mM cysteine, or 6.24 \( \mu \)g/ml thioglycerol was added. Thioglycerol is a thiol which has been shown to transport cystine rapidly into the cell (Ohmari and Yamamoto, 1983). Cultures were initiated at both high (70,000 cells/ml) and low (7000 cells/ml) cell levels. Cell level measurements were taken every 10 to 11 hours from 17 to 61 hours after inoculation. The growth rates for each culture were calculated from the five data points. Table 26 presents the growth rates resulting from each condition as an average of the growth rates of replicate cultures. The addition of cysteine increased the growth rate from 0.016 to 0.024 \( h^{-1} \), whereas, there was no increase when cystine was added. Based on a student - t test analysis, there is a greater than 99.9 % probability that this difference is significant. Thus, it is clear that cysteine, not cystine, can stimulate the growth rate at both high and low initial cell levels.
More importantly, the difference in the growth rates observed between cultures initiated at high cell levels and cultures initiated at low cell levels was significantly reduced when cysteine was added (0.005 vs. 0.009 h⁻¹). These is a greater than 99.9% probability that this difference in growth rate is significant. Additionally, thioglycerol stimulated growth slightly compared to the controls (99 % certainty). However, the growth rates of both the high and low cell level cultures were stimulated by the same extent, and consequently, there was no significant decrease in the difference in the growth rate between the two cell levels.

Table 26. Growth rates are presented for cells cultured in DMEM at 1% FCS supplemented with cysteine, cystine, or thioglycerol at two different initial cell levels. Each entry represents the average of replicate cultures.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Initial Cell Level (cells/ml)</th>
<th>Addition</th>
<th>Growth Rate h⁻¹</th>
<th>Standard Deviation h⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>70,000</td>
<td>None</td>
<td>0.025</td>
<td>0.002</td>
</tr>
<tr>
<td>E2</td>
<td>7,000</td>
<td>None</td>
<td>0.016</td>
<td>0.001</td>
</tr>
<tr>
<td>E3</td>
<td>70,000</td>
<td>0.2 mM cystine</td>
<td>0.026</td>
<td>0.002</td>
</tr>
<tr>
<td>E4</td>
<td>7,000</td>
<td>0.2 mM cystine</td>
<td>0.015</td>
<td>0.002</td>
</tr>
<tr>
<td>E5</td>
<td>70,000</td>
<td>0.2 mM cysteine</td>
<td>0.029</td>
<td>0.002</td>
</tr>
<tr>
<td>E6</td>
<td>7,000</td>
<td>0.2 mM cysteine</td>
<td>0.024</td>
<td>0.002</td>
</tr>
<tr>
<td>E7</td>
<td>70,000</td>
<td>6.2 µg/ml thioglycerol</td>
<td>0.027</td>
<td>0.001</td>
</tr>
<tr>
<td>E8</td>
<td>7,000</td>
<td>6.2 µg/ml thioglycerol</td>
<td>0.019</td>
<td>0.002</td>
</tr>
</tbody>
</table>

The results indicate that cysteine, or a similar thiol, is limiting growth at 1% FCS, and that the enhancement of the growth rate with increasing initial cell concentrations might be at least partially attributable to cell-mediated reduction of spontaneously
oxidized cystine-carrying thiols in serum. The observation that thioglycerol shows no
cell level - dependent effect on the growth rate may indicate that the thioglycerol
concentration was not high enough to circumvent the need for cell-mediated reduction
of oxidized thiols.

IV.D.3 Growth Rate Profiles

To this point, only initial growth rates had been measured and related to the initial
environmental conditions. To be useful, any mathematical description must be able to
predict the growth rate at all subsequent times. Figure 14 presents the growth rate as
a function of time for cells grown in DMEM with 1% FCS at initial cell levels of 5000
and 50,000 cells/ml. Cell levels were measured every 6 to 8 hours and growth rates
were estimated using three successive data points. It is seen that initially, the growth
rate of the high cell level culture is larger than that of the low cell level culture, but that
the growth rates of both cultures decline drastically 40 to 60 hours after inoculation.

This decline in the growth rate might be due to the depletion of a nutrient in
DMEM. However, if the data in Figure 14 is re-plotted as a function of the instan-
taneous cell level at every time during growth, as was done in Figure 15, this is seen
not to be the case. The growth rate profile of a culture grown in 10% FCS at an initial
cell level of 5000 cells/ml is also shown in Figure 15 for comparison. Since the growth
rate of the cells grown in 10% FCS remained relatively constant until 500,000 cells/ml,
it can be concluded that the decline in the growth rate of cultures grown in 1% FCS is
not due to cellular consumption of nutrients supplied in standard DMEM. Note that for
the culture initiated at 5000 cells/ml and 1 % FCS, the growth rate began to decline as
the cell level reached 40,000 cells/ml. However, for the culture initiated at 1 % FCS
and 50,000 cells/ml, the growth rate was higher and remained constant at cell levels
more than three times the level at which the growth rate of the culture initiated at 5000
cells/ml started to decline. Thus, it seems unlikely that the decline in the growth rate is
due to the cellular consumption of a limiting component supplied in serum. This
decline in the growth rate for cells grown in 1% FCS appears to be a time dependent
phenomenon, independent of the cell level.
Each point is an average of two cultures. Calculated from three successsive cell levels measured at 1 to 12 hour intervals. Supplemented to 1% FCS initially at the given cell levels. Growth rates were Figure 14. Growth rates are plotted as a function of time for cultures grown in DMEM.
Figure 15. The growth rate of CRL-1666 cells in DMEM at 10% FCS inoculated at 5000 cells/ml is plotted against the instantaneous cell.

Figure 16. Level in the growing cultures.
One explanation for this phenomenon is that a serum nutrient or growth factor is inactivated over time. An example of this last point is the oxidation and subsequent inactivation of serum thiols that transport cystine into the cell. Support for this hypothesis is presented in Figure 16. Cultures were initiated at cell concentrations of 5000 and 50,000 cells/ml in 1% FCS, in either DMEM or DMEM/F-12 supplemented to 6.24 μg/ml thiglycerol. Recall that DMEM/F-12 contains approximately 0.1 mM cysteine and 0.1 mM cystine. However, all of the cysteine has likely been oxidized to cystine prior to the addition of serum or cells into the medium, since the rate of oxidation of cysteine is very rapid (Torchinsky, 1981). The cells grown in DMEM/F-12 with thiglycerol exhibits a slower rate of decline in the growth rate than do the cells grown in DMEM. This supports the hypothesis that the decline in the growth rate for cultures grown at low serum levels is due to oxidation and subsequent inactivation of cystine transporting serum thiols and that the addition of thiglycerol and cysteine can reduce this rate of inactivation. Previous cultures grown in DMEM/F-12 without addition of thiglycerol showed that the addition of F-12 medium to DMEM alone, without thiglycerol, did not stabilize the growth rate of cultures grown in low serum medium (Figure 16a). In fact, the growth rate appears to decline more rapidly in F-12/DMEM medium. Since F-12 has a eight times higher iron concentration than DMEM, and since iron catalyzes thiol oxidation (Torchinsky, 1981), this more rapid rate of decline observed in F-12/DMEM medium might be due to the increased rate of iron-catalyzed oxidation of thiols. It will be shown in the next section that the growth-promoting activity of serum does decline with time, and that the rate of deactivation may be decreased with the addition of thiols.

IV.D.4 Degradation of Serum Activity and Thiol Chemistry

From the data presented in Figure 15 and 16, and Table 26, the following working hypothesis was formulated:

Serum thiols limit growth in low serum cultures, and the spontaneous oxidation of these thiols is responsible for the observed decline of the
has been shown to have no stimulatory effect on the growth rate (Figure 16).

The culture with thymidine was grown in DME/F-12 medium. The cultures were inoculated with and without the addition of 50 mM thymidine. The cultures were inoculated with cell concentration in growing cultures. Cells were cultured in DME at 10 °C.

Figure 16. The growth rate of CRL-1606 cells are plotted against the instantaneous

GROWTH RATE (1/hr)

CELL LEVEL (cells/mL)
hours for the DMEW and the DMEW/F-12 grown cultures was 60,000 and 30,000 cells/ml, respectively. The cell levels at 120 hours is the average of two growth rates from replicate cultures. The cell levels are shown as a function of time. The cultures were initiated at 5000 cells/ml. Each point is the average of two growth rates of cells grown in 1% FCS in either DMEW or DMEW/F-12. The growth rates of cells grown in 1% FCS in either DMEW or DMEW/F-12 are shown as a function of time. The cultures were initiated at 5000 cells/ml.
growth rate with time. The cells may be able to re-reduce the oxidized serum thiols, thereby accounting for the observed stimulation of the initial growth rate with increasing initial cell levels.

The first step in characterizing this phenomenon is to determine the growth response of CRL-1606 hybridomas to both cystine and cysteine. Cultures were grown in DMEM at 0.7% FCS with varying levels of cystine and cysteine. The initial cell level was 5000 cells/ml. The growth rate was calculated from four cell counts taken at 12 hours intervals between 16 and 52 hours after inoculation. The results are shown in Figure 17. Each point in the figure represents the average from replicate cultures. Note that cysteine monomer, in either the thiol or disulfide form, is an absolute requirement for growth, since no growth occurs without either cystine or cysteine. It is clear that cysteine thiol is more effective in stimulating growth than the corresponding disulfide, cystine. The response reaches saturation at 75 μM or less for both compounds. The addition of cystine to medium containing cysteine imparts no stimulatory effect above that of cysteine.

Figure 18 shows the results from a similar experiment in which the initial cell level was increased to 70,000 cells/ml. All of the growth rates in Figure 18 are higher than those in Figure 17. However, it is clear that the stimulatory effect of cysteine is not as great for cultures initiated at high cell levels as it is for cultures initiated at low cell levels. This conclusion is reinforced by the data presented in Figures 19 and 20. Higher initial cell concentrations in DMEM at 0.7% FCS increased the initial growth rate by approximately 0.015 h⁻¹ at all cystine levels tested (Figure 19), whereas higher initial cell levels did not have as great of an effect on cultures grown with cysteine (Figure 20, = 0.005 h⁻¹). This data is consistent with the working hypothesis.

It may be concluded from the preceding data that cysteine or an equivalent thiol is limiting in low serum, low cell level cultures. How may this limitation be a function of the serum level? Figures 21 and 22 illustrates the extent of the stimulatory action of cysteine as a function of the serum level for cultures initiated at 5000 and 70,000 cells/ml, respectively. It is clear that cysteine thiol preferentially stimulates growth at
Each culture was inoculated at 5000 cells/mL and is the average of replicate cultures. Between 16 and 52 hours after inoculation, and 4 cell measurements were made at equal time intervals. All cultures were grown in DMEM supplemented to 0.7% FCS.

Figure 17. The growth rate of cultures grown in DMEM supplemented to 0.7% FCS.

- 0.15 mm Cystine, Variable Cystine
- Without Cystine, w/o Cystine
- With Cystine, w/o Cystine
Figure 18. The effect of the level of cysteine and/or cystine on the growth rate of cultures inoculated at 75,000 cells/ml. The experimental procedure is identical to that described in Figure 17.
Figure 19. The growth rates of cells grown in 0.7% FCS at various cystine concentrations are presented for cultures initiated at two different initial cell levels. The values shown are averages of replicate cultures.
Cell yields. The values are averages of replicate cultures.

Concentrations of cysteine in the growth medium for cultures harvested at two different intervals are presented for cultures enriched at various cysteine concentrations.

**Figure 20:** The growth rates of cells grown in 0.7% FCS at various cysteine concentrations.
Figure 21. The stimulatory effect of cysteine on the growth rate of hybridoma cultures in DME/F12 shown as a function of the serum level. The initial cell level was 5000 cells/ml. Each value represents the average of replicate cultures.
The growth rate of cultures inoculated at 7.5,000 cells/ml is presented. The experimental data is shown in Figure 22. The effect of the level of cysteine and/or cystine on the growth rate is depicted.

Figure 22. The effect of the level of cysteine and/or cystine on the growth rate.

- 0.15 mM cysteine, variable cystine
- 0 mM cysteine, variable cystine
- 0.25 mM cysteine, variable cystine
low serum levels. At low initial cell levels, cysteine stimulates the growth rate approximately 0.01 h⁻¹ at 0.5% and 0.75% FCS, while boosting the rate by only 0.003 h⁻¹ at 2.5% FCS. However, at high initial cell concentrations, the addition of cysteine increased the growth rate by only 0.005 h⁻¹ in low serum medium, and by only 0.001 h⁻¹, if at all, at the higher serum levels. Thus, it may be concluded that the rate limiting component in low serum cultures at low initial cell levels are either serum thiols or serum components whose growth promoting activity may be replaced by cysteine. Again, this is consistent with the working hypothesis.

If this hypothesis is indeed correct, one would expect other thiols to stimulate growth in low serum medium at low cell levels. Figure 23 demonstrates that this is indeed the case, with glutathione showing the greatest stimulatory activity of all the thiols added to cultures growing in DMEM at 1% FCS. The growth rates of cultures grown in 1% and 3% FCS without additional thiols are shown for comparison. Although all the thiols shown stimulate the growth rate, the character of the responses are different. As previously shown, the stimulation by cysteine exhibits saturation at approximately 40 μM, whereas saturation was not reached for cultures dosed with glutathione or dithiothreitol (DTT) at 240 μM.

It has been hypothesized that the declining growth rates observed in cultures initiated at low cell levels in low serum medium was due to progressive oxidation, and hence, inactivation of vital serum thiols. This implied that serum - supplemented medium incubated at 37°C without cells should lose its growth promoting activity over time, and that the addition of a reducing agent should slow this rate of inactivation. To test this, DMEM without glutamine or cystine and with 1% FCS was incubated in the dark for four days at 37°C and 10% CO₂. Prior to incubation, 200 μM cystine and/or 50 μM DTT (with 100 μM EDTA) was added to various fractions of the medium. Each fraction was placed in a one - liter bottle on a platform shaker rotating at 30 RPM. Glutamine was left out of the medium to avoid ammonium accumulation. Recall from Figure 23, that 50 μM DTT did not appreciably affect the growth rate. However, since DTT is a powerful reducing agent, this concentration should provide sufficient
1% FCS and 3% FCS is shown for comparison. The growth rate of cultures grown in DMEM at 37°C and 5% CO2 is shown as a function of the concentration of glutathione, cysteine, and DTT. The initial cell level was 8500 cells/mL. Each point is the average from replicate cultures. The growth rate of cultures grown in DMEM supplemented to 1% FCS is shown as a function of the concentration of glutathione, cysteine, and DTT.
reducing power. EDTA at 100 μM has been found not to either stimulate or inhibit growth, although higher concentrations (333 μM and above) does slightly inhibit the growth rate (data not shown). EDTA was added to chelate any soluable iron or copper which might be present, since both catalyze thiol oxidation (Torchinsky, 1981).

Two 40 ml samples were periodically collected aseptically from each bottle and frozen at -15°C. The samples were later thawed and supplemented to 4 mM glutamine from a 320 mM stock solution. Medium incubated without cystine was supplemented to 200 μM cystine just prior to inoculation of the medium with cells. Medium incubated with cystine was diluted with an equivalent volume of PBS just prior to inoculation. Cultures were inoculated at approximately 5000 cells/ml and the growth rates were measured from 4 points taken every 12 hours between 16 and 52 hours. The results are shown in Figure 24.

As hypothesized, the growth-promoting activity of typical DMEM (with 200 μM cystine) at low serum levels does decline with time, and this rate of decline may be reduced by the addition of a strong reducing agent, DTT. However, what was not covered by the working hypothesis, was the observation that the incubation of DMEM with 1% FCS and without cystine does not exhibit any loss of growth-promoting activity. Thus, while it is very clear that thiols do play a role in stabilizing the activity of serum, the data suggests that oxidation of serum thiols alone cannot explain the observed decline in the growth rate of cells grown in low serum medium.

Can other thiols stabilize the growth activity of serum-supplemented medium? Low serum medium (1 % FCS) was incubated with either 200 μM cystine, 300 μM glutathione (this time, without EDTA), or without supplements and tested for growth activity (Figure 25). Again, the addition of glutathione stimulated the initial growth rate in low serum medium (compare the zero hour time points). Glutathione also appeared to stabilize the growth rate activity after an initial sharp drop in the activity occurred between 0 and 30 hours of incubation, which again, supports the working hypothesis. Note also that, although the decline in the growth-promoting activity of medium incubated without cystine is still much less than medium incubated with cystine, there is a perceptible decline in the activity with incubation time, in contrast to the data
Without CDS, cultures CDS was added to 200 µM for cultures grown in medium incubated and with 50 µM DTT. Each point represents the average of replicates. Growth rate. Medium was incubated with and without 200 µM cystine dihydride (CDS) at 5000 cells/mL. The growth-promoting activity was taken as the result of the samples. Cells were then thawed, supplemented to 4 mM glutamine, and incubated without CDS and without glutamine was incubated for the specified time. Then frozen. Cells without glutamine was incubated at 37°C and 10°C. Medium without glutamine was shown as a function of incubation time at 37°C and 10°C. Figure 2. The growth-promoting activity of DPM supplemented to 18 FCS is shown.
Experimental procedure was as outlined in Figure 24. CDS and 300 µM glutathione, and without CDS, the serum level was 1%. The figure shows growth-promoting activity of serum-supplemented DMEM is shown as a function of time for medium incubated with 200 µM cysteine dithiolethione (CDS) with or without 300 µM glutathione.
presented in Figure 23. The reason for this discrepancy is unclear.

Is the rate of loss of the growth-promoting activity of serum-supplemented DMEM a function of the serum level? This is addressed in Figure 26, which presents the activity as a function of the incubation time of medium supplemented to different serum levels. Medium at high serum levels exhibited a slower rate of deactivation of growth-promoting activity than low serum medium. This is consistent with the observation that cultures grown in high serum medium exhibit a more stable growth rate over time compared to cultures grown in low serum medium (Figure 16).

This deactivation of serum activity may be expressed mathematically. From the 0 hour time points (i.e., no incubation) given in Figure 26, the growth rate of the three cultures may be related to the serum level from a non-linear regression using equation (42) and a zero ammonium concentration, to give:

\[ \mu = \frac{0.046S}{S + 0.67} \]  

(61)

Rearranging, we may develop an expression for the units equivalence of serum activity (UESA) as a function of the growth rate, as follows:

\[ \text{UESA} = \frac{0.67\mu}{0.46 - \mu} \]  

(62)

The data in Figure 26 may then be expressed as UESA based on the growth rate at the various incubation times, as shown in Figure 27. In an effort to mathematically describe the inactivation kinetics, the transformed data in Figure 27 was fitted to the following simple equation:

\[ \frac{dS}{dt} = k_d S^\gamma \]  

(63)

The best fit values for the parameters were: \( k_d = -0.016 \) (\% FCS)\(^{0.3}\) and \( \gamma = 0.7 \). By integrating equation (63) and substituting into equation (61), the growth-promoting activity of the medium at various serum levels may be described as a function of the
In the text, each value is the average of replicate cultures. The experimental procedures were as described in Figure 24. The solid lines as a function of incubation time for serum levels of 0.7%, 1%, and 2.5% FCS. Figure 26: The growth-promoting activity of serum-supplemented DMEM is shown.

TIME (h)

GROWTH RATE (1/hr)

2.5% FCS
0.7% FCS
1% FCS

100 80 60 40 20 0

0.05 0.10 0.15 0.20 0.25 0.30 0.35
The 0.7 power, and the rate constant, \( k \), was taken to be 0.016 (\%FCS)\(^{-0.34} \). Lines represent the rate equation given in equation (63), where the serum degrades to 62% as a function of time from the data presented in Figure 26. The solid line is plotted as a function of serum activity (UESA) as defined by equation (27). The units equivalence of serum activity (UESA) is given in Figure 27.
incubation time. The solid lines in Figure 26 represent such a simulation. This mathematical description is seen to reflect the actual kinetics reasonably well, although the rate of loss of the serum activity is overpredicted for medium incubated with 2.5% FCS. The standard deviation of the fit was 0.003 h⁻¹.

That thiol oxidation is not the whole story is also reflected in the rapid rate with which thiols react in DMEM. The reaction rate at 37°C of the free sulphydryl groups of cysteine and thioglycerol in DMEM prepared with and without iron and with and without 3.5% FCS is shown in Figure 28. After just three hours, almost all of the free-SH groups have reacted, regardless of the medium conditions. This does not imply that all of the thiols have been oxidized. Thiols are very reactive, and may readily participate in thiol-disulfide and disulfide-disulfide exchanges with other thiols and disulfides, such as cystine. In light of this data, the growth-stimulatory activity of thiols can not be due to the thiol moiety itself, but to some thiol reaction product or to a slowly reacting sulphydryl-containing compound yet to be identified.

All the data presented thus far tend to support the working hypothesis, except for the observation that the absence of cystine in the medium stabilizes the growth-promoting activity of serum-supplemented medium. This may be explained by one or both of the following hypothesis:

1. A compound in serum irreversibly binds cystine, thereby removing it from the medium. Recall that the cystine was added just prior to inoculation for those cultures that were grown in medium that had been incubated without cystine.
2. The oxidation-reduction potential of the medium is the relevant property that determines the stability of the growth-promoting activity of serum-supplemented medium. Note that cystine is an oxidized molecule, so that its absence from the medium constitutes a more reduced state.

To test the hypothesis, medium at 1% FCS was incubated with 200 μM cystine, as before. Prior to inoculation of the incubated media, some of the cultures were dosed with 100 μM cystine. If cystine is indeed sequestered by a serum compound
FIGURE 2B. The kinetics of thiol instability in DMEM supplemented with various media conditions:

- a) with adherent cells;  
- b) with medium 199;  
- c) with and without serum (10% FBS) and 300 IU/ml penicillin;  
- d) with and without 150 μM catalase.
during incubation, the addition of cystine just prior to inoculation should restore any lost growth-promoting activity lost due to cystine binding. The results, presented in Figure 29, indicate that the addition of cystine to the culture medium just prior to inoculation was not able to restore the growth-promoting activity lost during incubation of medium containing cystine.

IV.D.5 Metabolic Response Time

Another aspect of metabolic kinetics not included in the initial rate expressions is the possibility that mammalian cells do not respond instantaneously to their environment. An indication that metabolic response time may be significant is reflected in Figure 30. This figure presents the growth rate as a function of time for cultures grown in 4 mM and 16 mM glutamine. Both cultures were inoculated in 3% FCS at low cell levels, so that the bulk of the ammonium formed should be due to glutamine degradation. Therefore, the volumetric ammonium productivity for the cultures grown with 16 mM glutamine should be greater than for the cultures grown with 4 mM glutamine. Consequently, the growth rate of the cultures grown at the higher glutamine level should decline relative to the other cultures as time progresses and ammonium is formed. As seen in Figure 30, this indeed does occur. However, divergence of the growth rate profiles occurs much later than expected based on the predicted rate of ammonium formation from spontaneous glutamine decomposition. For example, at 60 hours, 25% of the glutamine should have degraded to ammonium and pyrrolidone-carboxylate. Thus, the high glutamine culture should have about 4 mM ammonium, while the low glutamine culture should have around 1 mM. Therefore, the growth rates should be very different. On the contrary, they are essentially identical. This discrepancy may be due to a delay in the response of the organisms to the change in the environment.

To determine the character of this presumed metabolic lag, cells were inoculated into medium at the conditions specified in Table 27. Using equation (44), the initial cell level in each culture was chosen such that the final cell level in each of the cultures 65 hours after inoculation would be approximately 70,000 cells/ml. The
Figure 29: The growth-promoting activity of serum-supplemented DMEM of replicate cultures. 100 μM CDS, while the other was not. The growth rates shown are averages cystine disulphide (CDS). Prior to inoculation, one culture was dose with 1% FCS is shown as a function of time for medium incubated with 200 μM CDS.
measured from three consecutive points and is an average of replicate cultures.

The initial cell concentration was 7500 cells/ml. Each growth rate was
and 16 M. The initial cell concentration was 7500 cells/ml. Each growth rate was
in DMEM with 3.5 % FCS, but with two different initial glutamine concentrations: 4 M

Figure 30. The growth rates as a function of time are compared for two cultures grown
growth rates were calculated from four points taken every 11 hours between 35 and 64 hours. At 65 hours, 15 mls of each cell suspension represented in Table 27 was inoculated into 45 mls of fresh medium formulated as shown in Table 28.

The fresh medium was equilibrated in 37°C in a 10% CO₂ environment for 3 hours, to minimize any lag that might occur due to brief exposures to lower temperatures. The cell levels were then measured every 8 hours. The log of the ratio of the cell levels at each time to the initial cell level is shown in Figures 31 - 33. Figure 31 represents a step changes in the serum level at 0 mM ammonium, while Figure 32 presents a step change in the ammonium concentration in high serum (3.5 % FCS) medium. Figures 31 and 32 clearly show that for the first 14 - 17 hours, all of the cultures grew at rates indicative of their inoculum environment. After this initial time period, there is an abrupt change in the growth response reflecting the change in the cell's environment. The same trend is observed when step changes in the ammonium concentration are made in low serum medium, except that the response is slower

Table 27. Stock cultures of CRL-1606 cells were maintained in 100 mls of medium formulated as specified in the table. The initial cell levels were different in each culture, ranging from 10,000 to 20,000 cells/ml. The growth rates shown were determined from 5 points taken 10 hours apart. These cells were used to inoculate medium formulated as in Table 28.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Serum(%)</th>
<th>Ammonium(mM)</th>
<th>Growth Rate (h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>3.2</td>
<td>0</td>
<td>0.038</td>
</tr>
<tr>
<td>M2</td>
<td>0.8</td>
<td>0</td>
<td>0.028</td>
</tr>
<tr>
<td>M3</td>
<td>3.2</td>
<td>4</td>
<td>0.031</td>
</tr>
<tr>
<td>M4</td>
<td>3.2</td>
<td>1</td>
<td>0.037</td>
</tr>
<tr>
<td>M5</td>
<td>0.8</td>
<td>4</td>
<td>0.011</td>
</tr>
<tr>
<td>M6</td>
<td>0.8</td>
<td>1</td>
<td>0.027</td>
</tr>
</tbody>
</table>
(Figure 32). Again, the growth rates started to reflect the cell’s new environment at approximately 14 - 17 hours, but the rate of change of the growth rate to the new value was slower. The transient response was not fully damped until approximately 30 hours. The relatively long transient response may be due to the large difference in the growth rate between the inocula cultures M5 and M6 (.011 vs. .027 h⁻¹, Table 27) compared to the other inocula cultures (.038 vs. .028 h⁻¹ and .031 vs. .037 h⁻¹).

Table 28. Cells from cultures grown in medium represented in Table 27 were used to inoculate medium prepared as noted below. Replicate cultures were run for each condition.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Serum Level Fresh Medium (%)</th>
<th>Ammonium Level Fresh Medium (mM)</th>
<th>Cell Inocula Source</th>
<th>Final Serum (%)</th>
<th>Final Ammonium (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>3.2</td>
<td>0</td>
<td>M1</td>
<td>3.2</td>
<td>0</td>
</tr>
<tr>
<td>N2</td>
<td>0</td>
<td>0</td>
<td>M1</td>
<td>0.8</td>
<td>0</td>
</tr>
<tr>
<td>N3</td>
<td>4.2</td>
<td>0</td>
<td>M2</td>
<td>3.2</td>
<td>0</td>
</tr>
<tr>
<td>N4</td>
<td>0.8</td>
<td>0</td>
<td>M2</td>
<td>0.8</td>
<td>0</td>
</tr>
<tr>
<td>N5</td>
<td>3.2</td>
<td>4</td>
<td>M3</td>
<td>3.2</td>
<td>4</td>
</tr>
<tr>
<td>N6</td>
<td>3.2</td>
<td>0</td>
<td>M3</td>
<td>3.2</td>
<td>1</td>
</tr>
<tr>
<td>N7</td>
<td>3.2</td>
<td>5.3</td>
<td>M4</td>
<td>3.2</td>
<td>4</td>
</tr>
<tr>
<td>N8</td>
<td>3.2</td>
<td>1</td>
<td>M4</td>
<td>3.2</td>
<td>1</td>
</tr>
<tr>
<td>N9</td>
<td>1</td>
<td>4</td>
<td>M5</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>N10</td>
<td>1</td>
<td>0</td>
<td>M5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>N11</td>
<td>1</td>
<td>1</td>
<td>M6</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>N12</td>
<td>1</td>
<td>5.3</td>
<td>M6</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
Average of two cultures. There was no ammonium added to the medium. Each value is the change in the serum level in DMEM at 1606 hybrids upon a step.

Figure 31. The response of the growth rate of GRL-1606 hybrids upon a step.

TIME (hr)

LOG (X(t)/X(0))
Figure 32. The response of the growth rate of CRl-1606 cells upon a step change in the ammonium level in DMEM at 3.2% PCS is presented. The cells were inoculated at 8500 cells/ml. Each value is the average of replicate cultures.

KINETICS OF STEP CHANGE IN AMMONIUM LEVEL IN DMEM AT 3.2% PCS
Figure 33. The response of the growth rate of CRL-1606 cells upon a step change in the ammonium level in DMEM at 0.6% PCS is presented. The cells were inoculated at 8500 cells/ml. Each value is the average of replicate cultures.

KINETICS OF STEP CHANGE IN AMMONIUM LEVEL IN DMEM AT 1.6% PCS
These transients, in control theory terminology, reflect first order response kinetics with transportation lag (Coughanowr and Koppel, 1965). The kinetic description may be formulated as follows:

\[
\frac{dX}{dt} = \mu X \rightarrow \frac{d(\ln X)}{dt} = \mu \quad (64)
\]

Let \( Y = \ln X \), so

\[
\frac{dY}{dt} = \mu \quad (65)
\]

Now, suppose at some point in time, the system is at steady state, i.e., the growth rate, \( \mu \), is a constant, \( \mu_s \). Then

\[
\frac{dY_s}{dt} = \mu_s \quad \text{where} \quad Y_s = Y_0 + \mu_s t \quad (66)
\]

Subtracting (66) from (65) gives

\[
\frac{dY}{dt} - \frac{dY_s}{dt} = \frac{d(Y - Y_s)}{dt} = \mu - \mu_s \quad (67)
\]

Let \( \bar{Y} = Y - Y_s \) and \( \bar{\mu} = \mu - \mu_s \), then

\[
\frac{d\bar{Y}}{dt} = \bar{\mu} \quad (68)
\]

Assume at \( t = 0 \), the system was at steady state so that \( \bar{Y} = 0 \), and \( \bar{\mu} = 0 \). The Laplace transforms may now be obtained:
\[ s\bar{V}(s) = \bar{\mu}(s) \Rightarrow \bar{V}(s) = \frac{\bar{\mu}(s)}{s} \]  

(69)

Thus, we now have a transfer function with \( \bar{\mu}(s) \) as the input and \( \bar{V}(s) \) as the output. As stated previously, Figures (31) - (34) imply that the growth rate response may be represented by a first order system with transportation lag (Coughanowr and Koppel, 1965). That is:

\[ \bar{\mu}(s) = \frac{e^{-\tau_L s} F(s)}{\tau s + 1} \]  

(70)

where  
\[ \tau = \text{first order time constant, h}^{-1} \] 
\[ \tau_L = \text{transportation lag parameter, h} \] 
\[ F(s) = \text{forcing function} \]

In this case, \( F(s) \) represents a step change from medium that would result in a growth rate of \( \mu_s \) to a medium that would result in a growth rate of \( \mu^* \). This step change is represented in Laplacian space by:

\[ F(s) = \frac{\mu^* - \mu_s}{s} \]  

(71)

So, \( \bar{V}(t) \) is now described by:

\[ \bar{V}(t) = (\mu^* - \mu_s)u(t - \tau_L)\left[ (t - \tau_L) + \tau e^{-(t - \tau_L)\tau} - \tau \right] \]  

(72)

where \( u(t - \tau_L) = \text{unit step function} \)

\[ = 0 \text{ for } t \leq \tau_L \]
\[ = 1 \text{ for } t > \tau_L \]
Converting back to the expression for the cell level gives:

\[
\ln \left[ \frac{X(t)}{X_0} \right] = \mu_a t + (\mu^* - \mu_a)u(t - \tau_L) \left[ (t - \tau_L) + \tau e^{-\tau(t - \tau_L)} - \tau \right]
\]

(73)

Note that in the limit as \( t \) approaches infinity, that

\[
\lim_{{t \to \infty}} \left[ \ln \left( \frac{X(t)}{X_0} \right) \right] = \mu^* t \quad \text{as expected}
\]

We may use the results of this mathematical treatment to determine the transportation lag constant, \( \tau_L \) as well as the first order response constant, \( \tau \), for each of the step changes in Figures 31 - 33. The values \( \mu^* \) and \( \mu_a \) may be determined from each figure from the growth rates of the two cultures which did not experience a step change. For example, for the cultures that experienced a step change from 3.2% FCS to 0.8% FCS (culture N2 in Table 29), \( \mu_a \) was taken to be the growth rate of the cultures grown in 3.2% FCS during the entire experiment (culture N1 in Table 29), while \( \mu^* \) was taken to be the growth rate of the cultures grown in 0.8% FCS the entire time (culture N4 in Table 29). The values of the time constants were calculated from a non-linear regression of \( \ln[X(t)/X(0)] \) vs. time using equation (73), and are presented in Table 29.

Table 29 confirms the observation that the response after the initial lag, for cultures shifted in the serum level or in the ammonium concentration at 3.2% FCS was very rapid, whereas the ammonium shift at low serum levels resulted in a relatively sluggish response. The reason for the difference is not clear.
Table 29. The transportation lag constants $\tau_L$ and the first order response time constants, $\tau$, calculated from the cultures experiencing step changes in Figures 31 - 33, are presented. The values of $\mu_s$ and $\mu^*$ are calculated from the cultures not subjected to step changes in Table 28. The constants were calculated via a non-linear regression of the combined transient response of replicate cultures.

<table>
<thead>
<tr>
<th>Culture</th>
<th>$\mu_s$ $h^{-1}$</th>
<th>$\mu^*$ $h^{-1}$</th>
<th>$\tau_L$ h</th>
<th>$\tau$ h$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>0.034</td>
<td>0.020</td>
<td>17.4</td>
<td>0.005</td>
</tr>
<tr>
<td>N3</td>
<td>0.020</td>
<td>0.034</td>
<td>18.3</td>
<td>0.083</td>
</tr>
<tr>
<td>N6</td>
<td>0.023</td>
<td>0.032</td>
<td>11.7</td>
<td>0.011</td>
</tr>
<tr>
<td>N7</td>
<td>0.032</td>
<td>0.023</td>
<td>11.4</td>
<td>0.011</td>
</tr>
<tr>
<td>N10</td>
<td>0.000</td>
<td>0.020</td>
<td>12.5</td>
<td>14.8</td>
</tr>
<tr>
<td>N12</td>
<td>0.020</td>
<td>0.000</td>
<td>7.8</td>
<td>13.5</td>
</tr>
</tbody>
</table>
IV.E.1 Formulation of the Optimal Control Problem for Fed-Batch Cultures of CRL - 1606 Hybridomas

A pictorial representation of a fed-batch cultivation of hybridoma cells is presented in Figure 34. Note from the figure that the inlet medium feed would contain glutamine at a concentration, $G_i$, but no serum. Thus, the serum level would be a monotonically decreasing function of time. This strategy was chosen, a priori, so that the greatest amount of product could be produced using the least amount of serum in the least amount of time. The relatively high serum level of the medium during the initial phase of the process should stimulate rapid growth to high cell concentrations. The serum level would be reduced during the latter, production phase of the culture, thereby minimizing the amount of serum used per total product produced.

\[ \text{DMEM w/o SERUM} \]

\[ [\text{GLN}] = G_i \]

\[ O_2 \]

\[ V_{\text{max}} \]

\[ X_0, S_0, G_0, A_0, V_0 \]

Figure 34. A fed-batch culture of hybridoma cells.
The rate of change of cell, serum, glutamine, ammonium, and antibody concentrations in a fed-batch culture operated as given in Figure 34, may be mathematically described as follows (see Appendix A for legend of symbols):

\[
\frac{d(XV)}{dt} = \mu XV
\]  
(74)

\[
\frac{d(SV)}{dt} = 0
\]  
(75)

\[
\frac{d(GV)}{dt} = FG_i - kGV - (m + q_A)\alpha XV
\]  
(76)

\[
\frac{d(AV)}{dt} = kGV + q_A\alpha XV
\]  
(77)

\[
\frac{d(PV)}{dt} = q_p\alpha XV
\]  
(78)

\[
\frac{dV}{dt} = F(t) \quad 0 \leq F(t) \leq F_{\text{max}}
\]  
(79)

\[
\mu = \frac{\mu_{\text{max}}SG}{\left[\left(K_0 + X\right) + S\right]\left[1 + \frac{A^2}{K_A}\right]\left[K_G + G\right]}
\]  
(80)

\[
\alpha = f(\mu)
\]  
(81)

\[
X_0, S_0, G_0, A_0, V_0, \text{ and } P_0 \quad \text{all given at } t = t_0
\]

Inherent in the preceding statements and the subsequent optimization using optimal control theory are the following assumptions:

a) **No lactate is formed.** This obviously is not correct, but was assumed so that the optimal control theory could be employed. Any additional equations to the system would make the optimal control problem practically insolvable.
As it is, the assumed system contains two more state equations than is usually assumed when fed-batch cultures of microbial systems are optimized (Modak et al., 1986). Recall, that while lactate exerts little influence on the growth rate, lactate may significantly inhibit the specific antibody productivity (Figure 10). The omission of lactate accumulation in the mathematical description may not be serious in practice, since it has been shown that lactate accumulation can be drastically reduced by either implementing glucose control (Glacken et al., Appendix B), or by utilizing alternate carbohydrate sources, such as galactose or fructose (Eagle, 1958).

b) **No loss in serum activity occurs over time.** Again, this is known not to be correct (Figure 24). However, if degradation of serum activity is included in the optimization process, the growth rate would be *explicit* in time. This also would make the optimal control problem very difficult to solve, since the time derivative of $\partial H/\partial F$, the derivative of the Hamiltonian with respect to the control variable, would not necessarily be zero along the singular arc (Bryson and Ho, 1975).

c) **Glutamine is the rate-limiting nutrient.** This may indeed be realized in practice by simply increasing the concentrations of glucose and amino acids in the medium. It has been shown that glucose levels up to three times the normal level in DMEM and amino acid levels up to four times the normal level have no effect on the growth rate of the cells (Table 11).

d) **Non-viable (i.e., trypan-blue stained cells) do not consume or produce nutrients or products.** Note in equations (76) - (78), the nutrient utilization rates and the product excretion rates are proportional to the total number of viable cells, given by $\alpha X V$.

e) **Nutrient utilization rates and product excretion rates are constant.**
It is desired to maximize the yield of either cells or antibody with respect to the costs of time, serum and inoculum, i.e.,

\begin{align}
J &= \frac{P_t V_f}{C_t + C_s S_0 V_0 + C_x X_0 V_0} \quad \text{(81a)} \\
J &= \frac{X_t V_f}{C_t + C_s S_0 V_0 + C_x X_0 V_0} \quad \text{(81b)}
\end{align}

where \(C_t\), \(C_s\), and \(C_x\) are the unit costs of time, serum, and cells, respectively.

The system may be reduced by solving equation (75) for \(SV\) to give:

\[ S = \frac{S_0 V_0}{V} \quad \text{(82)} \]

The differential equation for glutamine may be eliminated by combining equations (76), (77), and (78) and integrating as follows:

\[
\frac{d(GV)}{dt} = G_i \frac{dV}{dt} - \frac{d(AV)}{dt} - \frac{m}{q_p} \frac{d(PV)}{dt} \quad \text{(83)}
\]

\[
GV = G_0 V_0 + G_i (V - V_0) - (AV - A_0 V_0) - \frac{m (PV - P_0 V_0)}{q_p} \quad \text{(84)}
\]

The dimensionality of the system is now reduced to four. However, Modak et al. (1986) suggests adding a state variable to the system when the terminal time, \(t_f\), is included in the objective function, \(J\). This new state variable, \(y\), is equivalent to the time. The system now is represented by the following equations:

\[
\frac{d(XV)}{dt} = \mu XV \quad \text{(74)}
\]
\[
\frac{d(AV)}{dt} = k \left[ G_0 V_0 + G_i(V - V_0) - (AV - A_0 V_0) - \frac{m(PV - P_0 V_0)}{q_p} \right] + q_A \alpha V \tag{85}
\]

\[
\frac{d(PV)}{dt} = q_p \alpha V \tag{78}
\]

\[
\frac{dy}{dt} = 1 \quad y = 0 \quad @ \quad t = t_0 \tag{86}
\]

\[
\frac{dV}{dt} = F(t) \quad 0 \leq F(t) \leq F_{\text{max}} \tag{79}
\]

\[
\mu = \frac{\mu_{\text{max}} \left[ \frac{S_0 V_0}{V} \right]}{\left[ \frac{K_G + G_0 V_0 + G_i(V - V_0) - (AV - A_0 V_0) - \frac{m(PV - P_0 V_0)}{q_p}}{\left( K_0 \right)_0 X^\beta + \frac{S_0 V_0}{V}} \right] \left[ 1 + \frac{A^2}{K_A} \right]}
\]

\[
\alpha = f(\mu) \tag{81}
\]

Now that the system is defined, what method should be used to determine the optimal control policy? The most rigorous method is that of Modak et al. (1986). The authors take advantage of the fact that HF and all the time derivatives of HF equal zero on the singular arc, and use these equations to solve for the adjoint variables and the flow rate directly. By taking advantage of some fortuitous aspects of the systems in question, Modak et al. and others (San and Stephanopoulos, 1983; San and Stephanopoulos, 1984; San and Stephanopoulos, 1986) were able to circumvent having to solve for some of the adjoint variables. Consequently, they were not required to evaluate derivatives of HF of orders higher than 2. If this method were attempted for the present system, derivatives of HF up to order four would have to be
evaluated. Since the present system is extremely complex, determining the analytical solution, via the method of Modak et al. (1986) would be extremely difficult and time consuming, if indeed it could be solved at all. Since this is only the first exploratory step in mammalian cell bioreactor optimization, and since the mathematical descriptions of the hybridoma metabolic kinetics is still in the development process, it is felt that such a rigorous optimization methodology is not warranted.

A method that simplifies the system somewhat is Kelley's Transformation of variables (Kelley, 1962). This procedure converts a singular problem with respect to a given control variable to a non-singular problem in another control variable. Hong (1986) used this method to determine the optimum feeding policy for ethanol production by yeast. Ohno et al. (1978) used Kelley's Transformation to determine the optimum operating mode for a lysine fermentation.

If the state equations are linear in the control variable, then the system may be transformed to new variables $Z_k$, such that

$$Z_k = g_k(x_1, \ldots, x_n) \quad k = 1, \ldots, m < n$$  \hspace{1cm} (88)

$$\sum_{i=1}^{n} \frac{\partial g_k}{\partial x_i} q_i = 0 \quad k = 1, \ldots, m < n$$  \hspace{1cm} (89)

where $q_i$ are the linear multipliers of the control variable, $F$, in the state equations. The new control variable is chosen as one of the state variables, $x_k = Z_n$. The set of $q_i$ are determined by expanding the differential equations in (74), (85), and (78):

$$\frac{dX}{dt} = \mu X - \frac{XF}{V}$$  \hspace{1cm} (90)

$$\frac{dA}{dt} = -\frac{k \left[ G_0 V_0 + G_1 (V_0 - V) - (AV - A_0 V_0) - \frac{m (PV - P_0 V)}{q_p} \right]}{V} + q_A \alpha X - \frac{AF}{V}$$  \hspace{1cm} (91)
\[ \frac{dP}{dt} = q_p \alpha X - \frac{PF}{V} \quad (92) \]

\[ \frac{dy}{dt} = 1 \quad y = 0 \quad @ \quad t = t_0 \quad (86) \]

\[ \frac{dV}{dt} = F(t) \quad 0 \leq F(t) \leq F_{\text{max}} \quad (79) \]

So, for all \( g_k \)

\[ \frac{\partial g_k}{\partial X} \left[ \frac{X}{V} \right] + \frac{\partial g_k}{\partial A} \left[ \frac{A}{V} \right] + \frac{\partial g_k}{\partial P} \left[ \frac{P}{V} \right] + \frac{\partial g_k}{\partial V} = 0 \quad (93) \]

which implies the following transformations:

\[ Z_1 = \ln(XV) \quad (94) \]

\[ Z_2 = \ln(AV) \quad (95) \]

\[ Z_3 = \ln(PV) \quad (96) \]

\[ Z_4 = y \quad (97) \]

\[ Z_5 = V \quad (98) \]

The system may now be described in terms of the transformed variables as follows:

\[ \frac{dZ_1}{dt} = \mu \quad (99) \]

\[ \frac{dZ_2}{dt} = k \epsilon \cdot Z_2 \left[ G_0 V_0 - G_i (Z_5 - V_0) - \left( Z_2 - A_0 V_0 \right) \frac{m (Z_3 - P_0 V_0)}{q_p} \right] + q_A \alpha \epsilon \cdot Z_1 \cdot Z_2 \quad (100) \]
\[ \frac{dZ_3}{dt} = q_p \alpha \theta z_3 - z_3 \]  
(101)

\[ \frac{dZ_4}{dt} = 1 \]  
(102)

\[ \mu = \mu_{\text{max}} \left[ \frac{S_0 V_0}{Z_5} \right] G_0 V_0 + G_L (Z_5 - V_0) - \left( e^{z_2} - A_0 V_0 \right) \frac{m(e^{z_3} - P_0 V_0)}{q_p} \]  
(103)

\[ \left[ K_G + G_0 V_0 + G_L (Z_5 - V_0) - (e^{z_2} - A_0 V_0) \frac{m(e^{z_3} - P_0 V_0)}{q_p} \right] \left[ 1 + \frac{e^{2z_2}}{K_A Z_5^2} \right] \left( \frac{z_1}{Z_5} \right)^{-\beta} \left[ \frac{S_0 V_0}{Z_5} \right] \]  

Note that the control variable for the transformed system is now the liquid volume, Z_5.

Before proceeding further, a form for the objective function must be chosen. In this case, it is desired to maximize the amount of antibody produced per unit cost (equation (81a)). In terms of the transformed variables, Z_i, this objective function may be represented as:

\[ J = \frac{e^{z_3}}{C_i Z_4 + C_s S_0 V_0 + C_x X_0 V_0} \]  
(104)

At this point, an expression relating the culture viability to the growth rate must be selected, as suggested by equation (81). An expression for \( \alpha \) was developed in section IV.B.2 (equation (49)). However, this relation is discontinuous, and discontinuities can create pathological difficulties when solving the optimal control problem. Therefore, equation (49) is approximated by the following continuous expression:

\[ \alpha = 46.2 \mu^{-46\mu} + 1.12 \ e^{-0.019\mu} \]  
(105)

The Hamiltonian may now be formulated:
\[ H = \lambda_{Z_1} \mu + \lambda_{Z_2} \left[ k e^{-z_2} \left( G_0 V_0 + G_I (Z_3 - V_0) - \left( e - A_0 V_0 \right) \frac{m(e - P_0 V_0)}{q_p} \right) + q_A \alpha e^{z_1 \cdot z_2} \right] \]

\[ \lambda_{Z_4} + \lambda_{Z_3} q_p \alpha e^{z_1 \cdot z_3} = 0 \] (106)

where the adjoint variables, \( \lambda \), are defined as follows:

\[ \dot{\lambda}_{Z_1} = -\lambda_{Z_1} \frac{\partial \mu}{\partial Z_1} - k \lambda_{Z_2} e^{-z_2} \frac{\partial G}{\partial Z_1} - e^{z_1} \left[ \frac{\partial \alpha}{\partial Z_1} + \alpha \right] \left[ \lambda_{Z_2} q_A e^{z_2} + \lambda_{Z_3} q_p e^{z_3} \right] \] (107)

\[ \dot{\lambda}_{Z_2} = -\lambda_{Z_2} \frac{\partial \mu}{\partial Z_2} - \lambda_{Z_2} e^{-z_2} \left[ k Z_5 e^{-z_2} \frac{\partial G}{\partial Z_2} - G \right] + q_A e^{z_1} \left[ \frac{\partial \alpha}{\partial Z_2} - \alpha \right] - \lambda_{Z_3} q_p e^{z_1 \cdot z_2} \frac{\partial \alpha}{\partial Z_2} \] (108)

\[ \dot{\lambda}_{Z_3} = \lambda_{Z_3} \frac{\partial \mu}{\partial Z_3} - \lambda_{Z_3} e^{-z_3} \left[ k Z_5 e^{-z_2} \frac{\partial G}{\partial Z_3} - q_A e^{z_1 \cdot z_2} \frac{\partial \alpha}{\partial Z_3} \right] - \lambda_{Z_4} q_p e^{z_1 \cdot z_3} \frac{\partial \alpha}{\partial Z_3} - \alpha \] (109)

\[ \dot{\lambda}_{Z_4} = -\lambda_{Z_1} \frac{\partial \mu}{\partial Z_4} - \lambda_{Z_2} \left[ k Z_5 e^{-z_2} \frac{\partial G}{\partial Z_4} - q_A e^{z_1 \cdot z_2} \frac{\partial \alpha}{\partial Z_4} \right] - \lambda_{Z_3} q_p e^{z_1 \cdot z_3} \frac{\partial \alpha}{\partial Z_4} \] (110)

At \( t = t_i \):

\[ \lambda_{Z_1} = \left[ \frac{\partial J}{\partial Z_1} \right]_{t_i} = 0 \] (111)

\[ \lambda_{Z_2} = \left[ \frac{\partial J}{\partial Z_2} \right]_{t_i} = 0 \] (112)
\[ \lambda_{z_3} = \left[ \frac{\partial J}{\partial z_3} \right]_{t_1} = J \]  

(113)

\[ \lambda_{z_4} = \left[ \frac{\partial J}{\partial z_4} \right]_{t_1} = \frac{C_t J}{C_t t_1 + C_s S_0 V_0 + C_x X_0 V_0} \]  

(114)

The Hamiltonian equals zero since the final time, \( t_1 \), is not specified in advance (Bryson and Ho, 1976). Note, that since both \( \mu, \alpha, \alpha \) and \( G \) are not explicit in time, \( t \), then:

\[ \frac{\partial \mu}{\partial z_4} = \frac{\partial \alpha}{\partial z_4} = \frac{\partial G}{\partial z_4} = 0 \Rightarrow \lambda_{z_4} = \frac{-C_t J}{C_t t_1 + C_s S_0 V_0 + C_x X_0 V_0} \text{ for all } t \]  

(115)

Thus, \( \lambda_{z_4} \) is a non-zero constant at all times. This fact may be used to eliminate \( \lambda_{z_4} \) from the system by transforming the adjoint variables as follows:

\[ \bar{\lambda}_{z_1} = \lambda_{z_1} / \lambda_{z_4} \]  

(116a)

\[ \bar{\lambda}_{z_2} = \lambda_{z_2} / \lambda_{z_4} \]  

(116b)

\[ \bar{\lambda}_{z_3} = \lambda_{z_3} / \lambda_{z_4} \]  

(116c)

\[ \bar{H} = H / \lambda_{z_4} \]  

(116d)

Using these new definitions, equation (110) and \( \lambda_{z_4} \) may be eliminated from the problem, and the redefined Hamiltonian may be described with only three adjoint variables, as follows:
\[ \bar{H} = \bar{\lambda}_Z \mu + \bar{\lambda}_Z \begin{bmatrix} \kappa e^{-Z_2} \\
 G_0 V_0 + G_1(Z_5 - V_0) - (e^{Z_2} - A_0 V_0) \frac{m(e^Z - P_0 V_0)}{q_p} \end{bmatrix} + q_A e^{Z_1} \cdot Z_2 \]
\[ + \bar{\lambda}_Z q_p \alpha \theta e^{Z_1 - Z_3} + 1 = 0 \] (117)

The three adjoint variables in equation (117) are defined by differential equations (107), (108), and (109), and by the definitions (116a), (116b), and (116c).

The problem may be simplified further by recalling that for the control to be optimal, then \( \bar{H} = 0 \) and \( \partial \bar{H} / \partial Z_5 = 0 \). This implies that \( \bar{H} = 0 \) and \( \partial \bar{H} / \partial Z_5 = 0 \). With this being the case, \( \bar{\lambda}_Z \) may be removed from the Hamiltonian via the following manipulations:

\[ \frac{\partial (\bar{H} \mu^{-1})}{\partial Z_5} = \mu^{-1} \frac{\partial \bar{H}}{\partial Z_5} + \bar{H} \frac{\partial \mu^{-1}}{\partial Z_5} = 0 \] (118)

\[ \frac{\partial (\bar{H} \mu^{-1})}{\partial Z_5} = \frac{\partial \mu^{-1}}{\partial Z_5} + \bar{\lambda}_Z \begin{bmatrix} \kappa e^{-Z_2} \\
 G \mu^{-1} + Z_5 \frac{\partial (G \mu^{-1})}{\partial Z_5} \end{bmatrix} + q_A e^{Z_1} \cdot Z_2 \frac{\partial (\alpha \mu^{-1})}{\partial Z_5} \]
\[ + \frac{\bar{\lambda}_Z q_p \alpha \theta}{\bar{\lambda}_Z} e^{Z_1 - Z_3} \frac{\partial (\alpha \mu^{-1})}{\partial Z_5} = 0 \] (119)

Thus, the solution for the optimal control problem is obtained by solving the split two-point boundary value problem given by equations (99)-(102) and (107)-(109). The necessary terminal conditions for the redefined adjoint variables \( \bar{\lambda}_Z \) and \( \bar{\lambda}_Z \) are still zero. However, the necessary terminal condition for \( \bar{\lambda}_Z \) is:

\[ \bar{\lambda}_Z = \frac{C_t + C_S 0 V_0 + C_a X_0 V_0}{C_t} \] (120)

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The optimal control problem is now completely characterized. The solution may proceed as follows:

1. Select a value of $\lambda_{Z_2}$ at time $t_0$.

2. From the requirement that $\frac{\partial (H\mu^{-1})}{\partial Z_5} = 0$, solve for $\lambda_{Z_3}(t_0)$.

3. From the requirement that $H = 0$, solve for $\lambda_{Z_1}(t_0)$.

4. Solve the differential equations (99) - (102) and (107) - (109) with definitions (116a) - (116c).

5. The final time, $t_f$, is determined when $\lambda_{Z_2} = 0$. Then the values of $\lambda_{Z_1}(t_f)$ and $\lambda_{Z_3}(t_f)$ are checked to determine if the boundary conditions specified in equations (111) and (120) are satisfied. If not, a new value of $\lambda_{Z_2}(t_0)$ is chosen until the boundary conditions are satisfied.

A listing of the computer program used may be found in Appendix C.

IV.E.2 Optimization Results - Batch Simulation

Before examining the solution of the optimal control problem outlined in the preceding section, it might be instructive to first determine the optimal starting conditions for batch operation. That is, determine the optimal initial glutamine, serum, and cell concentration that maximize the objective function presented by equation (81a). For illustrative purposes only, suppose that it is desired to produce monoclonal antibody in a 2000 liter vessel, and that the costs of time, serum and inoculum are as follows:

\[ C_t = \$60/h \quad (\$30/h \text{ labor, } \$30/h \text{ capital costs}) \]
\[ C_s = \$100/l \quad (D. Girard, Director of M.I.T. Cell Culture Center, personal communication) \]
\[ C_x = \$100 \text{ per } 10^{10} \text{ cells} \quad \text{(D. Girard, personal communication)} \]

The differential equations (74) - (79) were solved with \( V = 2000 \text{ l and } F = 0 \) using a 4th-order implicit Runge-Kutta method with variable stepsize. The initial ammonium and antibody concentrations were taken to be zero. The value of the metabolic rate parameters in equations (74) - (79) were taken to be the following:

\[
(K_s)_0 = 6.5 \% \text{ FCS} \\
\beta = 0.21 \\
K_A = 26 \text{ mM}^2 \\
q_p = 1.7 \text{ pg/cell-h} \\
q_A = 5.0 \times 10^{-11} \text{ mmol/cell-h} \\
m = 2.0 \times 10^{-11} \text{ mmol/cell-h} \\
\mu_{max} = 0.055 \text{ h}^{-1} \\
K_G = 0.15 \text{ mM} 
\]

A numerical optimization employing a Simplex search routine (Beveridge and Schecter, 1972) was used to determine the values of \( X_0, G_0 \) and \( S_0 \) that maximized the objective function \( J \). The optimization program may be found in Appendix D. The initial cell, serum and glutamine levels determined as optimal via the computer program are as follows:

\[
X_0 = 2.4 \times 10^8 \text{ cells/l} \\
S_0 = 1.5 \% \text{ FCS} \\
G_0 = 13.8 \text{ mM} 
\]

The results of a batch simulation with these initial conditions are shown in Table 30. The cost of producing a gram of antibody was estimated to be \$30.50/g. The results clearly predict that ammonium accumulation would limit the productivity of a batch process. Higher initial glutamine levels would serve only to increase the rate of
ammonium accumulation via glutamine decomposition, thereby prematurely halting
growth before all the glutamine could be utilized by the cells. This would result in
lower final cell and antibody levels. Conversely, lower glutamine levels would result in
premature nutrient exhaustion.

Table 30. A representation of an optimized batch culture of CRL-1606 hybridoma cells
determined by a numerical Simplex search procedure is presented below.
The initial serum level, $S_0$, was 1.5%.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Cell Level (cells/l)</th>
<th>Glutamine (mM)</th>
<th>Ammonium (mM)</th>
<th>Growth Rate h$^{-1}$</th>
<th>Product Titer (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>2.4 x 10$^8$</td>
<td>13.8</td>
<td>0</td>
<td>0.041</td>
<td>0</td>
</tr>
<tr>
<td>12.2</td>
<td>3.9 x 10$^8$</td>
<td>12.8</td>
<td>0.9</td>
<td>0.040</td>
<td>6</td>
</tr>
<tr>
<td>20.6</td>
<td>5.5 x 10$^8$</td>
<td>12.1</td>
<td>1.6</td>
<td>0.037</td>
<td>13</td>
</tr>
<tr>
<td>34.8</td>
<td>9.2 x 10$^8$</td>
<td>10.6</td>
<td>2.9</td>
<td>0.028</td>
<td>31</td>
</tr>
<tr>
<td>55.7</td>
<td>1.6 x 10$^9$</td>
<td>8.2</td>
<td>4.9</td>
<td>0.017</td>
<td>73</td>
</tr>
<tr>
<td>87.0</td>
<td>2.8 x 10$^9$</td>
<td>4.1</td>
<td>8.1</td>
<td>0.009</td>
<td>157</td>
</tr>
<tr>
<td>110.0</td>
<td>3.6 x 10$^9$</td>
<td>1.6</td>
<td>10.0</td>
<td>0.005</td>
<td>217</td>
</tr>
<tr>
<td>125.0</td>
<td>4.0 x 10$^9$</td>
<td>0.3</td>
<td>10.8</td>
<td>0.003</td>
<td>250</td>
</tr>
</tbody>
</table>

Note, that a relatively low serum level is recommended. A higher serum level would
increase serum costs more than it would increase productivity, whereas a lower serum
level would result in such low growth rates that prohibitive ammonium levels would be
accumulated before a high cell level is achieved.

IV.E.3 Optimization Results - Determination of the Optimal Control
Policy In Fed - Batch Bioreactors

The optimal medium control policy for a fed-batch bioreactor of CRL - 1606
hybridomas was determined as outlined in section IV.E.1, using, for illustrative purposes only, the following initial conditions:

\[
X_0 = 2 \times 10^8 \text{ cells/l} \\
S_0 = 2.6 \% \text{ FCS} \\
G_0 = 1.3 \text{ mM} \\
G_i = 33 \text{ mM} \\
V_0 = 930 \text{ l}
\]

The values of the metabolic parameters used in the simulation are the same as the values used to determine the optimal initial conditions for batch operation (section IV.E.2). The method of solution was as given at the end of section IV.E.1. The computer used is given in Appendix C. Different values of \(\lambda_{Z_2}(t_i)\) were selected until all of the boundary conditions given in equations (111), (112), and (120) are satisfied. Note that every term in the boundary condition for \(\lambda_{Z_3}(t_i)\) is constant except the terminal time, \(t_i\), which is not known from the outset. However, at the terminal point of the process, both of the other adjoint functions must be zero, if the solution is optimal. Thus, a possible value for the terminal time is given whenever the first two adjoint functions are zero. The required value of \(\lambda_{Z_3}(t_i)\) given by equation (120) is then checked against the value of \(\lambda_{Z_3}(t_i)\) resulting from the solution of the differential equations of the state and adjoint variables. If the two are equivalent, a stationary point for the problem has been found and the optimal control problem has been solved.

The initial value of \(\lambda_{Z_2}\) that satisfied the terminal conditions was 0.12. The terminal time in this case was 192 h and the subsequent boundary condition for \(\lambda_{Z_3}\) was -260. The volume, ammonium and glutamine profiles resulting from the solution of the optimal control problem is shown in Figure 35. The predicted cell, serum and antibody profiles are given in Figure 36. The resultant antibody cost was $19.30, which is 60 % of the cost estimated from an optimized batch culture. It is predicted that the final total cell and antibody levels would be \(6.1 \times 10^9\) cells/l and 405 mgs/ml, respectively. Note
The cost of the product would be $19.30.

The predicted total cell and antibody concentrations would be 6.1 million cells/ml and 405 mg/l, respectively. It is predicted that the glutamine and ammonium profiles are also shown. The resultant plot of glutamine cells is shown as a function of time. The resultant plot of glutamine cells is shown as a function of time.
Figure 36. The total cell level ($X$), the antibody concentration ($P$), and the growth rate ($\mu$), are predicted as a function of time for a fed-batch bio-reactor controlled as shown in Figure 35.
from the volume profile given in Figure 35, that the control is bang-singular-bang, which is typical of the solution of the optimal control problem for fed-batch cultures of microbial cells (Modak et al., 1986).

This control strategy is reasonable and intuitive. The initial glutamine concentration, chosen randomly, was higher than it should have been, resulting in a control strategy that would initially operate batch-wise. This would allow the glutamine concentration to decrease somewhat. This lower glutamine concentration would result in a 15% lower growth rate than if very high glutamine levels were used. However, since the initial serum level would be 2.6% FCS, the cells would still grow at a reasonably rapid rate (≈ 0.04 h⁻¹), while at the same time, the formation of excess ammonium would be minimized because of the low glutamine levels present initially. Medium would flow into the bioreactor at 13 hours. Medium would be fed slowly to the culture so that the glutamine level is kept at a relatively low level while the serum is diluted and ammonium accumulates. When the liquid volume approaches 1300 liters, the flow rate would decrease to an extent that the glutamine level would again begin to decline. Again, the control strategy is striking a balance between keeping the glutamine level low to minimize ammonium accumulation, and operating as close the saturated portion of the Monod curve as possible.

Soon after, medium would flow rapidly into the vessel until the maximum volume is attained. This would accomplish two things. First, the ammonium level would be diluted. Second, since the last portion of a fed-batch culture would be operated batch-wise, sufficient nutrient must be added prior to batch operation to maintain growth and achieve high cell levels during the final batch phase.

Figure 37 presents the adjoint variables as a function of time for the optimal solution. It is clear that the boundary conditions are indeed satisfied. A non-optimal solution is presented in Figure 38. Note that the boundary conditions are not satisfied. The resultant antibody cost is consequently greater ($20.40). Figure 39 presents the corresponding non-optimal volume, glutamine and ammonium profiles.
that the boundary conditions are indeed satisfied.

Figure 37. The time profiles of the adjoint variables of the optimal control problem

\begin{equation}
\frac{d^2 \alpha}{dt^2} + \beta \frac{d \alpha}{dt} + \gamma \alpha = f(t)
\end{equation}

subject to the initial conditions

\begin{align*}
\alpha(0) &= a_0 \\
\frac{d \alpha}{dt}(0) &= a_1
\end{align*}

and the terminal conditions

\begin{align*}
\frac{d \alpha}{dt}(T) &= g(T) \\
\alpha(T) &= b_0
\end{align*}

where \( \alpha \) is the control variable, \( f(t) \) is the forcing function, and \( a_0, a_1, b_0, a_1, b_0, \) and \( g(T) \) are constants.
FOR $\alpha_2 = 0.02$, note that the boundary conditions are not satisfied. The initial value solution is shown for a sub-optimal adjoint variables are shown for a sub-optimal.
The resultant antibody cost is $20.40. The diagram represents the concentration profiles for the suboptimal solution.
IV.E.4.1 Sensitivity Analysis - Method:

The fed-batch culture simulated in Figure 35 and 36 represents a feed-forward control scheme. No information gathered during the production of the hybridoma cells will be fed-back to adjust the nutrient feed rate. Thus, it must be known how small changes in the metabolic kinetic parameters would affect the bioreactor performance given the control scheme presented in Figure 35. It would also be useful to determine how the bioreactor performance is affected if some of the model assumptions are relaxed (i.e., including lactate formation, serum degradation, etc.). To rigorously determine the sensitivity of the optimal control scheme, numerical simulations of fed-batch cultures controlled as presented in Figure 35 were initiated after varying the metabolic kinetic parameters and the model assumptions as determined from a $2^{12-6}$ factorial experimental design, presented in Table 31. The simulations were performed by solving equations (74) - (80) using an implicit fourth-order Runge-Kutta integration routine with a variable step size. The sensitivity of the objective function to each model parameter or assumption both singularly and collectively may then be calculated via the methods of Box, Hunter, and Hunter (1975).

The range of values used in the simulation for each parameter, as well as an explanation of the relaxation of the model assumptions, are given as follows:

$K_s$ (at 8500 cells/ml): The Monod constant for serum has been determined at low initial cell levels from five different sets of experiments. The values determined ranged from 0.75 - 1.0 % FCS for all experiments except one, where the value was calculated to be 1.6 %FCS. This one experiment used a different lot of serum. Nevertheless, the a range from 0.75 to 1.6 % FCS was used in the sensitivity analysis.

$\beta$: This parameter has been calculated from two different sets of experiments in which the growth rates of cultures at various initial cell and serum levels were determined. The value of $\beta$ ranged from 0.21 to 0.30. In the simulation for the sensitivity analysis, the given Monod constant at 8500
cells/ml and the given value of $\beta$ were used to calculate $(K_s)_0$ (recall that $K_s = (K_s)_0 X^{-\beta}$).

$K_A$: The inhibition constant for ammonium has been determined from three sets of experiments and ranged from 26 to 45 mM$^2$.

$\dot{Q}_A$: The specific antibody productivity at low lactate levels has been calculated from many cultures and ranges from 1.2 to 2.2 pg/cell-h. The variation between cultures grown at similar times is usually much less. Hybridoma cells are known to lose their antibody production capacity upon subculturing, and this may be responsible for the variation.

$\dot{Q}_G$: The specific rate of ammonium production has been determined from sixteen cultures grown at various serum and ammonium levels and ranges from 3.2 to $7.1 \times 10^{-11}$ mmol/cell-h.

$\dot{Q}_G$: The specific rate of glutamine utilization has been determined from sixteen cultures grown at various serum and ammonium levels and range from 5.4 to $9.1 \times 10^{-11}$ mmol/cell-h.

$\mu_{\text{max}}$: The maximum growth rate has been determined from five different sets of experiments and ranges from 0.053 - 0.061 h$^{-1}$.

$K_G$: The Monod constant for glutamine has been determined for one experiment and was 0.15 mM. The standard deviation from the non-linear regression was 0.02 mM, so the range is taken to be 0.13 - 0.17 mM.

response time: It has been demonstrated that the cells do not respond to external changes in the environment instantaneously. It requires 11 to 12
hours for the growth rate of the cells to begin to respond to an external change in the medium; i.e., a decrease in the serum level via dilution, or the addition of ammonium chloride from a concentrated stock solution. After this lag of 11 hours, the cells respond to the new environment via first order kinetics, with a time constant, $\tau_L$, of 0.01 hours. Thus, for the fed-batch simulations, any decrease in the serum or ammonium level via dilution, or any increase in the ammonium level via glutamine decomposition (since this may be considered an external addition of ammonium not produced by the cells) was not immediately reflected in the growth rate equation. This may be simulated by including the following equation (see Section IV.D.5):

$$\frac{d\mu}{dt} = \frac{\mu(t - \tau_L) - \mu(t)}{\tau}$$

(121)

In Table 31, a +1 value in column headed "lag" represents the assumption of instantaneous metabolic response, whereas a value of -1 means that the response time, as given by equation (121), was included in the simulation.

**serum degradation**: It has also been demonstrated that the growth activity of serum decreases with time via the following kinetics (see Section IV.D.4):

$$\frac{dS}{dt} = -0.016S^{0.7} \quad (% \text{ FCS})$$

(63)

In Table 31, a value of +1 in column headed "serum deg." represents no serum degradation, whereas a value of -1 indicates serum degradation was included.

**lactate production**: The production of lactate was included in the simulation by incorporating the following expressions (see sections IV.A.2 and IV.B.2):
\[
\frac{dL}{dt} = q_L X - \frac{LF}{V} \tag{122}
\]

\[
q_L = \frac{64 + 0.25L}{37 + L} \tag{48}
\]

\[
\mu = \frac{\mu_{\text{max}} SG}{\left[ S + (K_{e0} X^\beta) \right] \left[ G + K_G \right] \left[ 1 + \frac{A^2}{K_A} \right] \left[ 1 + \frac{L^2}{K_L} \right]} \tag{47}
\]

A -1 in the column headed "lactate" of Table 31 means that lactate production was included in the simulation.

**viability:** The relation for the culture viability given in expression (105) in Section IV.E.1 is an approximation of the relation presented in Figure 9 and equation (49) in Section IV.B.2. A -1 in the column headed "α" of Table 31 indicates expression (49) was used in the simulation, whereas, a +1 indicates expression (105) was used.

### IV.E.4.2 Sensitivity Analysis - Results:

The values of the objective function for each of the 64 simulations are shown in the final column of Table 31. The values of the objective function ranged from 10 $/g to nearly 100 $/g, with the average value of the objective function being 36 $/g. The final antibody titers ranged from 60 mg/l to 700 mg/l. The linear sensitivity parameters (i.e., the difference in the objective function between the high and low level of a given parameter) calculated via the method of Box, Hunter, and Hunter (1978) are given below:

\[
\begin{align*}
ks: & \quad a_1 = 7.2 \\
\beta: & \quad a_2 = -3.8 \\
k_a: & \quad a_3 = 1.0
\end{align*}
\]
qp: \quad a_4 = -22.0 \\
qa: \quad a_5 = -7.6 \\
viability: \quad a_6 = 0.4 \\
qg: \quad a_7 = 6.0 \\
\mu_{max}: \quad a_8 = -1.4 \\
lag: \quad a_9 = -2.2 \\
serum \\
degradation: \quad a_{10} = -11.0 \\
kg: \quad a_{11} = 2.0 \\
lactate: \quad a_{12} = -11.2 \\

Thus, the product cost is most sensitive to the specific antibody productivity. The inclusion of serum degradation and lactate production also significantly increased the product cost. The simulation is also moderately sensitive to changes in the Monod constant for serum, and the cellular ammonium productivity and glutamine utilization rates.

IV.E.5 Experimental Results From a Fed-Batch Bioreactor with Nutrient Control Determined via Optimal Control Theory

An experimental fed-batch culture of CRL-1606 hybridoma cells with a volume profile represented in Figure 35 was implemented to determine the validity of the mathematical model presented in equations (74) to (80). The experimental methods were given in detail in Section III.E. Briefly, exponentially growing cells were introduced at 2.0 \times 10^5 \text{ cells/ml into 932 mls of DMEM medium at initial serum, glutamine, and glucose levels of 2.6 \% FCS, 1.3 mM, and 12.5 mM, respectively. Medium without serum and with 33.6 mM glutamine and 165 mM glucose was added to give the volume profile determined optimal from control theory (Figure 35). The amino acid levels (excluding glutamine and cystine) in the initial medium and the feed medium was four times that of standard DMEM. The cystine level was twice the standard concentration.
Table 31. A factorial statistical design for determining the sensitivity of the simulation with respect to the system parameters and assumptions is presented. The units for each variable is given in the text. Note that columns 6 and 8 are multiplied by $10^{12}$, while column 5 is multiplied by $10^{12}$.

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Table 31. (Continued) A factorial statistical design for determining the sensitivity of the simulation with respect to the system parameters and assumptions is presented. The units for each variable is given in the text. Note that columns 4 and 5 are multiplied by 10^11.

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The dissolved oxygen concentration was maintained between 20 and 70 % of air saturation at all times. The pH was controlled via CO2 addition. The growth rate of the seed culture prior to inoculation was 0.03 h\(^{-1}\). Samples were taken every 7-8 hours and assayed for cells, viability, antibody, ammonium, glutamine and lactate.

Figure 40 compares the experimental growth rate profile of the experimental fed-batch culture with the growth rate predicted from the results of the solution to the optimal control problem presented in Section IV.E.1 and IV.E.3. Recall that in solving the control problem, the model for the culture kinetics did not include metabolic response time (equation 121), serum degradation (equation 63) or lactate accumulation (equations 47, 48, 122). Each growth rate in the figure was calculated from three
Simulation
not the degradation of the growth-promoting activity of serum was included in the
the solution of the optimal control problem. Neither the metabolic response time
as indicated in Figure 35 is compared with the growth rate profile predicted by
Figure 40. The experimental growth rate profile from a fed-batch culture controlled

TIME (hr)
contiguous total cell measurements. Figure 40 shows that the predicted growth rate suggests the qualitative behavior of the actual growth rate profile very well. The actual growth rates calculated between 50 and 110 hours agree very well with the expected values. However, the simulation over-predicts the growth rate at both the beginning and the end of the culture. This over-prediction accounts for discrepancy between the predicted maximum total cell level (6.1 x 10^6 cells/ml) and the actual maximum total cell level obtained (3.1 x 10^6 cells/ml).

As mentioned previously, the cells do not respond instantaneously to changes in their environment (Section IV.D.5). Also, recall that the growth rate of the stock culture prior to inoculation of the fed-batch bioreactor was 0.03 h^{-1}. Thus, if this initial growth rate is incorporated into a fed-batch simulation including metabolic response kinetics, as represented in equation (121), the growth rate profile shown in Figure 41 is obtained. It must be emphasized that no parameters were adjusted to obtain the profile. The parametric values used were determined from experiments presented in section IV.D.5. The volume profiled assumed is identical to that in Figure 35 and to that used in the experiment.

Although there is an improved agreement between the predicted and experimental profiles during the beginning of the culture, a considerable discrepancy still exists at times greater than 130 hours. The resultant predicted viable and total cell levels are compared with the actual viable and total cell concentrations over time in Figure 42. The maximum predicted viable cell level of 3.0 x 10^6 cells/ml is slightly higher than the maximum viable cell concentration of 2.6 x 10^6 cells/ml actually obtained. The predicted total cell level was still much higher than the actual total cell level (5.5 vs. 3.1 x 10^6 cells/ml). This is because the area under the predicted growth rate profile curve is greater than the area under the actual growth rate profile curve.

Could the low growth rates at times greater than 130 hours be due to the degradation of serum activity demonstrated in Section IV.D.4 and represented by equation 63? The growth rate profile resulting from such a simulation is presented in Figure 43. Again, no parameters were adjusted. There is very good agreement.
Included in the simulation, no adjustable parameters were added to the simulation.

Figure 41. The experimental growth rate profile is compared with the growth rate predicted from the simulation of the optimal control problem, including metabolic response time. Degradation of the serum activity with time was not accounted for.
The simulation response time. The solid lines represent the total and viable cell levels predicted by predicted from the simulation of the optimal control problem, including metabolic process. The experimental total and viable cell levels are compared with that experimental total and viable cell levels are compared with that.
No adjustable parameters were added to the simulation.

Metabolic response time and degradation of the growth-promoting activity of serum.

Profile predicted from the simulation of the optimal control problem including both

Figure 43. The experimental growth rate profile is compared with the growth rate

TIME(HI)

GROWTH RATE(1/HI)

predicted

actual
between the predicted and actual growth rates at all times. The viable and total cell concentration are compared in Figure 44. The simulation under-predicts the viable cell concentration slightly at the end of the culture. This is due to the under-prediction of the culture viability by equation (105). Thus, it appears that the inclusion of metabolic response and serum degradation in the simulation represents the actual data reasonably well. This is further verified by comparing the actual ammonium, glutamine, and antibody profiles with that predicted from a simulation including serum degradation and metabolic lag (Figures 45, 46 and 47). The simulation slightly under-predicts the final ammonium level and over-predicts the final glutamine level. Again, this is due to the under-prediction of the culture viability. The simulation slightly over-predicts the antibody titer because the assumed specific antibody productivity (1.7 pg/cell-hr) is slightly higher than the actual value (Figure 48). The accumulation of lactic acid does not appear to significantly inhibit the antibody productivity.
and viable cell levels predicted by the simulation. The solid lines represent the total metabolic response time and serum degradation. The predicted from the simulation of the optimal control problem, including both the predicted with that experimental total and viable cell levels are compared with that.

Figure 44.
and the degradation of serum activity.

The simulation of the optimal control problem including both metabolic response time

Figure 45. The experimental ammonium profile is compared with that predicted from

TIME (hr)

AMMONIUM (mM)

Actual
Predicted
and the degradation of serum activity.

The simulation of the optimal control problem including both metabolic response time

Figure 46. The experimental glutamine profile is compared with that predicted from

actual glutamine (GLN)
predicted glutamine (GLN)
and the degradation of serum activity.

the stimulation of the optimal control problem including both metabolic response time

Figure 47. The experimental antibody profile is compared with the predicted from

TIME (hr)

0 40 80 120 160 200 240

ANTIBODY (mg/l)

Actual
Predicted
Figure 48. The experimental specific antibody productivity is compared to the model and actual lactate profiles. The value assumed for all of the stimulations also presented.
V. DISCUSSION AND CONCLUSIONS

V.A. SIGNIFICANCE

The dramatic increase in the commercial potential of products derived from mammalian cells will require more cost-efficient cultivation processes be developed at increasingly larger scales. The rate-limiting step in the realization of this goal is the lack of systematic and rigorous methodologies for obtaining quantitative descriptions of mammalian cell culture kinetics. Once such descriptions are obtained, design and control of the production process may be implemented as with any other commercial endeavor. This thesis represents the culmination of an experimental and theoretical research plan designed to develop such methodologies.

This work has shown that the culture kinetics of hybridoma cells could be both qualitatively and quantitatively predicted from mathematical expressions derived from initial rate data taken at low cell levels. These expressions were used to determine the optimal control strategy for a fed-batch bioreactor. Using this strategy, twice the level of viable cells ($2.6 \times 10^6$ cells/ml) and monoclonal antibody (210 mg/l) was obtained compared to a typical batch culture ($1.2 - 1.5 \times 10^6$ cells/ml, and 100 mgs/ml, respectively).

However, the significance of this research lies not in the fact that the culture kinetics of CRL - 1606 hybridomas cells could be accurately predicted from empirically derived mathematical relations. Nor does it lie in the fact that these relations were successfully used to develop an optimal control strategy which increased antibody productivity relative to serum, inoculum and time costs. The primary utility of this thesis is its value as both a guide and a reference for others that desire to develop more efficient production processes for their given cell line and production system. To this end, a summary of the five major issues that were found to be most significant for developing both the mathematical relations of the culture kinetics, as well as the optimal control policy, are outlined as follows

1. **Variable Reduction via Statistical Design:** Cell culture medium
contains approximately 30 different chemical species in addition to the waste products that the cells may excrete into the medium. The concentration of these components constitute variables, which either singularly (linear) or on concert with other variables (co-linear), may affect the culture kinetics. This variable set is clearly much too large to attempt to mathematically relate the metabolic rate parameters to each variable. This work demonstrated that well designed factorial experiments can reduce the variable set to more manageable dimensions. The resulting reduced variable set may then be examined fully in developing new mathematical descriptions. Variables may be lumped together to reduce the factorial designs. For example, amino acid, vitamin, and mineral salt levels relative to the normal levels found in DMEM were examined together (b-DMEM) and were found to affect the growth rate only at levels below 60% of that of standard DMEM. Also, these experiments were initiated at low cell levels. This detail was important to the experimental design, since it assured that any metabolic rates measured were directly attributable to the initial conditions. In this way, the rates intrinsic to the initial environmental conditions were "de-coupled" from any and all cellular influences.

2.) Mathematical Description of the Initial Metabolic Rates as a Function of the Reduced Variable Set: Experimental designs for the development of mathematical descriptions of metabolic rates would be much more efficient if the approximate form of the relation was known a priori. At the initiation of this research, the availability of quantitative relationships in the literature to serve as guides in this work was nonexistent. With the conclusion of this thesis, such a guide is now available (equation (47)). As a general starting point in developing mathematical descriptions of new systems, the following may be
assumed *a priori*: i) nutrients such as glutamine and glucose may exhibit Monod-type kinetics with respect to the growth rate. The Monod constants are probably low(< 0.2 mM); ii) inhibitors of growth, such as ammonium and lactate, may exhibit non-competitive type inhibition, except that the apparent inhibition constant may be inversely proportional to the concentration of the inhibitor; iii) the growth rate may also demonstrate Monod-type kinetics with respect to the concentration of serum or specific growth factors. The Monod constant in this case may be a decreasing function of the cell concentration; iv) each relation in i) - iii) is independent of the other; that is, the form for the growth rate may be developed by simply multiplying relations i) - iii) together; v) the uptake of nutrients may be dominated by maintenance energy requirements, and may not be a function of the growth rate; and vi) lactate may inhibit excretion of molecules (i.e., antibody and ammonium) non-linearly.

3.) **Coupling the Initial State to Other Culture States - Cell Level:** Simply put, this work would have ended in failure if the mathematical descriptions developed from initial rate data at low cell levels could not be applied to other culture states, especially states at higher cell levels. One mechanism which coupled the initial state to states at higher cell levels was equation (46), this cell level-dependent Monod constant for serum in the growth rate equation. In developing mathematical descriptions of new cell systems, the possible cell level-dependency of the metabolic kinetics should be kept in mind. Note that there may be many ways to achieve such a state-to-state coupling. From the data presented herein, it is likely that equation (46) is a lumped representation of the hybridoma's ability to reduce oxidized, and hence inactive, serum thiols (see section V.D.). Obviously, this lumped representation would be unnecessary if the
details of the thiol chemistry in cell culture medium were known.

4) **Coupling the Initial State to Other Culture States - Serum Degradation:** The discovery that the growth-promoting activity of serum decays with time is not reflected in the initial rate data. The simple kinetic relation describing serum inactivation presented in equation (63) provides the state coupling. Again, this equation is a lumped representation of more complex kinetics, and may be replaced by a more detailed description, if one is known. The possibility that growth factors may become inactivated with time should always be considered, especially in serum-free medium culture.

5.) **Simplification of Process Description for Optimal Control Solution:** In general, determining the optimal control policy for fed-batch mammalian cell bioreactors is more difficult than it is for microbial systems. There are many reasons for this. First, the dimensionality of realistic mathematical models of mammalian cell bioreactors is higher than it is for microbial ones. Additionally, with microbial systems, metabolic rates, such as growth rates, are usually only functions of, at most, two state variables, whereas it has been shown with this work that the growth rate of hybridoma cells was dependent on the values of five state variables. Thus, it may often be prudent to introduce assumptions into the model that simplify the system, even if it is at the expense of realism. Recall that the degradation of serum activity was ignored, since this would have made the growth rate an explicit function of time, which would result in a problem that was orders of magnitude more difficult. A sensitivity analysis should be performed, however, after the optimal control problem is solved to determine how greatly the assumptions may affect the solution. It may then be
determined which assumptions should be relaxed in order to obtain an more accurate solution of the optimal control problem.

V.B. Optimal Control of Mammalian Cell Bioreactors

The productivity of the fed-batch bioreactor described in section V.E.5 was clearly limited by either ammonium accumulation and/or serum degradation. Nutrient limitation could not have been a factor since the addition of 600 mls of DMEM medium with elevated glucose, glutamine and amino acid levels stabilized, but did not increase, the growth rate from 0.010 h⁻¹, a value the growth rate had fallen to from an initial rate of 0.04 h⁻¹ (Figure 43). It is certainly possible, however unlikely, that the accumulation of an unidentified cellular waste product could be responsible for this decline. Ammonium, however, is the logical and likely culprit since it is quite clear from this work that ammonium does indeed greatly inhibit growth (Figure 4).

If ammonium accumulation was the only reason for decline in the growth rate, the growth rate of the culture should have been much higher than it was during the final batch period of growth (Figure 40). This is especially true at the point when 600 mls of medium was introduced. At that time, the ammonium level was immediately diluted from 8 to 6 mM. The growth rate should have increased significantly. This did not happen, although that rapid decrease in the growth rate that was occurring prior to this point was halted, and the growth rate remained approximately constant for 40 hours. Since serum was not added to the culture at any time after inoculation, it may be concluded that some component in serum was either utilized or degraded as the culture progressed, and became rate-limiting during the terminus of the process. That the inclusion of the serum degradation kinetics (equation (63)) in the process simulation of the fed-batch culture resulted in a growth rate profile that nearly parallels the experimental profile, especially at the terminus, is a strong indication that serum degradation also limits growth (Figure 43). Recall that serum degradation was also able to account for the rapid decline in the growth rate in low serum batch cultures (Figures 14 and 15).

Given the results presented in this thesis, what general comments may be made
concerning optimal mammalian bioreactor performance? It is clear that the key to
optimizing productivity is to minimize ammonium accumulation and serum
degradation. Controlling the glutamine level at a constant low value would reduce
ammonium accumulation, but at the expense of adding complexity to the system, since
a method to either measure or estimate glutamine on-line would be required. This
would be in contrast to the fed - batch culture performed in this research, which was
very easy to operate. The liquid volume profile was determined beforehand so that the
rate of the medium feed pump was simply adjusted periodically to provide the desired
amount.

Serum could also be added during the later portion of the fed - batch process as
a replacement for the serum activity lost due to degradation. The increased cell and
antibody levels would have to be weighed against the cost of providing additional
serum. Determining the optimal control policy for serum addition would be extremely
difficult, however. Note that the benefits of adding additional serum decreases as the
ammonium level increases. This is because, at any point, no matter how much
additional serum is added, the maximum growth rate that could be achieved at a given
ammonium level is the following:

\[
\mu = \frac{\mu_{\text{max}}}{1 + \frac{A^2}{K_A}}
\]

A perfusion system would address many of the limitations inherent in batch
cultures. By adjusting the dilution rate, the ammonium level can be maintained at an
arbitrarily low level. In addition, fresh serum is continuously provided. However, the
total serum usage could be prohibitively high. The optimal control policy could be
determined for this system to determine the most appropriate dilution rate that
balances productivity against serum usage. For example, Ohno et al. (1978) used
Kelley's Transformation method to determine the optimal dilution rate in a continuous
lysine fermenter.
A repeated fed-batch strategy could potentially be a very productive, and very simple process. To illustrate, suppose the fed-batch culture represented in Figure 42 was stopped at 130 hours, when the total number of viable cells in the bioreactor was the highest (3.0 x 10^9 cells). At this point the ammonium level was 6.5 mM and the antibody level was 140 mg/l. Now suppose medium was removed until 186 mls were left, and that medium was then added to restore the liquid volume to the original initial liquid volume (932 mls). The cell, ammonium and antibody level would then be 3.0 x 10^8 cells/l, 1.3 mM, and 28 mg/l, respectively. Compare this to the 20 hour point in the original fed-batch culture, where the cell, ammonium and antibody level was, 3.0 x 10^8 cells/l, 1.0 mM, and 8 mg/l, respectively. Thus, the two points are essentially equivalent, except in the former there is 20 mg/ml more antibody. Also, what took 20 hours to achieve in the latter case, was achieved instantly in the former. Thus, if serum is added to a level of 2.6 % FCS, it would be expected that the new fed-batch system would reach an antibody level of 160 mg/l in 110 hours. The procedure could then be repeated. Each time, a little more antibody could be "squeezed-out" of the system in a little less time. Obviously, the incremental improvement would decrease each time.

The preceding illustration presented but one of many possible operating scenarios that could be implemented in a repeated fed-batch process. There is considerable choice in selecting the following:

1) When should a given fed-batch phase be stopped?
2) How much should the medium be diluted when starting a new fed-batch phase?
3) What should the initial volume be for each fed-batch phase?
4) What should be the new serum level?
5) What should be the optimal feeding policy for each fed-batch phase?

Weigand (1981) addressed some of these points in applying optimal control theory to repeated fed-batch microbial fermenters.

The solution of the optimal control problem is obviously dependent on the
optimality criterion. In the present case, the antibody cost, based on time, serum, and inocula costs, was minimized. The serum and inocula costs did not significantly enter into the control solution since these costs are fixed once the initial serum, cell and volume levels are selected. The initial serum, glutamine, cell, and volume levels were selected as 2.6% FCS, 1.3 mM, 2.0 x 10^5 cells/ml and 932 mls by using a Simplex search procedure similar to that used in the batch optimization (section IV.E.4.2, Beveridge and Schecter, 1970). Several different functions relating flow rate of medium to the fed-batch bioreactor were tried in the simulations, and the best one was selected. The selected flow rate profile was very close to the one determined from the solution to the optimal control problem (Figure 35). The initial flow rate into the vessel was initially very slow, but increased very rapidly until the maximum allowable volume was reached at 93 hours. The maximum volume was reached at 113 hours for the optimal control solution.

If inocula and serum costs are not included in the objective function, the Simplex search routine would select the highest possible values for the initial cell and serum levels, since this would maximize volumetric productivity. This would not represent a realistic solution, however. Also, since the performance of the simulated fed-batch bioreactor would be greatly exaggerated, the objective function would be relatively insensitive to the control profile of the medium flow rate.

If purification costs are included into the optimality criterion, the optimal control profile would be drastically different. The importance of achieving very low final serum levels and very high final product concentrations becomes even more important in this case. The initial volume would likely be low so that high serum and cell levels could be used initially, even though the total amount of serum used could be much less, and the final serum level could be quite low. The solution of the optimal control problem would likely keep the glutamine level much lower than was the case in Figure 35. The lower growth rates that might occur due to the low glutamine levels initially would be outweighed by the lower ammonium levels that would accumulate near the end if the process when the cell levels and the volumetric productivity should be the greatest. Indeed, sub-optimal control schemes were found while solving the optimal control
problem in section IV.E.5 which predicted higher final antibody titers than the optimal control solution (simulations not presented). These higher antibody levels were obtained at a cost, however, since these titers were not achieved until 225 - 250 hours, which is 40 - 60 hours longer than the terminal time for the optimal solution given. These sub-optimal solutions would be nearly optimal, also, if the optimality criterion was simply to maximize the total product produced.

Inhibition of the antibody productivity by lactate may also affect the solution of the optimal control problem. The results of the sensitivity analysis (section IV.E.4.2) supports this, since the inclusion of lactate inhibition for the simulation of the optimally controlled system was shown to greatly increase the product cost. The problem is, however, that very little independent control may be exerted over lactate accumulation. Any control scheme that reduced lactate accumulation would also reduce antibody accumulation. This is seen from the following relations:

\[
\frac{d(LV)}{dt} = q_L X V \tag{122}
\]

\[
\frac{d(PV)}{dt} = q_P X V \tag{78}
\]

which implies:

\[
d(PV) = \begin{bmatrix} q_P \\ q_L \end{bmatrix} d(LV) \tag{124}
\]

If \( q_p \) is not related to the lactate concentration, and the initial lactate and antibody levels are both zero, then:

\[
P = \begin{bmatrix} q_P \\ q_L \end{bmatrix} L \tag{125}
\]

Thus, it can be seen that minimizing lactate accumulation in this case implies minimization of antibody accumulation. This characteristic is offset somewhat if \( q_p \) is
a strong function of the lactate level, but the general idea is still the same. The best solution would be to reduce lactate accumulation by other means, such as substituting fructose for glucose in DMEM medium. Fructose has been shown to reduce lactate accumulation by 90% compared to glucose from CRL-1606 hybridoma cultures (E. Adema, personal communication).

The preceding discussion was a brief attempt to summarize some general aspects regarding optimal mammalian cell bioreactor design and control based on the results from this thesis. However, no matter what production system is actually used, be it fed-batch, repeated fed-batch, perfusion, hollow-fiber, encapsulated, etc., the most important consequence of this work is the following:

The quantitative description of mammalian culture kinetics determined via the methodology developed and experience acquired in this thesis provides, for the first time, the tools necessary to rigorously design and control mammalian cell bioreactors in an optimal fashion.

V.C. Initial Metabolic Rates

A mathematical expression has been developed relating concentrations of serum, glutamine, ammonium, lactate, and cells to the growth rate (equation (47)). The equation represents multiple substrate limitations (glutamine and serum) via coupled Monod expressions, superimposed with non-competitive inhibition by ammonium and lactate. At this point it would be instructive to compare the mathematical descriptions of the metabolic rates developed in this thesis with that in the literature. Unfortunately, there exists a paucity of similar data which with to compare. For the most part, only qualitative observations coupling metabolic rates with environmental variables are given. For example, it has long been known that ammonium accumulation may inhibit growth (Ryan and Cardin, 1963). However, no quantitative descriptions relating the extent of inhibition to the ammonium level has been found.

Recall that the growth rate could be related to the serum level via a Monod-type equation. The growth rate of the hybridoma cell line HL-60 demonstrated a similar
relationship with respect to the serum level (Breitman and Keeen 1982). That such a simple relationship may be found was somewhat surprising. Serum contains many growth factors that could stimulate growth. In general, each growth factor would exist in serum at different concentrations, and each would exhibit different Monod constants. Thus, it would be expected that the graph of the growth rate versus the serum level would resemble a combination of Monod curves, and that the predominant rate-limiting growth factor would change as the serum level is changed. That this indeed may occur is supported by the decrease in the Monod constant that occurs when either cysteine, (Figure 21), or cells (Figure 14) are added to cultures grown in low serum medium and at low initial cell levels. That is, cysteine (or other thiols) was only rate-limiting at low serum and cell levels, and thus, was only able to increase the growth rate of cultures at these conditions. Other growth factors must be rate-limiting at higher serum levels.

Other researchers have observed cell level dependent Monod constants for growth on various substrates. Contois (1959) first observed this phenomenon with *Aerobacter aerogenes* growing on glucose. Pareilleux and Chaubet (1980) used the Contois equation to describe the growth of apple fruit cells on sucrose. However, in both of these cases the exponent of the cell level in the equation for the Monod constant was 1, not -0.21, as was found to be the case with CRL - 1606 hybridomas. A physiological explanation for this is given in detail in section V.D.

**V.D. Degradation of Serum Activity, Redox Potential, and Thiol Chemistry**

One of the significant features of this work is the identification and characterization of the importance of thiol/disulfide chemistry, redox potential, and their subsequent effect on the stability of the growth - promoting activity of serum - supplemented media. Although a precise mechanistic picture of the interactions at this point can not be conclusively drawn, the following statements may be made with certainty:
- Thiols are rate-limiting for cultures growing in low serum medium at low initial cells levels;
- The growth-promoting activity of serum decays with time, especially at low serum levels;
- The addition of reducing agents, i.e., thiol compounds, slow this rate of deactivation;
- The stimulatory effect of high initial cell concentrations on the growth rate may be replaced, at least in part, by the addition of thiols.

Based on these statements, the growth rate decay observed in low serum cultures, and the resultant stabilization of the growth rate upon addition of thioglycol, may be explained by the following working hypothesis:

Thiol compounds in serum which are rate-limiting in low serum cultures are spontaneously oxidized and subsequently inactivated with time. This is responsible for the observed decay in the growth rate of these cultures. The addition of any component that provides a more reduced environment, such as thiols, or the elimination from the medium of any compound whose inclusion would increase the oxidation state, such as cystine (see Figure 24), stabilize the growth-promoting activity of the medium. The hybridoma cells, themselves, may act to reduce the surrounding medium, thereby explaining the observed stimulation of the growth rate with increasing initial cell levels.

The question now becomes, what are the serum components that are oxidized, and what is the biochemistry involved? One problem with the hypothesis given above is that thiols are oxidized very rapidly within hours (Torchinsky, 1981). Figure 28 demonstrates that thiols are indeed extremely reactive. However, the rate of deactivation of the growth-promoting activity of serum exhibits much slower kinetics.
REDUCTION OF OXIDIZED DITHIOLS BY THIOLS

R—R' \[ \xrightarrow{SLOW} \] \[ \xrightarrow{O_2} \] S—S

+ R—SH \[ \xrightarrow{FAST} \] S—R

OXIDATION OF DITHIOLS (DTT) BY DISULFIDES

RS—RS + HS—CH(CHOH)_2CH—SH_2

RSH \[ \xrightarrow{FAST} \] S—R

\[ \xrightarrow{O_2} \] S—S

H_2C \[ \xrightarrow{SLOW} \] HOHC

Figure 49. Hypothesized role of dithiols in mammalian cell culture medium
Figure 50. The oxidation of the biological thiol, lipoic acid.

(Figure 24). How can this discrepancy be reconciled?

A possible molecular explanation is given in Figure 49. Suppose the rate limiting component is a special class of thiols that contain two sulfhydryl groups. An example of such a dithiol is lipoic acid (Figure 50). Lipoic acid is widely distributed in animal tissues (Torchinsky, 1981), and is a prosthetic group of enzymes involved in oxidative decarboxylation (Reed, 1960; Schmidt et al., 1965). The best known enzyme of this type is pyruvate carboxylase. The reduction potential of dithiols is, in general, greater than thiols. For example, the reduction potential of lipoic acid is -325 mV, and is greater than that of cysteine (-220 mV), or glutathione (-240 mV), and is of a par with
in general, much slower than that of thiols, and is more indicative of the serum inactivation kinetics presented in Figure 24.

Another problem with the working hypothesis given above, was that it was unclear how unstable compounds like thiols could stimulate the growth rate of cultures for such long periods of time. Once gain, the inclusion of dithiols into the hypothesis provides an explanation. Again, assume a dithiol, such as lipoic acid, is rate-limiting in low serum medium. Some of the dithiol will be in the reduced form, while some will be in the oxidized form. The addition of a thiol, such as cysteine, would perform two functions. First, the added thiols would lower the oxidation-reduction potential of the medium, reducing the favorability of dithiol oxidation. Second, and more importantly, the addition of thiol would catalyze the cleavage of all disulfide compounds, including that of the oxidation product of the rate-limiting dithiol (Figure 49). Once the oxidized disulfide is reduced, it would be slow to re-oxidize. In essence, the addition of thiols initiates an exchange mechanism: the reducing power is exchanged from unstable thiols to more stable dithiols.

Assuming that dithiols are rate-limiting in low serum medium provides an explanation as to why the elimination of cystine from the medium stabilizes the growth-promoting activity of serum. Aside from increasing the oxidation-reduction potential of the medium, the addition of disulfides, such as cystine, also catalyze the oxidation of dithiols (Figure 49, bottom). If a thiol-disulfide exchange occurs between one of the sulphydryl groups on a dithiol and a disulfide bond of cystine, a new compound is formed that can easily be oxidized to form a ring structure. This is the basis of DTT’s strong reducing action (Figure 49). The formation of two free thiol compounds drives the reaction is far to the right (Torchinskiy, 1981). Lipoic acid catalyzes the cleavage of disulfide bonds as a cofactor for oxidative enzymes in this manner.

In summary, although it can not be conclusively stated, there exists extremely strong evidence to suggest that the rate-limiting components in low serum cultures are dithiol compounds, and that the degradation of the growth-promoting activity of serum observed is due to oxidation of these dithiols. The data also suggests that hybridoma cells may exert some reducing action on their environment. Ohmari et. al.
(1983) have put forth a similar suggestion. Indeed, it is known that glutathione reductase activity is present in the cytoplasm of lymphocytes (Mannervik, et. al., 1975). This activity would also have to be at the membrane, however, to be able to reduce the environment. Bannai et. al. (1980) has also shown that human fibroblasts excrete sulfydryl compounds into the medium, which also would have the effect of reducing the culture environment. This may partially explain why the Monod constant for serum was a decreasing function of the cell level (equation (46)).

V.E. Waste Product Inhibition - Possible Molecular Mechanisms

It was found that ammonium inhibited growth in a very non-typical fashion. Ammonium inhibited growth non-competitively, but the apparent inhibition constant was found to be inversely proportional to the ammonium concentration. What could be a possible explanation for this phenomenon? This question may be answered by first considering why ammonium is toxic, which, to date, is not known. As discussed in section II.B.2, it was hypothesized that ammonia's toxicity may be the result of the destruction of electrochemical gradients that may occur due to ammonia's basic nature. Considerable evidence exists to support this hypothesis. Implicit in this hypothesis is the premise that un-ionize ammonia is the actual inhibiting moiety, and not the ionized ammonium. Gaseous ammonia can diffuse rapidly through the cytoplasmic membrane, whereas the ionized form can not (Benjamin et. al., 1978). Once the un-ionized molecule diffuses across the membrane, it may pick-up a hydrogen ion to form the ionized ammonium. In this way, the pH of the cytosol may be increased.

The same scenario could occur across the vesicle membranes. For example, transferrin - iron complexes are shuttled from the cytoplasmic membrane to acidic vesicles (Dautry-Varsat and Lodish, 1984). Once inside the vesicles, the low pH stimulates the release of iron from the transferrin. Any shift upward in the pH of this vesicle could inhibit release of the iron. Indeed, it has been shown that the addition of ammonium does inhibit iron release inside the vesicle (Karin and Mintz, 1980). Thus, ammonium toxicity is the result of complex multi-step mechanisms involving acid -
ammonium does inhibit iron release inside the vesicle (Karin and Mintz, 1980). Thus, ammonium toxicity is the result of complex multi-step mechanisms involving acid-base equilibrium, diffusion, and iron-transferrin binding characteristics. It should not be unexpected, therefore, that the affect of ammonium on the growth rate would be atypical and highly non-linear.

Lactate was found to inhibit both antibody and ammonium productivity of CRL-1606 cells. Lactate is a very potent chelator of calcium (Gosh and Nair, 1970). Calcium levels have been found to be intimately involved in the excretion of proteins (Orci, et. al., 1972). Adema has suggested that via chelation of calcium, lactate may act to decrease antibody productivity by lowering the intracellular calcium levels and has also suggested that increased calcium levels in the culture medium could mitigate the adverse affects of lactate (personal communication). Also, it has been shown that in the rat brain, calcium stimulates glutaminase activity (Benjamin, 1981). Again, lowered calcium levels caused by lactate binding could result in reduced glutaminase activity, and hence, reduced ammonium productivity.

V.F. Modeling Transients With Steady State Relations

Mathematical descriptions of initial rate data coupled with transient growth rate kinetics (equation (121)) and serum instability (equation (63)) gave the best prediction of the growth response in a fed-batch culture for over 180 hours and five population doublings. However, the initial rate or steady state relations describe the actual growth rate profile reasonably well also, especially after the end of the lag phase (Figure 40). This is intriguing, since steady state relations have been shown not to be able to describe transient kinetics in microbial cultures (Mateles et. al., 1965; Cooney and Wang, 1978; Maaloe and Kjeldgaard, 1966). Why do steady state relations provide reasonable predictions of the transient hybridoma growth response in a fed-batch culture? The answer may be found by examining the time constants in the system. This is done by scaling time with respect to the transportation lag constant from Table 29, and by scaling serum, cell, glutamine and ammonium levels by their initial values in equations (74), (75), and (77) to form:
\[
\frac{dX^*}{dt^*} = (\mu \tau_L) X^* - \left[ \frac{\tau_L F}{V} \right] X^* \quad (126)
\]

\[
\frac{dS^*}{dt^*} = \left[ \frac{-k_d \tau_L}{S_0^{0.3}} \right] S^{*0.7} - \left[ \frac{\tau_L F}{V} \right] S^* \quad (127)
\]

\[
\frac{dA^*}{dt^*} = (k \tau_L) G^* + \left[ \frac{\tau_L q_A X_0}{G_0} \right] X^* - \left[ \frac{\tau_L F}{V} \right] A^* \quad (128)
\]

where
t^* = t/\tau_L
S^* = S/S_0
X^* = X/X_0
A^* = A/G_0
G^* = G/G_0

By examining the time constants in these differential equations, insight may be gained as to the relative rate of change of each of the state variables. The value of the time constants for the fed-batch experiment presented in Figure 40 are as follows:

\[(\mu \tau_L) = 0.71 - 0.26 \quad (\text{for} \ \mu = 0.055 \Rightarrow 0.020 \text{ h}^{-1})\]

\[
\frac{-k_d \tau_L}{S_0^{0.3}} = 0.08 \quad (\text{for} \ S_0 = 2.6 \% \ FCS, \ \text{the initial serum level})
\]

\[(k \tau_L) = 0.06\]

\[
\frac{\tau_L q_A X_0}{G_0} = 0.11 \quad (\text{for} \ X_0 = 2 \times 10^8 \text{ cells/l}, \ G_0 = 1.3 \text{ mM})
\]
\[ \frac{\tau_L}{\nu} = 0 - 0.07 \quad \text{at times prior to 113 hours} \]

Thus, it appears that rate of change of both the serum and ammonium levels are much slower than the rate of growth of the cells. Since the growth rate equation presented in equation (47) is dominated by the serum and ammonium terms, this relatively slow rate of change of these state variables may indicate the effect of the environment on the growth rate of the cells may indeed be close to steady state.

Figures 31 - 33 indicate that it takes the cells approximately 12 - 17 hours before they start to respond to either a shift-up or shift-down in the ammonium and serum levels, but that once they do start to shift, they shift instantaneously (Table 29). With microbial systems, the growth rate of the cells may respond instantaneously to shifts in nutrient levels, although one to two doubling times were required to reach a new steady state (Mateles et. al., 1965). Again, what are the reasons for this discrepancy? Maaloe and Kjeldgaard (1966) observed that in E. Coli, the RNA and protein synthesis rates responded almost instantaneously to a shift-up in the growth rate, while it took about 60 - 70 minutes (or about a doubling time) for the DNA synthesis rate to increase. Recall that all of the growth rates measured in this thesis were determined on a per unit cell basis, and not on a cell mass basis. A possible explanation for the data of Figures 31 - 33, is that while the RNA and protein production rates in the hybridoma cells may respond quickly to shifts, it may take 12 - 14 hours for the DNA synthesis rates to shift, so that changes in the growth rate determined by measuring cell number would not be seen immediately. However, at the point at which the DNA synthesis rate begins to respond, the RNA and protein synthesis machinery is already in full gear, and may aid in accelerating the shift - up in DNA synthesis, thereby accounting for the quick response following the initial lag.

This hypothesis illustrates a potentially important point: The time-honored practice of monitoring the progress of mammalian cell cultures by measuring cell
number may mask certain kinetic effects. Consideration should be given in abandoning this practice in favor of other methods, including the measurement of dry cell weight, cellular protein, etc..
VI. SUGGESTED FUTURE WORK

This thesis should be but the beginning of efforts to develop accurate mathematical descriptions of mammalian culture kinetics for the purpose of optimal bioreactor design and control. Much work remains to be done. This remaining work may be grouped into two general categories: 1) Improvements to the mathematical descriptions of the metabolic rates; and 2) development of more sophisticated control strategies based on more realistic process models.

VI.A.1 Improvements to Mathematical Descriptions: Death Rate Kinetics

Without question, the weakest part of the mathematical model describing hybridoma culture kinetics was the representation of cell death as the growth rate ventured below 0.02 h⁻¹. Indeed, the mathematical expression for the culture viability, \( \alpha \), presented in equation (105) was only intended to provide an approximation of the culture kinetics at these conditions. Obviously, inaccurate depictions of cell viability compromises the solution of the optimal control problem, since typically, the culture viability begins to decrease rapidly around the point of greatest volumetric productivity (Figures 44 and 47).

It is suggested that the death rate and the growth rate of CRL - 1605 cells be investigated as a function of the environment conditions at growth rates (based on total cells) below 0.02 h⁻¹. This could be done via statistically designed experiments at low cell levels as was done in Table 3. A practical problem to be overcome, however, is the accurate estimation of non-viable cells at such low cell levels, since there would not be enough cells for microscopic examination. One possibility is to use a Coulter Counter with a channelizer which provides a size distribution of the population. It has been observed that non-viable (i.e., trypan blue stained cells) are smaller than viable cells (personal observation). The kinetics may then be related to the following:

\[
\frac{dX_v}{dt} = \mu_v X_v - \mu_d X_d
\]  

(129)

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\[
\frac{dX_d}{dt} = \mu_d X_d \tag{130}
\]

These equations may then replace equation (74) in the set of equations from (74) to (81), and the optimal control problem could be solved.

**IV.A.2 Improvements to Mathematical Descriptions: Thiol Chemistry**

It has been clearly shown that the activity of serum degrades with time and that this degradation can be slowed considerably by the addition of thiols. Also, the addition of thiols and or cells was found to stimulate the growth rate at low serum and cell levels. The Monod constant relating the growth rate to the serum level was found to be a decreasing function of the cell level. Furthermore, there was strong indications that serum degradation limited the productivity of a fed-batch culture of hybridoma cells. To explain these results, an hypothesis has been formulated relating the observed loss of serum activity with a molecular model of thiol chemistry in mammalian culture medium (section V.D, Figures 49 and 50). It is recommended that this hypothesis be vigorously explored.

Equation 63 describing the kinetics of serum degradation is in all likelihood a gross simplification a very complicated system. This may be surmised from the fractional exponent in the equation, as well as from Figure 26, which demonstrates that the relation provides a qualitative, not quantitative representation of the observed kinetics. The cell level dependent Monod constant in equation (46) is also probably a lumping of more complicated cell/thiol interactions. Elucidation of these interactions could yield many benefits. First, more accurate descriptions of the culture kinetics would obviously lead to a more satisfactory solution of the optimal control problem, consequently resulting in a more efficient process. Second, knowledge of thiol interactions could provide insight into how to improve productivity. Should thiols be added, and if so, which ones, how much, and at what times during the process? Should redox potentials be monitored and controlled? Could the medium be reformulated to increase productivity?
The key to understanding thiol chemistry in mammalian cell culture is to identify which thiol(s) provided in serum are rate limiting and which thiol(s) are depleted upon incubation of the medium. HPLC or electrophoretic methods (Ohnori et al., 1983) exist that can separate and distinguish between different thiols, disulfides and mixed disulfides. It would also be important to determine how cell act to reduce their environment. Do they excrete reducing compounds as some fibroblasts have been found to do (Bannai, and Ishii, 1980), or is their reducing activity located on the membrane surface, as Ohmori et al. suggests (1983)?

VI.B.1 Development of Control Strategies - Theoretical Mathematics

The control strategy formulated in this work was successful in approximately doubling the antibody productivity, while reducing the amount of serum utilized. Despite the success, the control strategy was developed from false assumptions. It was assumed that no lactate was formed, and no serum activity was lost. This implies that both the specific antibody productivity and the total serum activity in the bioreactor was constant. This is known not to be true. Obviously, better control would be obtained if these assumptions were relaxed in solving the optimal control problem. This is easily said, but let the reader beware, that the inclusion of serum degradation converts the system from an autonomous one (i.e., implicit in time) to a non-autonomous one (explicit in time). Strategies for solving optimal control problems for non-autonomous systems have not been well developed.

Additionally, it should be noted that the solution of the optimal control problem presented in section IV.E.3 was very specific with respect to the assumptions made, and does not represent a general solution. If lactate accumulation and subsequent inhibition of antibody production was included, the solution method given in this thesis could probably not be used. Solution of the problem via the method of Modak et al., (1986) is possible, but not likely, for the reasons stated earlier. That is, the successive derivation of HF up to order five or six would lead to very complex calculations and expressions, which may not result in relations explicit for the adjoint variables. Thus, the method of Modak et al., as presented, would break down.
Therefore, breakthroughs in developing optimal control strategies for mammalian cell bioreactors necessitates the simultaneous development of the mathematical theory of optimal control.

IV. B.2 Development of Control Strategies - Incorporation of Feedback

The optimal control strategy presented in this thesis represents feed-forward control. Once the control strategy was determined, the culture was at the mercy of any perturbations to the system. Obviously, better performance could be achieved if current knowledge of the culture kinetics could be used to update the control strategy. Some of the feedback information that could be incorporated include estimates of cell, glutamine, ammonium, and antibody levels. The cell level may be estimated from the ATP production rate calculated from the oxygen uptake rate and the lactic acid production rate (Glacken et al., 1986, Appendix B). The lactic acid production rate may be estimated from the amount of base added to keep the pH constant. The ammonium level can be measured automatically off-line with an ammonium probe.

The measurement of glutamine and antibody on line will require new techniques. Little success has been achieved with glutamine probes because of the instability of glutaminase, and interference of other compounds (Rechnitz et al., 1979). However, equations (76) and (77) may be used to estimate the glutamine level from ammonium measurements by forming the following relation:

\[ \bar{G} = \frac{k(1 - Y_{a/g}) \int G \, dt - \int dA}{Y_{a/g}} \]  \hspace{1cm} (131)

where \( \bar{G} \) is the estimated glutamine level, whereas \( Y_{a/g} \) is the ratio of cellular ammonium excreted to cellular ammonium excreted; i.e. \( q_A/q_G \).

Additionally, if the specific antibody productivity is known as a rigorous function of the lactic acid concentration, and the specific lactate productivity is known, then the antibody titer may be estimated from equation (124):

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\[ d(PV) = \int \left[ \frac{q_p}{q_L} \right] d(LV) \]

which in many cases may be solved explicitly.

Optimal control strategies may then be developed from the methods described in Bryson and Ho (1976) or Ray (1981).

Additional control loops may also be added to improve productivity. For example, there can be separate control loops for glutamine, redox and serum control. The specific glutamine utilization rate should be known as a function of the glutamine level beforehand, however, so that the optimal glutamine set point can be selected. It has been shown that the glutamine utilization rate, and hence the ammonium production rate may be reduced at low glutamine levels (Glacken et al., 1986). The rate of degradation of the growth-promoting activity of serum should also be known as a function of the redox and DO level if redox control is to be implemented. The redox potential may be controlled with the addition of oxygen, or DTT (or other dithiols or thiols).

IV.B.3 Development of Control Strategies - Other Bioreactor Configurations

The mathematical descriptions developed from this work have applicability, not only to fed-batch systems but to other systems as well. These relations may be coupled to process design equations for perfusion, continuous, hollow-fiber, and encapsulated systems, as well. The latter two systems would need to include mass transport relations. The optimal control problem could then be solved in each case. Ohno (1978) developed a strategy using Kelley's Transformation in which optimal control theory was used, not only to determine the nutrient feed rate profile, but the optimal operating mode (i.e., batch, fed-batch, continuous) as well. Issues involved in optimizing repeated-fed batch bioreactors was discussed in detail in section V.B.

This thesis clearly demonstrated that ammonium accumulation and serum degradation limited productivity in a fed-batch bioreactor. This limitation is clearly
overcome by perfusion or continuous systems. The main drawback to such processes is the presence of serum, the most expensive raw material, in the effluent. The advantages of overcoming ammonium accumulation and the loss of serum activity must outweigh the high rate of serum utilization.
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APPENDIX A - LEGEND OF SYMBOLS

A = ammonium level, mM
C_a = cost of serum, $/l
C_t = cost of time, $/h
C_x = cost of inoculum, $/cell
F = flowrate of medium into the bioreactor, l/h
G = glutamine concentration, mM
G_i = glutamine concentration in the nutrient feed, mM
H = Hamiltonian
J = objective function, cells/$ or g antibody/$
k = first order decomposition rate of glutamine, h^{-1}
K_A = growth inhibition constant of ammonium, mM^2
K_G = Monod constant for glutamine, mM
K_L = Growth inhibition constant, mM^2
K_S = Monod constant for serum, mM
L = lactate level, mM
m = maintainence energy coefficient, mmol/cell-h
P = antibody concentration, pg/cell-h
q_A = specific cellular ammonium production rate, mmol/cell-h
q_G = specific cellular glutamine utilization rate, mmol/cell-h
q_L = specific lactate production rate, mmol/cell-h
q_p = specific cellular antibody production rate, mmol/cell-h
S = serum level, l/l
T = time, hr
V = liquid volume, l
X = cell concentration, cells/l
y = time, hr
Z_l = transformed variables
\[\alpha = \text{percent viability}\]
\[\beta = \text{exponent of the cell level in the Monod constant for serum}\]
\[\lambda_i = \text{adjoint functions}\]
\[\mu = \text{specific growth rate, } h^{-1}\]
\[\mu_{\text{max}} = \text{maximum specific growth rate, } h^{-1}\]
\[\tau = \text{first order response time constant, } h\]
\[\tau_L = \text{transportation lag constant, } h\]
Reduction of Waste Product Excretion via Nutrient Control: Possible Strategies for Maximizing Product and Cell Yields on Serum in Cultures of Mammalian Cells

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Mammalian cells grown in culture excrete lactic acid and ammonium ions in quantities that may limit growth and reduce product synthesis. Frequent replenishment of the culture medium is often necessary to prevent waste product accumulation which could inhibit cell growth. Since increased medium replenishment results in increased usage of animal serum, the most expensive raw material, excessive production of waste products lowers the cell and product yield on serum, and hence increases production costs. Strategies for reducing the production of lactic acid and ammonium by mammalian cells via controlled addition of glucose and glutamine will be demonstrated. Mathematical relations coupling ammonium and glutamine kinetics will be described. Additionally, a method for automatic on-line estimation of the cell concentration was developed. This method involves calculating the ATP production rate from the oxygen uptake rate and the lactic acid production rate. Automatic on-line estimation of the cell concentration is critical if nutrient levels in large-scale mammalian cell cultures are to be accurately maintained via process control.

INTRODUCTION

Glucose is metabolized to pyruvate for energy in mammalian cells via the glycolytic pathway. If pyruvate is reduced to lactate, only 2 mol ATP are produced/mol glucose catabolized. Alternatively, pyruvate may enter the Kreb's cycle via acetyl CoA, typically producing 36 mol ATP/mol glucose catabolized. Glucose is also used to produce reducing equivalents, in the form of NADPH, and the ribose units in nucleotides, via the pentose-phosphate pathway.

Glutamine is also catabolized to produce energy as illustrated in Figure 1. The yield of ATP depends on the relative flux of glutamine between each of three possible pathways: 1) glutamine may be completely oxidized to CO₂, yielding 21 mmol ATP/mmol glutamine; 2) incomplete oxidation to aspartate yields 12 mmol ATP/mmol glutamine; and, 3) incomplete oxidation to lactate only yields 6 mmol ATP/mmol glutamine.

The relative amount of cellular energy provided by glucose or glutamine depends upon the cell line. For example, with HeLa cells (a transformed cell line), 80% of the glucose utilized is converted to lactate via glycolysis, while 35 and 13% of the glutamine utilized is converted via the Kreb's cycle to CO₂ and lactate, respectively. Consequently, 70% of the ATP produced is from glutamine metabolism. In contrast, glutamine provides only a maximum of 40% of the ATP produced in normal diploid fibroblasts.

Carbohydrate levels in culture medium can affect energy metabolism and, in particular, lactic acid production. For instance, Eagle demonstrated that substitution of more slowly utilized sugars, such as fructose or galactose, for glucose resulted in less lactic acid formation in the culture medium. Also, Reiter demonstrated that an increase in glucose concentration from 0.2 mM to 10 mM reduced the flux of glucose carbon through the pentose-phosphate pathway by 50%, while simultaneously increasing the flux of glucose carbon through glycolysis to lactic acid. Zielke has observed that increasing the concentration of glucose in the mammalian cell growth medium results in more rapid utilization of glucose. Thus, it appears that, as the rate of carbohydrate uptake increases, carbohydrate carbon chains are preferentially shunted through glycolysis towards lactic acid, rather than through the pentose-phosphate pathway. Slow rates of carbohydrate uptake have been shown to stimulate both respiration and oxidation of glutamine to CO₂. Additionally, at these conditions, since most of the carbohydrate is shunted into the pentose-phosphate pathway, a higher percentage of the lactic acid that is excreted is derived from glutamine.

Ammonium is an end product of glutamine catabo-
lism in mammalian cells (Fig. 1). Depending on the relative flux of glutamine through the three pathways shown, the ratio of the moles of ammonium excreted to the moles of glutamine catabolized can vary between 1 and 2. Ammonium also results from the spontaneous decomposition of glutamine to pyrrolidone-carboxylic acid at 37°C.6

Mammalian cells grow rapidly in vitro only over a very narrow pH range within 0.2 to 0.4 pH units of the optimum.7 Production of lactic acid often exceeds the buffering capacity of the medium, thereby lowering the culture pH and inhibiting cell growth. Additionally, the lactate ion itself (independent from changes in pH) has been shown to inhibit antibody production in hybridoma cells.8 Ammonium has been shown to inhibit cell growth for strain L mouse cells,9 choriocarcinoma tissue,10 mouse asitics tumor cells,11 and hybridoma cells.8 Ammonium has also been shown to inhibit interferon production from human fibroblast cells.12 Virus production may also be reduced by ammonium.11,13,14

The usual strategy for limiting the accumulation of waste products in mammalian cell culture is to replace the "spent" medium with fresh medium. This strategy, however, increases serum usage and therefore reduces the yield of both cells and product on serum, thus increasing production costs.

This article will introduce strategies for reducing lactic acid and ammonium accumulation by feeding glucose and glutamine into cultures of mammalian cells at controlled rates. Human foreskin fibroblasts (FS-4) grown on microcarriers were cultured in a 14-L (total volume) aerated vessel which was equipped to monitor and control pH, dissolved oxygen, and glucose levels via a computer. The volumetric ATP production rate was calculated indirectly and used to estimate the cell concentration on-line. The volumetric lactic acid production rate and the volumetric oxygen uptake rate were estimated at various glucose levels, and were compared with the results obtained from cultures grown in galactose (without glucose). The estimated cell concentration was used to calculate the glucose feeding rate. The culture in which the glucose was thus controlled showed a reduced lactic acid production. Additionally, glutamine and ammonium kinetics for Madin–Darby canine kidney (MDCK) cells grown on microcarriers were determined and described mathematically. From these mathematical relations, a glutamine addition schedule was formulated that main-
tained a relatively low concentration of glutamine in cultures of MDCK cells. Cultures maintained at these low glutamine levels exhibited reduced ammonium production compared to standard batch cultures. Although two different cell lines were used for the studies, the general principles and strategies that may be utilized to reduce waste product accumulation in cultures of mammalian cells can still be effectively illustrated. The strategies presented should, in general, reduce waste product accumulation, regardless of the cell line used.

MATHEMATICAL FORMULATIONS

ATP Production Rate (APR)

Calculation of the volumetric ATP production rate (\( \dot{A}_{\text{PR}} \)) (mmol/L h) may be used for estimating cell densities automatically on-line. If the specific ATP productivity (mmol ATP/g dry cell wt hr) is constant, the cell concentration can be estimated by calculating the APR (for FS-4 cells, 1 g dry cell wt is equivalent to 2.3 \( \times 10^6 \) cells). The volumetric rate of ATP production can be estimated by calculating the energy generation associated with the volumetric oxygen uptake (OUR) and volumetric lactic acid production rates (LPR). It is known that 0.5 mol O\(_2\) is needed to oxidize 1 mol of either NADH or FADH via the electron transport chain. The oxidation of 1 mol NADH provides enough energy for the creation of 3 mol ATP, while the oxidation of 1 mol FADH provides energy for the production of 2 mol ATP. Fortunately, FADH generation always occurs simultaneously with GTP generation in the Kreb’s cycle; thus, for ATP accounting purposes, the utilization of 0.5 mol O\(_2\) can be said to produce 3 mol ATP, regardless of whether NADH or FADH is oxidized.

If the only source of lactic acid production were from carbohydrate metabolism in cultured mammalian cells, one could conclude that 1 mol ATP was formed for each mole of lactate excreted. Figure 1 shows, however, that lactic acid produced from glutamine is a waste product of glutamine oxidation and is not representative of any additional production of ATP over that produced via respiration. Therefore, to estimate the APR properly, the fraction of the total lactic acid produced from the carbohydrate \( F_{\text{LIC}} \) must be known. The volumetric ATP production rate can then be calculated as:

\[
\text{APR} = 6 \text{OUR} + \text{LPR} (F_{\text{LIC}})
\]

where

\[
\dot{A}_{\text{PR}} \text{ is the volumetric ATP production rate (mmol/L h);}
\]

\[
\text{OUR} \text{ is the volumetric oxygen uptake rate, (mmol/L h);
}
\]

\[
\text{LPR} \text{ is the volumetric lactic acid production rate (mmol/L h); and}
\]

\[
F_{\text{LIC}} \text{ is the ratio of lactic acid produced from the carbohydrate to the total lactic acid produced (range of 0–1).}
\]

Glutamine

Glutamine spontaneously decomposes following first-order kinetics to pyrrolidone–carboxylate and ammonium at 37°C, and is transported into and utilized by mammalian cells. It is reasonable to assume that the rate of cellular utilization is related to the cell concentration by \( m_{\text{GLN}} \), the specific glutamine uptake rate, which may vary as a function of the medium conditions. Since mammalian cells catabolize glutamine, and since this catabolism results in ammonium excretion by the cells into the medium, it can be assumed that the ammonium excretion rate is related to the glutamine utilization rate. As shown in Figure 1, a maximum of two moles of ammonium may be excreted per mole of glutamine catabolized. Thus, glutamine metabolism may be quantitatively described as follows:

\[
-\frac{d[\text{GLN}]}{dt} = k[\text{GLN}] + m_{\text{GLN}}X
\]

\[
\frac{d[\text{NH}_4]}{dt} = k[\text{GLN}] + Y_{\text{AIC}}m_{\text{GLN}}X
\]

where

\[
[\text{GLN}] \text{ is the glutamine concentration (mM);}
\]

\[
[\text{NH}_4] \text{ is the ammonium ion concentration (mM);}
\]

\[
X \text{ is the cell concentration (cells/L);}
\]

\[
k \text{ is the first-order rate constant for glutamine decomposition (h}\text{)}^{-1};
\]

\[
m_{\text{GLN}} \text{ is the specific cellular glutamine uptake rate (mmol/cell hr); and}
\]

\[
Y_{\text{AIC}} \text{ is the yield of ammonium from glutamine (mmol ammonium excreted/mmol glutamine utilized).}
\]

MATERIALS AND METHODS

Stock Cultures

Human diploid foreskin cells (FS-4) were obtained from Jan Vilcek (New York University School of Medicine, New York, NY). Cells were obtained at approximately the 18th population doubling. They were frozen in one batch for the entire series of experiments and were used between population doublings 30 and 40. Stock cultures were maintained in 490-cm\(^2\) plastic roller bottles (Corning Glass Works, Corning, NY) at 37°C. For experimental purposes, microcarrier cultures were seeded from these stocks. The growth medium for stock cultures consisted of Dulbecco’s modified Eagle medium (DMEM, GIBCO Laboratories, Grand Island, NY) supplemented with 10% (v/v) fetal calf serum (FCS, Sterile Systems, Inc., Logan, UT).
Madin–Darby canine kidney (MDCK) cells, an anchorage-dependent cell line supplied from the American Type Culture Collection (ATCC, Rockville, MD) at passage number 69, were propagated as stock cultures in tightly capped roller bottles at 37°C in DMEM supplemented to 8% newborn calf serum (NCS, Hazelton Duthchland, Inc., Denver, PA), and 2% fetal calf serum (FCS, Hazelton Duthchland). The cells were passaged via trypsin treatment13 and diluted fivefold with DMEM (8% NCS, 2% FCS) every 5–6 days.

Ammonium and Glutamine Modeling Experiments in Microcarrier Cultures

Prior to experimentation, MDCK cells were harvested and inoculated into twice as many new roller bottles (1:2 split). The medium used was DMEM without glucose, but with 10 mM fructose and 5% FCS (Hazelton). Just prior to confluence, the medium was removed, and the bottles washed with phosphate-buffered saline (PBS) without magnesium or calcium salts (Flow Laboratories, Inc.). After the cells were removed from the surface of the bottle by a 0.2% trypsin–0.05% EDTA solution, the experimental medium (without glutamine, but with serum) was added and used as inoculum for the experimental microcarrier cultures.

The microcarriers were supplied in 20 g/L sterile suspensions in PBS from Flow Laboratories, Inc. The microcarriers were washed twice with PBS and once with serum-supplemented DMEM. The microcarrier suspension was then diluted to 5 g/L microcarriers with DMEM (5% FCS, without glucose, with 10 mM fructose, without glutamine) before addition to 200-mL spinner flasks (Wilbur Scientific, Boston, MA). Glutamine was added from a 160 mM stock solution in PBS. The spinners were gassed with CO₂, and allowed to incubate for an hour at 37°C and 10% CO₂ prior to inoculation. The spinners were inoculated at cell densities of 3.0–3.4 × 10⁵ cells/mL and placed on magnetic stirrers (Belco Glass, Inc., Vineland, NJ) in a 10% CO₂ incubator. Medium was replaced by first allowing the microcarriers to settle by stopping the agitation. The spinners were weighed on a top loading digital scale before the spent medium was withdrawn. Fresh medium was added to restore the previous weight.

Cultivation of FS-4 Cells with Controlled Feeding of Glucose

The initiation of microcarrier cultures has been previously described16 and essentially consisted of the following: microcarriers were suspended in a 14-L (total volume) New Brunswick fermentor in PBS (without Mg or Ca salts, Flow Laboratories, Inc., McLean, VA), pH 7.2, at a concentration of 10 mg/mL and were sterilized in situ by autoclaving. Following sterilization, the microcarriers were washed once with PBS and then with the growth medium (DMEM, with 25 mM glucose 5% FCS without NaHCO₃, with 5 mM tricine buffer and 7.14 g/L NaCl) and were suspended with growth medium to give a final concentration of 5 mg/mL. Antibiotics used were penicillin (100 μg/mL) and streptomycin (100 μg/mL) obtained from Sigma Chemical Co. (St. Louis, MO). Cells harvested from roller bottles were used to inoculate the fermentor at 3 × 10⁵–4 × 10⁵ cells/mL.

The measurement of the volumetric OUR has been described previously17 and is briefly outlined as follows: The oxygen probe (Instrumentation Laboratories, Lexington, MA) was calibrated in water saturated with air at several agitation rates. The culture was oxygenated using silicon rubber tubing. Air was passed through the tubing but was intermittently replaced with pure O₂ if the dissolved oxygen concentration (DO) of the culture fell below a determined set point. Except during OUR measurements, the DO of the medium was maintained at 60% of air saturation.

To measure the volumetric OUR, a transient was periodically induced, in which the gas flow in the headspace and in the syringe tubing, was replaced with a mixed gas consisting of 10% O₂ and 90% N₂. The resulting decrease in the DO was due to both mass transfer of oxygen out of the vessel and oxygen utilization by the cells. The volumetric OUR for a given time period was evaluated using the following equation:

\[
\text{OUR} = \left( C_0 - C_f \right) \frac{K_{a}}{t_f - t_0} + \int_{t_0}^{t_f} K_{a} \left( C^* - C_L \right) dt \quad (4)
\]

where \( C_0 \) is the initial oxygen concentration (mM); \( C_f \) is the final oxygen concentration (mM); \( C^* \) is the concentration of oxygen in the gas phase in equilibrium with the saturated concentration of oxygen in the liquid phase (atm); \( C_L \) is the concentration of oxygen in the gas phase in equilibrium with the actual concentration of oxygen in the liquid phase (atm), \( K_a \) is the mass transfer coefficient (mmol O₂/atm L h); \( t_0 \) is the initial time (h); \( t_f \) is the final time (h); and \( dt \) is the time interval between successive DO measurements. Values for the mass transfer coefficient had been calculated previously.17

The pH in the culture was maintained at 7.2 by addition of 200 mM NaOH using a pH controller. The amount of base added was quantitated using a calibrated Gould loadcell (model L-32-2, Oxnard, CA).

The concentration of glucose or galactose in the culture medium was determined by using the reaction of o-toluidine with aldohexoses.18 The method was automated for on-line determination as shown in Figure 2. The sample was mixed with the reagents in the proportions shown via a Technicon Autoanalyzer (Channucy, NY). The o-toluidine reagent was prepared as described by Fring.18 After allowing for reaction at 95°C for 5 min, the absorbance of the reaction mixture was measured via a flow-through colorimeter at 660
nm. The operation of the system was controlled and monitored by an Apple II computer. Samples were automatically withdrawn from the vessel every 4.5 h via a peristaltic pump turned on with a signal from the computer. The absorbance of standard solutions of glucose were determined automatically prior to each culture sample measurement.

Analytical Methods

Cell concentration was determined by enumerating stained nuclei on a hemocytometer via microscopic observation. Ammonium was assayed enzymatically with glutamate dehydrogenase as described by Lund. Glutamine was determined by high pressure liquid chromatography (Waters Associates, Milford, MA) after derivatization of the amino acids in the sample with o-phthalaldehyde. Lactate was determined enzymatically using lactate dehydrogenase. All chemicals were obtained from Sigma Chemical Company.

RESULTS

Ammonium and Glutamine Kinetics in MDCK Cell Cultures

Figure 3 shows that the growth rate of MDCK cells is inhibited with increasing concentrations of ammonium ion. The cells were cultured on microcarriers in DMEM without glucose but with 10mM fructose and 5% FCS. A 1-L culture was maintained in a 2-L spinner flask during lag phase (32 h after inoculation). Six hours after the onset of growth was detected, the culture was split 100 mls each into eight 200-ml spinner flasks, each supplemented to 0mM, 4mM, 7mM, or 10mM NH₄Cl from a 170mM stock solution. To maintain equivalent volumes, PBS was added to the 0mM, 4mM, and 7mM NH₄Cl cultures. Every 12 h, the cells were counted and the medium was replaced with fresh medium supplemented with the same concentration of NH₄Cl. This was done to avoid the accumulation of ammonium in the cultures. Growth rates were calculated from three successive cell concentration measurements.

It can be seen that while 4mM NH₄Cl does not inhibit growth greatly compared with the control culture, 7mM and 10mM NH₄Cl decreased the growth rate by ca. 50%. The decrease in the observed growth rate with time is typical of microcarrier cultures seeded with a high cell inoculum and is due to the decreasing surface area available as the cells grow. Since the initial growth rates at all ammonium concentrations are similar, it appears that the cells require significant exposure time to high concentrations of ammonium before growth is inhibited. It should be noted that 10mM ammonium chloride does not alter the pH of standard DMEM medium equilibrated with 10% CO₂. Thus, ammonium inhibition of growth cannot be attributed to an increase in pH.

The specific glutamine uptake rate, m_GLN, must be known before the proposed model can be tested. To determine m_GLN and its functional relationship with varying levels of glutamine and NH₄Cl, cultures were grown in fructose medium supplemented with 5% FCS at the initial conditions shown in Table 1. All cultures containing initial glutamine concentrations of 1mM and
below were replenished with glutamine from a 160mM stock solution every 12 h to avoid glutamine depletion. The cell, glutamine, and ammonium profiles were followed in each culture, with samples being taken every 12 h for 60 h. The specific glutamine uptake rate was calculated for each interval in each culture, and was plotted with respect to the average glutamine concentration over that interval (Fig. 4).

By assuming constant \( m_{\text{GLN}} \) over the small time interval between each sample, \( m_{\text{GLN}} \) was then calculated by:

\[
m_{\text{GLN}} = \frac{34 \left[ \text{Glutamine} \right]}{4.0 + \left[ \text{Glutamine} \right]} \ (\text{mmol/10}^{11} \text{ cells h})
\]

which is typical for substrate transport into cells. The correlation coefficient \( R^2 \) for this fitted equation was 0.83, while the significance level (the probability that the null hypothesis describes the data better than the fitted equation) is less than 0.01%. The glutamine uptake rate could not be correlated with the ammonium concentration \( R^2 = -0.01 \); data not shown). The scatter in the data is probably due to the differential method

Table I. Initial conditions of medium used to determine \( m_{\text{GLN}} \) as a function of glutamine and ammonium for MDCK cells.

<table>
<thead>
<tr>
<th>Glutamine (mM)</th>
<th>Ammonium (mM)</th>
<th>Number of cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0</td>
<td>0.3</td>
<td>4 (2 each)</td>
</tr>
<tr>
<td>2.0</td>
<td>0.4, 7, 10</td>
<td>4 (1 each)</td>
</tr>
<tr>
<td>1.5</td>
<td>0.4, 7, 10</td>
<td>4 (1 each)</td>
</tr>
<tr>
<td>1.0</td>
<td>0.4, 7, 10</td>
<td>4 (1 each)</td>
</tr>
<tr>
<td>0.6</td>
<td>0.3</td>
<td>4 (2 each)</td>
</tr>
<tr>
<td>0.5</td>
<td>0.4, 7, 10</td>
<td>4 (1 each)</td>
</tr>
<tr>
<td>0.25</td>
<td>0.4, 7, 10</td>
<td>4 (1 each)</td>
</tr>
</tbody>
</table>

Figure 3. Inhibition of growth of MDCK cells by ammonium. Every 12 h, medium was replaced in each culture with DMEM and without glucose, with 10mM fructose, 5% FCS and (O—O) 0mM NH\(_4\)Cl, (□—□) 4mM NH\(_4\)Cl, (Δ—Δ) 7mM NH\(_4\)Cl, (○—○), and 10mM NH\(_4\)Cl.
used to calculate $m_{\text{GLN}}$ [eq. (5)]. It is possible, due to the transient nature of the experiments performed, that depletion of nutrients and/or accumulation of end products may contribute to the data scatter. However, fructose utilization or lactate accumulation was not large, since only 4mM fructose was utilized (out of 10mM) and only 4mM lactate was accumulated after four days. Additionally, all cultures grown as per Table 1 had similar growth curves, implying that $m_{\text{GLN}}$ was not growth associated. It should be noted that all values of $m_{\text{GLN}}$ shown in Figure 4 were determined for cultures in either exponential or stationary growth, since $m_{\text{GLN}}$ calculated during lag phase were very much higher than those shown in Figure 4. It is possible that the cells need more energy during lag phase, and thus, require more glutamine.

To test the validity of the proposed model, glutamine and ammonium profiles from a batch culture of MDCK cells grown in fructose medium with 3mM glutamine and 5% FCS were compared to the results predicted from eqs. (2) and (3). The functional relationship for $m_{\text{GLN}}$ (Fig. 4, solid line) was inserted into differential eqs. (2) and (3), which were solved numerically, given the initial concentrations of glutamine and ammonium, and given the functional relationship of cell concentration with time (Fig. 5, top graph, solid line). The ammonium yield on glutamine ($Y_{\text{AG}}$) was calculated to be 1.0. This was estimated as the value which minimized the mean-squared error resulting from the variance between the experimental data and the ammonium profile predicted from eqs. (2) and (3). It can be seen from Figure 5 that the model accurately describes the kinetics, once exponential growth begins. Although Figure 4 showed some scatter, the fitted relation for $m_{\text{GLN}}$ used in eqs. (2) and (3) was satisfactory for describing the data in Figure 5, since the fitted relation represents a smoothing of the differential errors inherent in calculating $m_{\text{GLN}}$.

If ammonium production is coupled to glutamine utilization, the kinetics demonstrated in Figure 4 imply that ammonium production should decrease with decreasing glutamine concentrations. To test this hypothesis, ammonium production from a culture of MDCK cells kept at low glutamine concentrations via
controlled addition of glutamine every 12 h, was compared to an uncontrolled culture (Fig. 6). The amount of glutamine added to the controlled culture for each successive interval increased to account for exponential cell growth. Glutamine was added so that the concentration would not fall below 0.2 mM after 12 h. Figure 6 shows that glutamine was added to a level of 0.5 mM initially, while at the end, glutamine was added to a level of 1.8 mM. Equation (2) with the fitted relation for \( m_{GLN} \) and the cell growth data were used to formulate a feeding schedule. The uncontrolled culture was replenished with 4 mM glutamine from a 160 mM stock solution whenever glutamine depletion was suspected (estimated from eq. (2)). It can be seen that the low glutamine culture accumulated less ammonium than the uncontrolled culture (6 mM versus 10 mM), thus verifying the hypothesis. Both cultures reached the same final cell concentration (1.2 × 10^6 cells/mL) and had similar growth curves (data not shown). Since the ammonium levels in the cultures did not start to diverge greatly until late exponential phase and since the growth rates at 7 mM to 10 mM NH₄Cl are not drastically different (Fig. 3), little difference in the growth of the two cultures were expected.

Additionally, Table II demonstrates that while both the glutamine utilized by the cells and the glutamine lost due to spontaneous decomposition was significantly reduced for the low glutamine culture from Figure 6, the overall yield of ammonium from glutamine was similar in both cultures. It should be noted that when this experiment was repeated, the results were essentially identical (data not shown).

**Glucose Control and ATP Yield in Cultures of FS-4 Cells**

The volumetric ATP production rate (APR, mmol/L h) was calculated from the volumetric oxygen uptake rate (OUR, mmol/L h) and the volumetric lactic acid productivity (LPR, mmol/L h) and was used to estimate automatically the cell concentration on-line for FS-4 cells grown on microcarriers using both standard DMEM medium (25 mM glucose, 5% FCS) and DMEM medium (without glucose, 5% FCS) with 25 mM galactose. The volumetric OUR was measured directly (see the Methods Section), while the volumetric LPR was calculated from the amount of base required to maintain a constant pH. Via enzymatic determination of lactate concentration in the medium, it was determined that lactic acid is the sole source of titratable acid produced by the cells. Since tricine was used instead of NaHCO₃ as the buffer, little CO₂ is present as carbonic acid.

Before the volumetric APR can be calculated from the volumetric OUR and the volumetric LPR via eq. (1), the fraction of the total lactic acid excreted that results from the carbohydrate (\( F_{LIC} \)) must be known. Oxygen and carbohydrate consumption, as well as lactate and ammonium production from batch cultures of FS-4 cells grown on glucose and galactose in a 14-L New Brunswick fermentor is shown in Table III. Table III shows that little error is introduced if \( F_{LIC} \) is assumed to be 1. The maximum fractional amount of total lactic acid produced from glutamine (\( F_{L/\text{GLN}} \)) is calculated from the following stoichiometric relation:

\[
\text{Glutamine} + 3H_2O + FAD^+ + NAD^+ \\
+ GDP + P_i \rightarrow \text{lactate} + 2NH_4^+ + 2CO_2 \\
+ NADH + FADH_2 + GTP
\]  

(7)

Assuming \( F_{LIC} \) to be equal to 1, the volumetric APR was calculated for both cultures and divided by the measured cell concentration to determine the specific ATP productivity (\( q_{ATP}, \text{mmol/g dry cell wt h} \)) (Fig. 7). It can be seen that after lag phase (more than 40 h), \( q_{ATP} \) is approximately constant and is similar for both the glucose and galactose cultures (1.7 mmol ATP/g dry cell wt h), even though Table III shows that the energy metabolism for both cultures is drastically dif-