GROWTH AND INJURY OF FREELY SUSPENDED ANIMAL CELLS IN AN AGITATED AND SURFACE-AERATED BIOREACTOR

by

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ABSTRACT

Growth and Injury of Freely Suspended Animal Cells in an Agitated and Surface-Aerated Bioreactor

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The mechanism of animal cell damage due to agitation and aeration in a stirred bioreactor and the effect of media components on cell damage were investigated. Hybridoma cells ATCC CRL-8018 were grown in controlled bioreactors of 1-2 liter working volume, each agitated with a 7 cm-diameter impeller having pitched blades 1.8 cm wide by 7 cm tall. The experimental agitation speed was imposed at a consistent point in the exponential phase of batch growth in each culture. The apparent growth rate and cell yields on substrates and metabolites were used to assess the cell response.

In bioreactor liquid volumes of 1.0-1.2 liters in the presence of a gas headspace, severe cell damage was caused at agitation speeds of 190-220 rpm as 1-3 mm bubbles were entrained at the vortex tip, moved through the bulk liquid, then burst at the liquid surface. Cell damage in this agitation range was not caused by turbulent eddies in the bulk fluid or merely by the presence of entrained bubbles,
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CHAPTER 1

Introduction

Animal cells grown in vitro are an important source of biological molecules and, increasingly, an important product themselves. Although genetic engineering applied to prokaryotic cells can be used to produce some biological molecules, many molecules require complex folding, glycosylation, and other post-translational processing that prokaryotic cells cannot perform correctly. Likewise, production of biological molecules in vivo using mice or other animals is unsuitable because of difficulties with scale-up, consistency, and sterility [Birch et al., 1985]. The proliferation of products that are now being produced using animal cell culture is proof of the utility and importance of this exploding field. This variety of animal cell products includes monoclonal antibodies, growth factors and regulators, viral immunogens, and, increasingly, animal cells themselves. Compilations of the biologicals produced from animal cells have been presented in detail [Mizrahi, 1986; Glacken et al., 1983]. New uses for animal cells include routine, standardized test systems based on living cells in culture for determining the toxicity or carcinogenicity of industrial products. Additionally, clinical applications are now being developed that require
the isolation of cells from an individual, growth and possible modification in vitro, and re-introduction into the individual [Spier, 1988]. The demand for products that can only be provided using animal cells requires that large scale means for the culture of animal cells be developed and optimized.

Research in the manufacture of products from animal cells has been traditionally divided into two areas: growth of cells and production of product; and separation and purification of the product. Although the ease of separation and purification can have a profound influence on the method chosen for growing the cells, the research presented in this thesis is concerned primarily with the actual growth and metabolism of cells. However, to facilitate the separation and purification, achievement of high product concentration is an underlying consideration in the research of cell growth and product formation.

Concerning the production of cells and their products, two areas of research have received special attention. The first is the design of reactor configurations that allow effective mass transfer and scale-up. The second is the design of defined, inexpensive media for cell growth.
1.1 Fluid Mechanical Considerations in the Design of Cell Culture Systems

A large variety of "novel" reactor designs have been proposed for cell culture in an effort to provide effective mass transfer in an acceptable environment for cell growth [Brauer, 1988], [Bliem and Katinger, 1988], [Glacken et al., 1983]. Most of these novel reactor designs can be viewed as variations on two main themes: the hollow fiber reactor, and the mixed tank reactor. In the hollow fiber reactor, cells are contained in the spaces between hollow fibers of a certain porosity. Cells and, sometimes, products remain in the spaces between the hollow fibers while media flows through the fibers, providing nutrients and removing waste products via the fiber membrane [Knight, 1989]. Although high concentrations of product can be achieved, problems with scale-up and uniform reactor conditions may limit the usefulness of hollow fiber reactors for large-scale, reproducible production of animal cell products.

The basic theme of the mixed tank reactor, on the other hand, has been extensively used in a wide variety of chemical engineering processes and has already been successfully adapted for use in the fermentation of bacterial and fungal products. Existing industrial capacity and technology of the mixed reactor, along with the flexibility of applications makes it an attractive choice for the production of animal cell products. Within the mixed tank, conditions are
relatively homogeneous compared with hollow fiber reactors. The monitoring and control of reactor conditions, because of their homogeneity, can be readily accomplished within the reactor. Adequate mass transfer can be provided by agitation with an impeller or sparging of gas or both. In a stirred tank reactor (STR) mixing is provided by an impeller, with or without sparged gas. In a bubble-column or air-lift reactor, mixing is accomplished using only sparged gas, sometimes within a draught tube. Additionally, the STR or bubble-column can be operated in batch or continuous mode, whichever configuration provides the greatest productivity. In continuous operation, the principle of the hollow fiber reactor can be duplicated using a perfusion membrane that traps cells in the reactor while allowing the removal of spent media and product. The research in this thesis, then, deals with the analysis of the problems associated with operation of the stirred tank reactor as applied to animal cell culture.

Because they are larger in size and lack a cell wall, animal cells are more sensitive than microbial cells to the hydrodynamic forces within a stirred tank reactor. The forces necessary for transfer of nutrients and oxygen within the stirred tank reactor create a complex fluid-mechanical environment which can elicit a wide variety of responses from animal cells, the most dramatic being cell death. Liquid mixing is necessary to provide a homogeneous environment,
increase mass transfer, and prevent the clumping of cells or microcarrier beads. Understanding the responses of animal cells to lethal and sub-lethal hydrodynamic forces remains a major problem in the successful development of animal cell culture in a stirred reactor environment [Arathoon and Birch, 1986], [Bliem, 1989], [Nelson, 1988], [Glacken et al., 1986]. These forces are caused by power input via the impeller or power input via gas sparging or both. The following is a summary of the research up to this point in the field of animal cell responses to hydrodynamic stresses.

1.1.1 Effects of shear stress on animal cells

The biomedical engineering literature contains a wealth of information describing the effects of hydrodynamic forces on anchorage-dependent and freely-suspended cells associated with the circulatory system, including endothelial cells [Frangos et al., 1985; Diamond et al., 1989], erythrocytes [O'Rear et al., 1982], platelets [Hellums and Hardwick, 1981], polymorphonuclear leukocytes [Rhee and McIntire, 1986], and T lymphocytes [Chittur et al., 1988]. These studies have used controlled shear gradient devices to characterize the response of cells to various degrees of liquid shear stress. To subject freely suspended blood cells to well defined and controlled shear stress, a rotational viscometer was developed which allowed analysis of a relatively large sample at a range of shear stresses.
The utility of rotational viscometers, capillary viscometers, and other shear-generating devices has been described [Meiselman and Cokelet, 1973]. For anchorage-dependent cells, a parallel-plate flow chamber has been used to subject cells to uniform and controlled shear stress [Frangos et al., 1985].

For the anchorage-dependent cell types mentioned above, exposure to shear stresses causes a variety of changes in cells' morphology, metabolism, or product excretion. When exposed to shear stresses as low as 4 dyne/cm², vascular endothelial cells elongate in the direction of fluid flow but remain attached to their support [Levesque et al., 1989]. This elongation is accompanied by cytoskeletal restructuring, including formation of actin fibers as a response to shear [Franke et al., 1984]. In addition to changes in morphology, exposure to shear stresses causes changes in cellular metabolism of human umbilical vein endothelial cells in the form of increased secretion of prostacyclin [Frangos et al., 1985], increased secretion of tissue plasminogen activator [Diamond et al., 1989], and activation of arachidonic acid metabolism [McIntire et al., 1987]. The responses of endothelial cells may be initiated by a K⁺ selective, shear-stress-activated ionic current, which represents an extremely rapid response of endothelial cells to shear stresses formed in a capillary viscometer [Olesen et al., 1988]. Stretch-activated ion channels in endothelial cells may also mediate
the response to shear stress [Lansman et al., 1987], although the channels reported in that study were induced by membrane stretching due to suction.

Although high enough stresses cause lysis, freely suspended blood cells also exhibit a response to sub-lytic shear stress. As shown in a cup-and-bob viscometer, erythrocytes exhibit changes in the viscoelastic properties of their membrane in response to shear stresses of $1.0-1.3 \times 10^3$ dynes/cm$^2$ [O'Rear et al., 1982]. Exposure of platelets to shear stresses of 100 dynes/cm$^2$ for 5 min induces platelet aggregation [Hellums and Hardwick, 1981]. For polymorphonuclear leukocytes, shear stresses in the range of 75-800 dynes/cm$^2$ for 2-10 min exposure times cause many metabolic changes normally associated with the leukocyte response to circulatory system damage [McIntire and Martin, 1981]. In addition to the endothelial cells, platelets and leukocytes also exhibit increased arachidonic acid metabolism in response to shear stresses [McIntire et al., 1987]. Furthermore, exposure to sub-lytic shear stresses results in alterations that affect the proliferative response of human T cells [Chittur et al., 1988]. Since circulatory-system cells are subjected to various stresses in their native environment, a variety of responses to shear stresses are expected. However, the behavior of these cells may suggest the types of responses to expect from other non-vascular cell types.
Researchers have applied the methods developed for measuring shear stress effects on circulatory-system cells to cells commonly used in production of biological products. Surface-attached human embryonic kidney cells exhibit elongation and alignment in the direction of fluid flow when subjected to shear stresses of 6.5 dynes/cm² in a flow channel [Stathopoulos and Hellums, 1985]. The damage to freely-suspended hybridoma cells by shear stresses generated in viscometric flows has been examined by various researchers. In a cup-and-bob viscometer, shear stresses caused loss of viability of an insect cell line over a time period of 3 h, starting with stresses of 15 dynes/cm² [Tramper et al., 1986]. In their system, cell damage increased with increasing shear stress. For shear stress values between 50-1000 dynes/cm² and time of exposure from 1/2-3 h, lysis of hybridoma cells increased with increasing shear stress and increasing time of exposure [Abu-Reesh and Kargi, 1989]. In studies of the long-term (15 h) shear effects on murine hybridoma growth, shear stresses of 6.7 dynes/cm² were required to cause significant cell damage [Smith et al., 1987]. Murine hybridoma cells grown in batch culture exhibit sensitivity to well-defined shear which depends on the culture age, presumably due to the changes in nutrient and metabolite concentrations during batch culture [Petersen et al., 1988]. In addition, the cell cytoskeleton and energy metabolism play
an important role in mediating shear sensitivity [Petersen, 1989].

1.1.2 Effects of hydrodynamic turbulence on surface-attached cells

In a mixed bioreactor, surface-dependent cells grown on microcarrier beads as well as freely suspended cells are subjected not just to shear stresses, but to a turbulent hydrodynamic environment. Because a detailed analysis of the fluid velocity profiles, and associated stresses, within turbulence is not possible at the present time, researchers have related the growth and metabolism of cells in the bioreactor to some measurable indicator of the magnitude of turbulence in the reactor. For chick embryo fibroblasts grown on microcarrier beads, the maximum cell concentration was correlated with an "integrated shear factor" obtained by dividing the impeller tip speed by the distance between the impeller tip and vessel wall [Sinskey et al., 1981]. In work with human fibroblast cells grown on microcarriers in a stirred reactor, the final cell population and the growth extent did not correlate with impeller tip speed but did correlate with the integrated shear factor used by Sinskey et al. [Hu et al., 1985].

More recently, the theoretical framework developed by Kolmogorov to describe turbulence has been used to correlate reactor parameters with the growth of cells on microcarriers.
The following description and equations used to characterize turbulence within a bioreactor have been presented in some detail in the literature [Cherry and Papoutsakis, 1986; Croughan et al., 1987] and are repeated here for convenience. In the bioreactor, large fluid eddies are generated by external power input to the reactor via an impeller or sparged gas. The large eddies pass their energy to intermediate-sized, energy-containing eddies in which most of the kinetic energy of the fluid resides. Energy cascades from these eddies without loss to progressively smaller eddies until eddies of a characteristic size are formed, where the kinetic energy is dissipated viscously. In the course of energy transfer, the original orientation of the large eddies is lost, allowing the assumption of local isotropy when analyzing small length scales within the reactor. Assuming isotropic turbulence, the following equations were derived by Kolmogorov and can be used to describe the smallest scale of eddies in turbulent flow:

\[ \eta = \left( \frac{\nu^3}{\varepsilon} \right)^{1/4} \quad (1.1) \]

\[ \nu_e = (\nu\varepsilon)^{1/4} \quad (1.2) \]

Here \( \eta \) is the size of the smallest eddies (Kolmogorov-scale eddies); \( \nu_e \) is the velocity of the smallest eddies; \( \nu \) is kinematic viscosity of the fluid; and \( \varepsilon \) is the rate per mass of fluid of viscous energy dissipation in the turbulence.
The local power dissipation, $\varepsilon$, is taken to be the average power dissipation in the reactor due to power input via the impeller, given by the following correlation:

$$\varepsilon = \frac{P}{\rho_f V_d} = \frac{N_p n^3 d_i^5}{V_d}$$ (1.3)

Here $P$ is the power input through the impeller; $\rho_f$ is the fluid density; $N_p$ is the dimensionless impeller power number; $n$ is the impeller rotational rate; $d_i$ is the impeller diameter; and $V_d$ is the liquid volume in which the energy dissipation takes place. $N_p$, which is a function of the impeller geometry and agitation rate, can be obtained using an appropriate correlation [Nagata, 1975]. The volume available for energy dissipation, $V_d$, may be taken as the entire reactor volume, $V$, or as the volume in the vicinity of the impeller, $V_d = d_i^3$. Cherry and Papoutsakis [1988] found that the best correlation for their data was obtained by assuming that $V_d = d_i^3$. The shear stresses that correspond to the Kolmogorov-scale eddies are usually estimated as:

$$\tau = \mu_f \frac{\nu_e}{\eta} = \rho_f (\nu_e)^{1/2}$$ (1.4)

where $\mu_f$ is the fluid viscosity.

Using data on chick embryo fibroblasts [Sinskey et al., 1981], human fibroblasts [Hu et al., 1985], and freely-suspended protozoa [Midler and Finn, 1966] from other researchers as well as data on human fibroblasts from their
own research, Croughan et al. [1987] found that the growth of these cells could be better correlated with the Kolmogorov eddy size than with the integrated shear factor. Cell growth on 180 μm diameter beads was significantly reduced for characteristic eddy sizes below about 100 μm, as indicated by a sharp sigmoidal dependency of the growth extent or normalized growth rate on the Kolmogorov eddy size. In addition, dependence of cell growth on medium viscosity was consistent with the eddy-size correlation [Croughan et al., 1987; Croughan et al., 1989].

Likewise, the growth of bovine embryonic kidney cells on microcarrier beads in a stirred reactor decreased linearly starting at a Kolmogorov eddy size to bead diameter ratio (η/d) of 1.8 [Cherry and Papoutsakis, 1988]. If the power dissipation was assumed to take place in the vicinity of the impeller, Vd=di³, instead of in the entire reactor volume, then onset of growth rate reduction occurred when η/d equaled approximately 1.0. In addition, death rates were found for various impeller sizes and agitation speeds using a serum-free medium that did not support cell growth. Consistent with the growth rate data, the death rate was found to increase linearly for eddy/bead ratios between 1.0 and 0.6. Changes in growth rate due to variations in medium viscosity and in microcarrier diameter were shown to further support the correlation of cell damage with the eddy/bead ratio [Cherry and Papoutsakis, 1989].
1.1.3 Effects of hydrodynamic turbulence on freely suspended cells

Although the damage to attached cells on microcarriers in turbulent flow can be correlated to reactor parameters, the damage mechanism of freely-suspended cells in bioreactors is not known. In a rotational viscometer, the damage to hybridoma cells was greater in the turbulent flow than in laminar flow for the same magnitude of shear stress [Abu-Reesh and Kargi, 1989]. Calculations of shear stress in the turbulent regime were done using the empirical curves of Taylor, and high shear stresses in the laminar regime were obtained by addition of dextran polymer to the medium. In another effort to simulate reactor turbulence damage, murine hybridoma cells were exposed intermittently to turbulent flow in a capillary tube [McQueen et al., 1987]. The onset of cell lysis corresponded with a Kolmogorov eddy size similar to the cell size, and the probability of lysis increased with time of exposure and intensity of turbulence. In further investigations, an increase in the viscosity of the medium, which was calculated to increase the Kolmogorov eddy size by 36%, did not change the extent of lysis of cells in the turbulent flow of the capillary tube. From this information, McQueen and Bailey conjecture that the mechanism of cell damage of suspended cells is different from that for cells on microcarriers in a stirred tank [McQueen and Bailey, 1989].
This difference may be partially attributed to the intermittent nature of the turbulence exposure in the McQueen and Bailey work.

In spinner flasks, rapid agitation at a rate of 240 rpm decreased the growth rate of hybridoma cells during exponential growth [Dodge and Hu, 1986]. Agitation at a rate of 200 rpm in spinner flasks increased the decline in cell concentration during death phase of batch growth [Lee et al., 1988]. An agitation rate less than 60 rpm was necessary to successfully grow hybridoma cells in a bioreactor stirred with large, teflon-bladed paddles [de St. Groth, 1983]. On a larger scale, the use of a 23 cm diameter marine propellor in a 150 l hybridoma suspension culture caused significant cell damage at an agitation rate of 170 rpm [Backer et al., 1988]. In these agitated spinner flasks or bioreactors, the cause of suspended-cell damage at rates from 60-250 rpm has not been mechanistically examined. In preliminary bioreactor experiments in which bubble entrainment due to agitation was carefully avoided, agitation rates up to 450 rpm did not alter hybridoma cell growth [Al-Rubeai et al., 1989]. Oh and co-workers [1989] postulate that conflicting accounts of cell damage due to agitation may be caused because studies have not, in general, distinguished between the effects of agitation alone and those arising from sparging or the entrainment of bubbles [Oh et al., 1989]. Furthermore, the source and state of inoculum used in these studies have
usually not been presented as an important parameter in the growth of cells in stirred vessels, although several literature sources state the importance of "healthy" inoculum [Oh et al., 1989], [Backer et al., 1988], [Dodge and Hu, 1986].

1.1.4 Effects of gas sparging on animal cells

In addition to damage due to agitation, damage to animal cells due to sparging of gasses in bubble columns or stirred tank reactors has been demonstrated. Insect cells have been shown to decline in concentration in bubble column reactors when protective agents, such as Pluronic F-68, are not used [Murhammer and Goochee, 1988], [Maiorella et al., 1988]. In cultures containing a low serum concentration, gas sparging in stirred tank reactors damaged animal cells [Kilburn and Webb, 1968]. The addition of serum or Pluronic F-68 counteracted the damaging effects of gas sparging. In another study, bovine serum albumin was shown to limit damage due to sparging of hybridoma cells in airlift loop reactors [Hülcher and Onken, 1988].

The mechanism of cell damage due to sparging of gas has been investigated by several researchers. In a detailed study using bubble column reactors, the effect of different bubble sizes and frequencies on the growth of hybridoma and other animal cells were examined [Handa et al., 1987], [Handa-Corrigan et al., 1989]. Higher bubble frequencies
caused greater cell damage, and, for identical bubble frequencies, smaller bubbles caused greater hybridoma cell damage. Since retention of cell viability increased with increasing column height, Handa and co-workers argued that hydrodynamic effects due to bubble rise in the column do not cause cell damage, and that cell damage occurs only in the bubble-disengagement region at the top of the bubble column. The data, however, do not rule out the possibility of damage occurring at the bubble injection region of the sparger. Finally, two mechanisms of cell damage were presented: one due to rapid oscillations caused by bursting bubbles; the other due to shear forces in draining lamellae in foams.

In other research, cell damage to insect cells in suspension in a bubble column reactor was found to depend very little on bubble size. On the other hand, cell damage did correlate with the flow rate of gas through the bubble column, which is in agreement with the dependence of damage on bubble frequency seen by Handa et al. [Tramper et al., 1986]. In further work, Tramper and co-workers provide evidence that cell damage may occur in two areas in the bubble column: the bubble disengagement region at the reactor surface and the bubble injection region at the sparger. Cell damage was not caused by shear forces due to the bubble rise in the column. They also present a model based on a first-order death rate, which was suggested by their data. This death rate model is as follows:
where \( k \) is the first order death rate constant; \( F \) is the gas flow rate; \( D \) is the bubble column diameter; \( H \) is the bubble column height; and \( X \) is the hypothetical killing volume associated with a gas bubble. This model allows design of bubble-column reactors for cultivation of animal cells provided that the value of \( X \) is first experimentally determined [Tramper et al., 1988].

1.2 Media Considerations in the Design of Cell Culture Systems

A second major area of research in the production of products using animal cell culture is media development. Unlike microbial cells, animal cells require a large number of nutrients, minerals, and growth factors for successful growth. Traditionally, media contain a defined array of sugars, amino acids, vitamins, and minerals which are supplemented with serum. Serum, an undefined blood fraction, provides additional proteins, lipids, and growth factors. One major goal of cell culture research has been to create completely defined media that allow cell growth while eliminating the need for serum. Many formulations for defined, serum-free media are now available although further improvements are necessary. First, present formulations of serum-free media are cell-type specific, creating the need
for new media development for each different cell type. Second, most present serum-free media have been developed for cell culture in a stationary environment. Growth of cells in a complex hydrodynamic environment, such as in a stirred tank reactor, may require special media components for successful growth. These components may physically protect the cell from hydrodynamic damage or may provide additional nutrients needed by cells when exposed to complex fluid flow.

In early work in the field of serum substitution in cell culture media, it was found that the requirement for serum could be reduced or eliminated by addition of certain hormones and proteins [Hayashi and Sato, 1976; Guilbert and Iscove, 1976]. Since then, the influence of serum on growth and ways to reduce the dependence on serum have been extensively studied. Several reviews of the media components necessary for cell growth and their role in cell growth have been published [Mizrahi and Lazar, 1988; Butler and Jenkins, 1988]. Kovar and Franek have given a detailed description of a serum-free medium used in the culture of parent myeloma cells, including the effects of different concentrations of medium components [Kovar and Franek, 1986]. Table 1.1 lists their medium formulation, which is typical of many serum-free media. In an attempt to quantify the influence of serum concentration, Dalili and Ollis [1989] found that serum was responsible for kinetic as well as stoichiometric limitations on cell growth in quiescent culture [Dalili and Ollis, 1989].
Exactly which serum factors support cell growth, and how those factors interact with the cell, are not known and continue to be investigated.

Cells have been grown in media supplemented with serum fractions in order to simplify media and determine which serum components are necessary for growth [MacLeod and Thomson, 1985]. The use of "conditioned" media from murine macrophage cultures was successful in reducing the need for serum in hybridoma cultures [Sugasawara et al., 1985]. Hybridoma cells in a stirred bioreactor were adapted to grow in media containing serum concentrations as low as 1% when Primatone RL, an enzymatic digest of animal tissues, was added to the media [Reuveny et al., 1985]. Although serum fractions, conditioned media, or digested tissue reduce the need for serum, the complex composition of these substitutes makes identification of the exact media requirements for cell growth difficult.

In an alternative approach, researchers have investigated the effect of defined additives in substituting for serum. Compounds investigated include components found in serum, other biologically active compounds, and synthetic polymers. Insulin is one common medium additive found in serum that is used to partially substitute for serum, although its metabolic and growth-promoting effects are not completely understood. Because of the relatively high concentrations of insulin (compared to the normal
physiological concentrations found in serum) needed to stimulate growth, it is thought that insulin may promote growth by acting as an analog to insulin-like growth factors [Ellis, 1989]. Transferrin, another compound found in serum, has also been shown to be an essential component in most serum-free media [Kovar and Franek, 1985]. It is not known whether transferrin is necessary as a growth-promoting compound or whether it merely acts as a messenger for iron transport from media to cells. Another serum component, albumin, has been used widely as a partial substitute for serum, and several mechanisms of action of albumin have been suggested. Albumin may provide physical protection from hydrodynamic damage, particularly in sparged cultures [Hülcher and Onken, 1988]. Albumin may not be by itself necessary for growth but may instead act as a source of fatty acids or a transporter of fatty acids from media to cells [Birch and Cartwright, 1982]. Furthermore, proteins such as casein and albumin may act as scavengers of H$_2$O$_2$ in serum-free media [Darfler and Insel, 1983].

In stirred cultures, addition of soybean phospholipid fraction to stirred cultures allowed the growth of hybridoma cells in serum-free media, indicating that phospholipids may act to protect the cells from hydrodynamic damage [Murakami et al., 1983; de St. Groth, 1983]. The lipid composition of animal cells in culture varies with the composition of lipids in the culture media [Spéctor, 1972], which indicates a
possible mechanism for the protection of cells by phospholipid addition. Ethanolamine has been described as an essential element for the growth of hybridoma cells in serum-free media [Murakami et al., 1982], although many serum-free media that support cell hybridoma growth do not contain ethanolamine. Finally, cyclic nucleotides have been shown to influence hybridoma growth in media containing serum [Dalili and Ollis, 1988].

Synthetic polymers have been shown to promote animal cell growth and to substitute for serum in culture media, particularly in agitated or sparged cultures. In early work, Mizrahi and co-workers showed that media supplemented with carboxymethyl cellulose (CMC), hydroxyethyl starch, or pluronic polyol provide better lymphoblastoid cell growth in shake flasks than unsupplemented media [Mizrahi, 1984; Mizrahi, 1975; Mizrahi and Moore, 1970]. Polyethylene glycol (PEG), a fusogen for mammalian cells, stimulated the growth of mammalian cells in quiescent culture and in stirred culture under serum-free or low-serum conditions [Shintani et al., 1988]. Damage of mouse cells due to gas bubbling could be prevented by increasing the serum concentration from 2 to 10% or by adding 0.02% Pluronic F-68, a nonionic surface-active polymer of polyoxyethylene and polyoxypropylene [Kilburn and Webb, 1968]. The protective effect of Pluronic F-68 against cell damage due to sparging has been confirmed for several animal cell types, although the mechanism of
protection is not yet understood. For insect cells grown in low-serum or serum-free media in bubble-column or sparged reactors, addition of 0.1% Pluronic F-68 eliminated cell damage [Murhammer and Goochee, 1988; Maiorella et al., 1988]. Pluronic F-68 also eliminated damage to hybridoma cells seen in the bubble-disengagement region of bubble-column reactors [Handa-Corrigan et al., 1989]. Although Handa-Corrigan et al. attributed the protective effect of Pluronic F-68 to its stabilizing effect on the reactor foam layer, Murhammer and Goochee showed that the presence of a foam layer was not necessary for Pluronic F-68 to act as a protectant. Instead, they suggested that Pluronic F-68 interacts with the cell membrane to reduce sparging damage.

Different combinations of the aforementioned media components have been used successfully by many researchers to develop serum-free media. In serum-free media, hybridoma cells required a higher inoculum cell density than cells growth in serum-supplemented media [Tharakan and Chau, 1986]. Furthermore, hybridoma cells required higher inoculum densities when grown in agitated cultures than when grown in stationary cultures [Velez et al., 1986]. A large number of serum-free media have been specifically developed for hybridoma, myeloma, or other lymphoid cell lines [Low and Harbour, 1985; Kawamoto et al., 1983; Ossendorp et al., 1986; Cole et al., 1985; Mimura et al., 1986; Chang et al., 1980; and Cleveland et al., 1983]. In all of these studies,
optimization of the media was carried out in stationary culture. The utility of these media in agitated culture is not known, since additional media supplements are often needed for protection against agitation or sparging damage. In addition, each media developed is cell specific and of somewhat different composition, although a general consensus exists that media developed for a parental myeloma cell line may be applicable to cells derived from that parental line. Evidently, it would be possible to develop a completely-defined media that could be universally applied to any cell type, since fetal bovine serum added to media allows growth of all these cell types in a variety of reactor environments. Therefore, further research is needed to identify the components in serum that support cell growth as well as their mechanism of action.

1.3 Detailed Thesis Objectives

The research in this thesis, as has been mentioned, is concerned with the growth of cells and production of cellular products using animal cell culture in a stirred reactor environment. In particular, the mechanism of cell damage due to agitation or aeration or both and the effect of media components, including serum, on cell damage are the main focus of research in this investigation. Because they are representative of many lymphoid cells, a hybridoma cell has been used as the model animal cell in these studies.
addition, study of hybridoma cells in bioreactors has its own relevance, due to commercial interest in production of monoclonal antibodies.
Table 1.1 Typical supplements used in serum-free medium.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration in the Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transferrin</td>
<td>5 μg/ml</td>
</tr>
<tr>
<td>Insulin</td>
<td>10 μg/ml</td>
</tr>
<tr>
<td>Ethanolamine</td>
<td>20 μM</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>5 μg/ml</td>
</tr>
<tr>
<td>Albumin (fatty acid free)</td>
<td>1 mg/ml</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>3 μg/ml</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>2 ng/ml</td>
</tr>
<tr>
<td>Trace element compounds</td>
<td></td>
</tr>
<tr>
<td>CdSO₄·8/3H₂O</td>
<td>50 nM</td>
</tr>
<tr>
<td>CoCl₂·6H₂O</td>
<td>10 nM</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>10 nM</td>
</tr>
<tr>
<td>(NH₄)₆Mo₇O₂₄·4H₂O</td>
<td>0.5 nM</td>
</tr>
<tr>
<td>MnCl₂·4H₂O</td>
<td>0.5 nM</td>
</tr>
<tr>
<td>NiSO₄·6H₂O</td>
<td>0.25 nM</td>
</tr>
<tr>
<td>Na₂SeO₃</td>
<td>40 nM</td>
</tr>
<tr>
<td>Na₂SiO₃</td>
<td>200 nM</td>
</tr>
<tr>
<td>SnCl₂·H₂O</td>
<td>0.25 nM</td>
</tr>
<tr>
<td>NH₄VO₃</td>
<td>2.5 nM</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>1000 nM</td>
</tr>
</tbody>
</table>

[Kovar and Franek, 1986]
CHAPTER 2

Materials and Methods

2.1 Cell Culture

Hybridoma cells CRL-8018 from the American Tissue Culture Collection (ATCC), which produce an IgM antibody to hepatitis B surface antigen, were used in all experiments. These cells were first developed from an SP2/0 parent myeloma line [Zurawski and Chang, 1983]. Cells used in this research were donated by Dr. L. P. Rutsky at the Department of Organ Transplant, University of Texas Medical School, Houston, TX.

Using standard tissue culture methodologies [Zola and Brooks, 1982; Freshney, 1987], several dozen vials of cells (1.7 ml per vial of $3 \times 10^6$ cells per ml) were initially frozen and stored in a liquid nitrogen freezer. Cells were thawed from the initial frozen stock every few months and grown as starter cultures to be used as inocula for further experiments. The freezing and thawing procedures are detailed in Appendix A. These inoculum cultures were routinely maintained in T-flasks of 25 or 75 cm$^2$ size (Corning Glass Works, Corning, NY) or in spinner flasks of 100 or 250 ml size (Bellco Glass Co., Vineland, NJ). Flasks were kept in an incubator at 37 °C in an atmosphere of 9% CO$_2$ and 95% relative humidity and, in the case of spinner flasks, agitated using Bellco low-profile multistir 4 magnetic
stirrers. The spinner flasks were modified by removing the large, flat paddle from the stir bar assembly, thus making the flasks suitable for suspension culture rather than microcarrier culture. Cultures were maintained by diluting 1:5 or 1:10 with fresh culture media every 3 or 4 days.

2.2 Bioreactor Cultures

Larger scale experiments utilized two Setric Genie 2C bioreactors (Setric Genie Industriel, Toulouse, France), which could be operated with a 1 to 2 liter working volume. The hemispherical-bottomed, glass reactor vessel measured 11.8 cm inside diameter and was approximately 18 cm tall. The impeller used for agitation was 7 cm in diameter with four blades pitched at 57° to the horizontal, each blade being 7 cm by 1.8 cm in dimension. Controls of temperature, pH, and dissolved oxygen were either proportional or proportional, integral, differential (PID) depending upon which of the reactors was used. The reactors were stationed inside a laminar flow hood to minimize the risk of contamination. Temperature inside the reactor was controlled at 37 °C by circulating heated air through a jacket around the reactor vessel. Dissolved oxygen concentration was maintained above a setpoint of 65% saturation with air by flowing air sparged with oxygen through the headspace or, in some experiments, through Silastic brand medical grade tubing (Dow Corning Corp., Midland, MI) with an ID of 1.47 mm and an
OD of 1.97 mm. The tubing was submerged in the medium and supported near the vessel wall by a stainless steel wire cage. The pH was maintained at 7.2 by addition of CO$_2$ into the aeration system or 0.5 N NaOH to the culture medium. NaOH addition for pH control was not necessary until batch cultures reached a high cell density, approximately $10^6$ cells/ml, and was therefore not used in all reactor cultures.

2.3 Culture Media

As obtained, the cells were grown in a medium supplemented with 20% fetal bovine serum (FBS). Subsequently, the cells were adapted to grow and were routinely grown in Dulbecco's modified Eagle's medium (DME; Sigma Chemical Co., St. Louis, MO) supplemented with 1% v/v FBS (Hyclone Laboratories, Logan, UT), 1% v/v Nutridoma NS or SP (Boehringer Mannheim Biochemicals, Indianapolis, IN), 2 mM glutamine (Sigma), and 50 units/ml penicillin, 0.05 mg/ml streptomycin, and 0.1 mg/ml neomycin (PSN; Sigma). The serum was denatured at 56 °C before use. The medium was filtered through a 0.2 μm Sterivex-GV brand filter (Millipore Corp., Bedford, MA) and stored at 4 °C until use. All experiments, unless otherwise noted, were performed using this base medium. Midway through experimentation, Nutridoma type NS became unavailable from Boehringer Mannheim, who recommended switching to Nutridoma type SP. As shown in Fig. 2.1, the hybridoma cells grew equally well in both NS and SP. In this
experiment, cells were grown in duplicate spinner flasks at 120 rpm in medium containing 1% FBS and 1% of either Nutridoma NS or SP. Based on these results, no distinction is made between cultures containing the two different formulations of Nutridoma.

In addition to the base medium formulation, certain experiments were performed using media supplements. Serum was either added to the medium in the required concentration before filtering, or was filtered through a 0.2 μm filter and added to already filtered medium. Dextran of average MW 129,000 (Sigma) was added to the base medium in the required concentration and allowed to dissolve completely before filtering.

2.4 Experimental Procedure

Each bioreactor experiment was preceded by calibration and sterilization. The temperature probe was factory pre-calibrated and was found to remain calibrated throughout all experiments by occasionally (every 6 months or so) checking the temperature as measured with the reactor probe against the temperature as measured with an immersed mercury thermometer. Before each sterilization, the reactor pH probe was zeroed using a pH 7.00 buffer and calibrated using a pH 10.00 buffer. Because the pH probe calibration sometimes changed upon autoclaving and sometimes drifted during the course of an experiment, a means for re-calibration without
removing the probe was developed. A solution of phosphate buffered saline (PBS) plus 16 mg/l of phenol red (the same concentration as in DME medium) was adjusted to a pH of 7.20 and stored in a centrifuge tube. The color of this pH standard could then be compared to the color of a reactor sample taken in an identical centrifuge tube. Visual examination of the phenol red indicator easily allowed calibration of the reactor to within 0.01 pH units of the desired set point of 7.20. Because the medium was buffered using a sodium bicarbonate buffering system, the pH of the reactor sample could not be measured accurately using an external pH meter. Loss of CO\textsubscript{2} from the sample to the atmosphere during measurement resulted in a rise in pH before an accurate measurement could be obtained.

The dissolved oxygen (DO) probe was zeroed before sterilization in a saturated sodium sulfite solution containing a small amount of copper sulfate. The reactor was sterilized by autoclaving at 121 °C (21 psi) for 45 min with probes in place and with the reactor half filled with de-ionized, distilled water (ddH\textsubscript{2}O). After sterilization, the DO probe was calibrated within the reactor to 100% saturation in ddH\textsubscript{2}O at 37 °C and stirred at 60 rpm with air flow through the reactor headspace.

The bioreactors were inoculated using cultures from spinner flasks that were diluted 1:5 with the appropriate medium into the reactor. Samples were removed from the
reactor in one of two ways. First, samples could be withdrawn from near the bottom of the reactor through the sample port. Using this method, the sampling port was cleared of quiescent medium by flowing sterile air into the port from a specially designed sampling device. Air was injected into the sample port only until a small amount of air flowed out of the bottom of the sample tube. Alternatively, the sample port could be cleared by withdrawing the quiescent media into a centrifuge tube to be discarded. Then, a sample of medium could be drawn through the sampling port and into a sterile centrifuge tube.

Second, samples could be withdrawn through the fill port using a sterile pipet. Since both methods drew medium from well-mixed regions of the bioreactor, no distinction is made between samples taken using either method. Typically, the sample size was 4-5 ml, of which 1 ml was used immediately for cell counting. The remainder of the sample was centrifuged at 300 x g for 10 minutes, and the supernatant was frozen at -20 °C for future assays.

2.5 Cell Counts

Total cell concentrations were determined using a Coulter counter (Coulter Electronics, Hialeah, FL). The use of the Coulter counter provided more precise and reproducible cell counts than the use of a hemacytometer, although the viability of the cells could not be explicitly determined
using the Coulter counter. Originally, each total cell count
using the Coulter counter was corrected to a viable cell
count after viability of the sample was estimated using the
method of trypan blue dye exclusion in the hemacytometer. It
was found, though, that viability of the cells was
consistently above 95% when the Coulter counter "peak" of
cells was narrow and centered at 13.5-14 μm, which
corresponded to exponentially growing cells. Therefore, the
total cell concentration presented in this research may be
taken to be within 5% of the viable cell concentration. At
the end of the exponential growth within the batch culture,
the drop in cell viability corresponded to a substantial
decrease in average cell size (the "peak" shifted to the
left) as well as a broadening of the distribution of cell
sizes. The batch experiments in this research were
terminated before a decline in cell viability occurred, and
growth rates were determined from exponentially growing
cultures only. Sensitivity analysis showed that over-
estimating the viable cell concentration by 5 or even 10%
results in less than 0.5% over-estimation of the growth rate.

Typically, cells to be counted in the Coulter counter,
such as erythrocytes, platelets, and leucocytes, are diluted
in Isoton II (Coulter Diagnostics, Hialeah, FL), an isotonic
electrolyte solution. However, the concentration of cells
suspended in Isoton II decreased with time and was lower than
the concentration of cells suspended in fresh medium, as
shown in Fig. 2.2. Therefore, medium was used as the
dilutant when counting cells in the Coulter counter. Counts
of cells suspended in medium declined only slightly over the
time span necessary to count the cells (about 10 min).

The procedure for counting cells was as follows. 200 µl
of sample was diluted with 9.8 ml of culture medium in a
Coulter sampling cup, a 1:50 dilution. Using a 10 ml pipet,
the sample was pipetted three times to gently break up any
cell clumps and disperse single cells evenly throughout the
sample. Under microscopic examination, the diluted coulter
samples before pipetting were found to contain many particles
consisting of two, three, or several cells clumped together.
After pipetting, no cell clumps were observed in the sample.
For each count, all particles greater than approximately 10.5
µm were counted as one cell. Two 0.5 ml counts were made of
each sample and were averaged to give the total cell count.
Therefore, each cell-concentration measurement was based on a
count of between 2 x 10^3 and 24 x 10^3 individual cells.

2.6 Assays

Glucose and lactate assays were performed using a YSI
model 27 analyzer (Yellow Springs Instrument Co., Yellow
Springs, OH). The glucose or lactate in a sample is
catalyzed by either glucose oxidase or lactate oxidase in
reactions that produce hydrogen peroxide (H₂O₂). Since the
enzymes are membrane bound, the H₂O₂ is trapped against a
platinum electrode where its concentration is measured. For glucose measurement, the analyzer was calibrated using an 11.1 mM glucose solution in ddH₂O. For lactate measurement, the calibrating solution was 4.44 mM lactate in ddH₂O. 25 μl of thawed sample, diluted 1:10 in the case of lactate and undiluted in the case of glucose, was injected into the analyzer for analysis. The analyzer was re-zeroed after each measurement and re-calibrated every 10 measurements.

Glutamine was assayed using a colorimetric method available in kit form (Boehringer Mannheim, Cat. No. 139-092). By this method, any L-glutamate in the sample is first measured using the following two reactions:

\[
\text{L-glutamate} + \text{NAD}^+ \leftrightarrow \text{2-oxoglutarate} + \text{NADH} + \text{NH}_4^+ \quad (2.1)
\]

\[
\text{NADH} + \text{INT} + H^+ \rightarrow \text{NAD}^+ + \text{formazan} \quad (2.2)
\]

First, glutamate is deaminated oxidatively by nicotinamide-adenine dinucleotide (NAD) to 2-oxoglutarate in the presence of the enzyme glutamate dehydrogenase (GLDH). Second, the NADH formed converts iodonitro tetrazolium chloride (INT) to formazan, which has a peak absorbance at 492 nm. Although the equilibrium of the first reaction lies far on the side of glutamate, the reaction is driven to completion as NADH is reacted irreversibly with INT. To measure glutamine, then, glutaminase is added to convert any glutamine to additional glutamate by the following reaction:
L-glutamine + H₂O → L-glutamate + NH₃  \hspace{1cm} (2.3)

The enzyme L-asparaginase has a glutaminase side activity of approximately 2% and is used for this reaction. The Boehringer Mannheim procedure was modified by reducing the assay volume from 3 ml to 210 μl so that samples could be read using a microplate spectrophotometer. Because the microplate reader could read only at certain wavelengths, absorbance was read at 490 nm instead of 492 nm. After thawing, culture supernatant was diluted 1:10 in PBS of which 10 μl was added to each microplate well. Each sample was measured using duplicate wells. A standard curve was determined for each microplate. The modified procedure is detailed in Appendix B.

Ammonia was assayed using the procedure given in a standard kit (Sigma, Cat. no. 170-3) although the assay solutions were made in house. In this method, GLDH catalyzes the oxidation of NADH to NAD⁺ as ammonia is incorporated into 2-oxoglutarate to form glutamate by the reverse of Eq. (2.1). The decrease in absorbance at 340 nm due to the oxidation of NADH is proportional to the ammonia concentration. A sample size of 25 μl in a total assay volume of 1.5 ml were contained in acrylic cuvettes, which do not absorb strongly at 340 nm. The assay solutions and procedure are detailed in Appendix C.
The IgM antibody produced by the cells was assayed using an enzyme-linked immunosorbent assay (ELISA). The ELISA measured any IgM antibody in solution and was not specific for anti-hepatitis B antibody. The assay works as follows. First, an anti-IgM polyclonal antibody (ICN ImmunoBiologicals, Lisle, IL) is adsorbed onto the surface of styrene microplate wells. Any further adsorption by other proteins is blocked using bovine serum albumin (BSA; Sigma). Second, sample is added, and any IgM molecules adhere to the anti-IgM antibody and, hence, to the microplate. Third, a solution of anti-IgM antibody conjugated to the enzyme alkaline phosphatase (ICN ImmunoBiologicals) is added to the wells. The phosphatase conjugate clings to the IgM antibody held to the microplate in proportion to the concentration of IgM antibody originally in the sample. Last, a solution of p-nitrophenyl phosphate (Sigma) is added to the wells. The phosphatase catalyzes the hydrolysis of p-nitrophenyl phosphate. The change in absorbance at 405 nm due to accumulation of p-nitrophenol is read after a set time period. Triplicate wells containing 10 µl of sample diluted 1:100 were used for each measurement. A standard curve was determined for each plate. The ELISA procedure is detailed in Appendix D.
2.7 Growth Rate and Cellular Yields

An apparent growth rate, $\mu_{app}$, is defined by the following equation:

$$\frac{dC}{dt} = \mu_{app}C$$

where $C$ is the actual concentration of cells in the reactor. Integrating from $C = C_0$ to $C$ and $t = 0$ to $t$ gives the following equation:

$$\ln(C) = \ln(C_0) + \mu_{app}t$$

The linear portion of the growth curve, a plot of $\ln(C)$ versus $t$, is taken to be the period of exponential growth during batch culture. $\mu_{app}$ is the slope of the linear portion of the growth curve and is determined by linear regression. Since $C$ is the actual concentration of cells within the reactor, the growth rate, $\mu_{app}$, defined here is the apparent growth rate which may differ from the intrinsic growth rate of the cells. Under certain conditions, the actual concentration of cells in the reactor may be declining, causing a negative apparent growth rate, but the cells that remain may be growing with a positive intrinsic growth rate.

Likewise, an apparent cell yield, $Y_{C/S}$, is defined according to the following equation:

$$Y_{C/S} = \frac{\mu_{app}}{dS/dt}C$$
where $S$ is the concentration of substrate or product of interest. Defined in this manner, the cell yield does not distinguish between utilization of substrate for maintenance and product formation and utilization of substrate for cell growth alone. When $S$ is a substrate such as glucose or glutamine, $Y_{C/S}$ is the net number of cells produced per mole of substrate consumed. When $S$ is a product such as ammonia or lactate, $Y_{C/S}$ is the net number of cells produced per mole of product formed. Assuming that $Y_{C/S}$ is independent of $C$ and that $S = S_0$ when $C = C_0$, Eq. (2.6) can be integrated to yield the following equation:

$$S = \left(S_0 - \frac{C_0}{Y_{C/S}}\right) + \frac{1}{Y_{C/S}} C \quad (2.7)$$

Using data only from the exponential phase of batch growth, $Y_{C/S}$ is found by linear regression of the plot of $S$ versus $C$.

### 2.8 Statistical Treatment of Data

In this work, the error bars shown for the apparent growth rate and apparent cell yields represent the 95% confidence interval for a regression parameter [Bowerman et al., 1986]. The regression model for the calculation of both growth rate and yields is a linear model of the form:

$$y = \beta_0 + \beta_1 x \quad (2.8)$$

In the calculation of growth rate, $y$ is the natural log of the cell concentration, $x$ is the time, and the parameter $\beta_1$ is the apparent growth rate. When calculating cell yields, $y$ is
the metabolite or product concentration, \( x \) is the cell concentration, and the parameter \( \beta_1 \) is the apparent cell yield. Using matrix notation given by Bowerman et al. in which \( \mathbf{Y} \) is a column matrix of the \( y \) values and \( \mathbf{X} \) is a matrix containing a column of 1's and a column of the \( x \) values, the least squares estimates \( b_0 \) and \( b_1 \) of the model parameters \( \beta_0 \) and \( \beta_1 \) are computed using the following matrix formula:

\[
\begin{bmatrix}
  b_0 \\
  b_1
\end{bmatrix} = \mathbf{b} = (\mathbf{X}'\mathbf{X})^{-1}\mathbf{X}'\mathbf{Y}
\]

(2.9)

where

\[
\mathbf{X} = \begin{bmatrix}
  1 & x_1 \\
  1 & x_2 \\
  \vdots & \vdots \\
  1 & x_n
\end{bmatrix}
\]

(2.10)

and

\[
\mathbf{Y} = \begin{bmatrix}
  y_1 \\
  y_2 \\
  \vdots \\
  y_n
\end{bmatrix}
\]

(2.11)

The 95% confidence interval, \( E^{(95\%)} \), is then calculated using the following formula:

\[
E^{(95\%)}_j = t^{(n-2)} s \sqrt{c_{jj}}
\]

(2.12)

Here \( t^{(n-2)} \) is the appropriate t-table value for \( n-2 \) degrees of freedom and a 95% confidence interval; \( c_{jj} \) is the diagonal element of the matrix \( (\mathbf{X}'\mathbf{X})^{-1} \) corresponding to \( \beta_j \); and \( s \) is the standard error. The standard error is calculated using the following formula:
In some cases, such as in Table 4.2, the mean of several growth rates is presented. The confidence limit shown for the mean of $n$ number of growth rates is as follows:

$$E_{\text{mean}} = \frac{\sqrt{\sum E_i^2}}{n}$$

(2.14).

Because the t-table values increase in a non-linear fashion with decreasing numbers of data points, at least 5-6 points within the exponential phase of cell growth were required to calculate a growth rate with a confidence interval smaller than 10-15% of the growth-rate value.
Figure 2.1 Growth curves in 250 ml spinner flasks at 120 rpm containing 1% FBS and 1% Nutridoma-NS (o) or 1% Nutridoma-SP (Δ).
Figure 2.2  Cell counts using the coulter counter as a function of time using complete medium (o) or Isoton II (Δ) to suspend the cells.
Bioreactor Cultures of Freely Suspended Animal Cells: Experimental Protocol and Assessment

The procedure developed for monitoring the growth of bovine embryonic kidney (BEK) cells in the Setric Genie bioreactors [Cherry, 1987] was modified to allow the accurate investigation of the freely-suspended hybridoma cells. In this chapter, the development of the experimental protocol as well as the appropriate methods for analyzing the experimental results are presented. First, the need for consistent and "healthy" inoculum for each experiment is investigated; second, the influence of the changes in cells that take place during batch growth on the experimental procedure are examined; and third, the appropriate experimental controls are developed. Last, the methods used to assess the growth and metabolism in the experimental system are explained.

3.1 Development of Experimental Protocol

Due to the complex nature of living organisms and their responses to environmental factors, experiments using biological systems can be influenced by a large number of variables. To minimize the effect of extraneous variables on
the performance of animal cells in culture, strict procedures must be adopted.

3.1.1 Effect of concentration and "health" of inoculum on cell growth

Although not systematically documented, the cell culture literature indicates that a minimum inoculation concentration and a "healthy" inoculum taken from the exponential phase of batch growth are necessary for reproducible batch growth rates. Furthermore, the literature suggests that use of low inoculum concentration or inoculum from cultures that have passed exponential growth results in poor growth in the reactor that persists past the stage of lag phase. Experimental results from the present research also indicate that the state of the inoculum can effect the growth rate of cells throughout the batch culture.

The initial cell concentration has been shown by researchers to affect the growth rate of cells throughout the batch run. In a preliminary investigation, Murhammer and Goochee [1988] present the results of the effect of inoculum concentration on cell growth of insect cells grown in spinner flasks. They performed experiments to determine the minimum inoculation concentration needed to avoid increasing the population doubling time, implying that low inoculum concentrations can result in lower growth rates. They also state that reproducible growth curves were difficult to
obtain for inoculum concentrations below a certain value [Murhammer and Goochee, 1988]. Since these experiments were preliminary runs used to develop their experimental protocol, the cell concentrations and growth rate data were not included in their paper.

Velez et al. have also found that a minimum inoculum concentration is necessary for hybridoma-cell growth. Furthermore, cultures in spinner flasks required a higher inoculum density than cultures grown in stationary T-flasks [Velez et al., 1986]. Their data suggest that the growth rate of cells increases, to a certain extent, with increasing inoculum concentration. They indicate that the crowding of cells at the bottom of stationary cultures may allow the medium to become conditioned more easily in T-flasks than in the spinner flasks. This analysis assumes that conditioning of the medium by the cells is necessary before cell growth can occur.

Cell "health" in the inoculum has also been seen as an important parameter in the growth of animal cells. When referring to "healthy" cells, researchers usually are referring to cells that are growing exponentially at a normal rate. "Unhealthy" cells would be cells in the lag or decline phase of batch growth or cells growing at low growth rates. Partially due to this ambiguity in the characterization of cell "health", researchers have not, in general, quantitated the effects of the condition of the inoculum on cell growth
in batch culture. Although no proof was provided, Oh et al. [1989] postulated that some of the ambiguity of their results in the study of agitation effects on animal cells is due to the fact that cells are not introduced at exactly the same physiological condition in each experiment [Oh et al., 1989].

In industrial-scale culture of hybridoma cells, Backer et al. [1988] have developed a rapid and reliable method for the production of monoclonal antibody using batch cultures. In their technique, a portion of the culture from each batch run is left in the reactor to serve as inoculum for the following culture. Although the final antibody concentration in each batch run could be increased by extending the run beyond the period of increasing cell concentration, they found a trade-off in overall productivity due to the effect of the extended run on the inoculum for the following culture. They found that extension of the culture past the exponential-growth phase typically resulted in lower cell viabilities and a long lag phase for the next batch culture. They also stated that constantly maintaining the culture in exponential-growth phase may contribute to "the good long-term stability of cell growth and antibody characteristics observed in our system," [Backer et al., 1988]. Although not explicitly stated, this analysis indicates that cells exhibit an extended period of low growth rate if inoculum of poor "health" or low concentration is used.
In other work, low growth rates in a one-liter vessel were said to be caused by using non-exponentially growing cells as inoculum although, again, no experimental results were presented to clearly demonstrate a relationship between the growth phase of the inoculum and the growth rate of the batch culture [Dodge and Hu, 1986].

In summary, several researchers have shown that the cell concentration and the state of inoculum can affect the growth of cells throughout the batch culture. In early work for this thesis, results similar to those seen by other researchers were found for the batch growth of hybridoma cells, as discussed below.

Results from two pairs of reactor experiments performed prior to the use of a consistent inoculum are shown in Fig. 3.1. Cultures with 1% FBS and 10% FBS were grown in parallel in two bioreactors, inoculated at the same time with identical inoculum. Likewise, cultures with 1% and 2% added dextran (in addition to 1% FBS in the standard growth medium) were also grown in parallel, using identical inoculum. Cell concentrations shown are viable cell concentrations as determined using a hemacytometer and trypan blue dye exclusion, and, hence, are not as accurate as coulter counter measurements might have been. The reactor cultures were stirred at a rate of 80 rpm, which is shown in following chapters to be a gentle speed at which variations of 1 to 10% FBS and 0 to 2% dextran have no effect on growth rate.
Nonetheless, the apparent growth rate in the culture with only 1\% FBS was significantly lower than the growth rate in the other three media. Aside from the medium composition, which is shown in subsequent chapters to have no effect on cell growth at an agitation speed of 80 rpm, the main difference in the initial conditions between the reactor runs shown in Fig. 3.1 is the difference in initial concentration. In the cultures with an initial concentration of 200 x 10^3 cells/ml, the lag phases lasted approximately 25 hours. With the low inoculum concentration of 90 x 10^3 cells/ml, the lag phases in media containing 1\% and 10\% serum were considerably longer, approximately 45 hours. In addition, the initial decline in cell concentration following inoculation was much greater in the medium with 1\% serum than in the medium with 10\% serum.

Apparently, the cells did not recover from the combination of low initial concentration and low serum concentration during the entire course of the batch run. In 1\% serum, the growth rate remained low even after the cell reached a concentration equal to the satisfactory initial concentration of the 1\% and 2\% dextran cultures. In addition, the peak cell concentration in 1\% serum occurred at the point of glucose depletion but was less than 700 x 10^3 cells/ml. With 10\% serum, however, the cells recovered from the low inoculum concentration and grew at a normal growth rate (0.035-0.045 h^{-1}) to a peak cell concentration of
approximately $1.5 \times 10^6$ cells/ml. Likewise, cell concentration increased from 0.2 to over $1.5 \times 10^6$ cells/ml at a normal growth rate in the 1% and 2% dextran runs. Thus, even though enough nutrients were present to produce over $1.3 \times 10^6$ cells, slow growth persisted throughout the batch run containing 1% FBS, and fewer than $0.7 \times 10^6$ cells/ml were produced. These results suggest that medium composition and low inoculation concentration combine to produce a low growth rate that can persist even after conditions under which normal growth should occur are reached.

In addition to the cell concentrations used to calculate growth rates, the concentrations of several key substrates and metabolites—glucose, glutamine, lactate, and ammonia—were determined during the course of the reactor runs. Only the exponential portion of the batch growth curves were used to calculate the cell yields. Table 3.1 lists the growth rates and yields for each medium composition. As with the growth rate, the culture with only 1% FBS had significantly different yields than the other cultures. In Fig. 3.2, the concentration of IgM antibody over time is shown for each reactor culture. Despite the relatively large scatter in the data, it can be seen that the culture with 1% FBS had a lower final concentration of antibody than the other cultures.

As seen in Table 3.1, the slow growth in the 1% serum culture is characterized by low cell yields on glucose and lactate (fewer cells produced per mole of glucose utilized or
mole of lactate produced). For the 1% FBS run, cell yields on glutamine and ammonia were reduced to the same extent as the glucose and lactate yields. No significant difference was seen in the ratios of lactate produced to glucose consumed or ammonia produced to glutamine consumed, also shown in Table 3.1. Because they are ratios of two numbers with large confidence limits, these values in Table 3.1 have very large uncertainties. Any subtle differences in metabolism in the different media would not be revealed due to the large data uncertainties. However, data from these two tables indicate that the metabolic pathways for cells in the different media are similar, but that the carbon and energy sources are used less efficiently with slower growth. Two explanations exist for the greater energy requirement during slow growth: more energy is needed to produce each cell; or some cells that are produced die, changing the apparent yield.

Further evidence of persistent slow growth depending upon inoculum conditions is shown in Fig. 3.3. As before, the medium used in this run contained 1% Nutridoma supplement and 1% FBS. As will be shown in subsequent chapters, the gentle agitation speed of 60 rpm used in this run does not effect the growth rate of cells in 1% FBS. During the first part of the reactor run, cells were grown as a batch culture until the glucose was nearly depleted. From that point, the reactor was operated as a continuous stirred tank reactor
(CSTR), with fresh medium added at a dilution rate equal to the prevailing growth rate of the cells. For a continuous reactor with cell concentration in the inlet stream equal to zero, Eq. (2.4) becomes:

\[ \frac{dC}{dt} = (\mu_{\text{app}} - D)C \]  

(3.1)

Here, D is the dilution rate equal to the flowrate into (and out of) the reactor divided by the reactor volume. The apparent growth rate, \( \mu_{\text{app}} \), at any given time is determined by adding D to the slope of Fig. 3.3, which is a plot of \( \ln(C) \) versus t.

As shown, the cell concentration declined slowly from approximately \( 700 \times 10^3 \) to \( 300 \times 10^3 \) cells/ml while the dilution rate was set equal to 0.018 h\(^{-1} \), indicating a growth rate in the CSTR of 0.014 h\(^{-1} \). Again, the low inoculum concentration and low serum concentration, \( 110 \times 10^3 \) cells/ml combined to produce a long lag phase, 46 hours, in the initial batch culture. The cells then grew at a slow rate that persisted until the cells reached a concentration of \( 700 \times 10^3 \) and continued even after fresh medium was continually added to the reactor. Therefore, growth was limited not by a nutrient depletion, but by the physiological state of the cells, presumably due to the unfavorable initial conditions.

At 285 hours into the reactor run, the medium was spiked with 40 ml of serum to bring the total serum concentration up to 5%. As a result, the growth rate of the cells increased to a
value of 0.035 h\(^{-1}\) at hour 330, which resulted in the increase in cell concentration from that point as shown in Fig. 3.3. Based on the dilution rate and serum addition, the serum concentration in the reactor with time is shown in Fig. 3.4. As seen, a growth rate of 0.035 h\(^{-1}\) continued even after the serum had washed out of the reactor. Slow growth in the reactor persisted until the medium was spiked with serum, even though the continuous addition of fresh medium would indicate that no nutrient limitations were present. Later, normal growth continued even after the serum had washed out of the reactor. Thus, the growth rate in the reactor appears to have been dependent upon the state and concentration of the inoculum.

Both the literature accounts and the present experimental results indicate that the initial concentration in the reactor and the state of the inoculum can have an effect on the growth rate of cells. This effect can persist even after the conditions in the reactor have reached a state at which normal growth would be expected to resume. Some literature accounts have attributed this phenomenon to inadequate cells available at the onset of batch growth for proper conditioning of the culture medium. In the experiments presented here, the slow growth was coupled with reduced cell yields. In addition, the presence of serum enabled the cells to grow at normal rates, even if the initial cell concentration was low. The presence of serum
did not, however, reduce the lengthy lag phase associated with poor inoculum. In both batch and continuous culture, the effect of serum on growth was not immediate. These results suggest that FBS may substitute for compounds produced by the cells during conditioning of culture medium. Furthermore, the factors that affect the length of lag phase may not be the same as those that affect growth rate, since FBS affected only the ultimate growth rate and not the length of lag phase.

3.1.2 Effect of inoculum history (agitated versus stationary culture) on cell growth

Another property of the inoculum that may affect the results of experiments in agitated reactors is the source of inoculum. Studies have indicated that cells may be able to adapt to some extent to agitation by becoming more resistant to hydrodynamic damage [Petersen, 1989]. In Petersen's work, cells taken from stationary T-flasks and from spinner flasks were sheared in a rotating viscometer. Cells from the spinner flasks were more robust and more resistant to shear stress than cells taken from T-flasks. Two reasons for this difference were presented. In the first scenario, only the most robust cells live while the weakest cells die due to agitation in the stirred culture. Growth in spinner flasks selects for shear-resistant cells. In the second scenario, cells react to the hydrodynamic stresses within the stirred
vessel and become more robust due to changes in the cells composition, similar to the formation of "heat-shock" proteins in hybridoma cells in response to elevated temperatures. In either case, the history of the culture contributes to the physiological state of the cells. Cells taken from different sources may respond differently when used as inoculum in agitated bioreactors and may influence batch growth parameters such as the length of lag phase and the growth rate. Therefore, inoculum used in the investigation of the hydrodynamic effects on animal cells should be consistent as to whether or not cells have been previously adapted to growth in agitated cultures.

3.1.3 Changes in cellular response during different phases of batch growth

The phase of batch cell growth at which animal cells are tested for their response to high agitation intensity may affect that response. Petersen et al. [1988] have shown that the shear sensitivity of the hybridoma cells used in the present research (ATCC CRL-8018) depends on the phase of batch culture [Petersen et al., 1988]. Batch cultures of cells, such as cultures to be used as inocula, are more easily damaged by shear during lag phase and again at the end of exponential growth, during the stationary or decline phase. Several reasons were found for this dependence. Prolonged exposure to an inhibitory environment, such as high
ammonia concentrations and low pH, makes cells more sensitive to shear forces. Shear sensitivity increases substantially when the microfilament network is disrupted by cytochalasis or when respiration is inhibited by KCN [Petersen, 1989]. These results indicate that the accumulation of metabolic products and depletion of energy sources at the later part of batch growth enhance the cells' susceptibility to shear damage.

Petersen and co-workers suggested a strategy for improving the cell viability and growth in stirred cultures which entailed using mild agitation immediately following inoculation then increasing agitation as necessary once cells pass lag phase and are more resistant to shear stresses [Petersen et al., 1988]. Whether or not shear sensitivity is a factor in the lengthy lag phase and persistent low growth rates seen in the aforementioned experiments has not been determined. These results do indicate that cells can behave differently in response to external conditions depending on the phase of batch growth in which those conditions are applied.

### 3.1.4 Experimental protocol

In this work, the causes of poor growth induced by substandard inocula have not been investigated in detail. Instead, experimental procedures that avoid the detrimental effect of inconsistent inocula have been implemented. For
each batch run, inoculum was taken from spinner flasks to insure that the inoculum had a consistent growth history. Spinner-flask cultures used as inoculum were stirred at a gentle speed of 80-100 rpm to avoid adaptation of the cells to a high-agitation environment. Also, the use of spinner-flasks provided a large volume of homogeneous inoculum for each batch run.

As explained in Chapter 2, the reactor cultures were started by diluting cultures from spinner flasks by a 1:5 ratio with the appropriate medium. Therefore, an initial concentration for the reactors was chosen that was high enough to prevent a lengthy lag phase and slow growth yet was low enough so that the spinner-flask cultures could reach the desired inoculum concentration without surpassing exponential growth phase. An initial concentration of \(200-250 \times 10^3\) cells/ml was chosen as the desired starting concentration based on the results of the experiments in Fig. 3.1 as well as the observations of previous routine cell subculturing. Since the phase of exponential growth in the spinner flasks usually continued until the cell concentration reached approximately \(1.5 \times 10^6\) cells/ml, an inoculum culture of \(1.0-1.25 \times 10^6\) cells/ml could be easily obtained from the exponential phase of growth. By diluting the inoculum cultures of \(1.00-1.25 \times 10^6\) cells/ml one to five, initial concentrations of \(200-250 \times 10^3\) cells/ml of exponentially-growing cells were achieved in the reactors.
In addition, since the sensitivity to hydrodynamic damage in the reactor is dependent on the phase of growth of the cells, reactor cultures were initially stirred at a gentle speed of 60 rpm. Once cells had passed lag phase and entered exponential growth, the experimental condition to be tested could be imposed on the culture. Thus, to test the effect of high agitation intensities on cell growth, the increased agitation rate was imposed when the cells reached a concentration between $400$ and $500 \times 10^3$ cells/ml. This concentration range assured that the cells had passed lag phase and had entered exponential growth, yet the cells had not depleted the media of essential nutrients for growth. Thus, enough time was left in the batch run after the experimental condition was imposed to obtain an accurate estimate of the growth rate and metabolic yields at that condition. The cells were, therefore, tested at approximately the same point in exponential growth in each batch culture, and any differences in cellular response due to the phase of growth of the batch culture were minimized.

To assure that extraneous effects due to any differences in inoculum or growth phase were minimized, several control measurements and experiments were utilized. First, by allowing each culture to reach the same point in exponential growth ($400$-$500 \times 10^3$ cells/ml) before imposing the desirable agitation intensity, the growth rate of the culture before the agitation increase could be estimated. An "internal"
control value for the growth rate at 60 rpm could be obtained for each individual batch culture. However, two factors make it difficult to quantitate precisely the growth rate at 60 rpm using this protocol. In a typical run, 3-4 points were obtained after the end of lag phase and before the culture reached a density of 400-500 \times 10^3 \text{ cells/ml}. The use of only 3-4 points to estimate the growth rate results in large 95% confidence intervals. Also, the demarcation between the end of lag phase and start of exponential growth is not always obvious. This uncertainty necessitates a qualitative judgement as to which points represent exponential growth phase. Because the end of lag phase and beginning of exponential growth is not clearly defined, the growth rate found at 60 rpm may be somewhat underestimated due to the possible inclusion of points from lag phase when calculating the growth rate. Therefore, the growth rate determined at 60 rpm is probably a conservative estimate. This subjectiveness in quantification is not as great when determining the apparent growth rates following the increase in agitation, since several points (at least 5-6) are obtained in the portion of the growth curve that is clearly linear. The use of this protocol allows the intrinsic growth rate of the cells to be estimated independently for each and every batch culture, regardless of the apparent growth rate found after the experimental conditions are imposed. Differences in the growth rates at 60 rpm may be used to correlate or correct
any differences in the apparent growth rates at greater agitation intensities.

Second, for the majority of experiments two reactors were used in parallel, with inocula for both reactors taken from the same source at the same time, to further compensate for any differences in inocula between reactor runs. Therefore, any differences found between the two reactors due to different experimental conditions would be virtually independent of the initial reactor conditions. Typically, one of the parallel cultures would contain the medium additive being tested while the other parallel culture acted as an "external" control with no additives. In addition, in some of the batch runs a portion of the culture was removed from the reactor and placed in stationary T-flasks several hours after the start of the run. These T-flask cultures acted as parallel, zero-agitation controls for the agitated reactor cultures.

Third, by utilizing the aforementioned experimental protocol, any batch cultures can be compared to each other because of the minimization of experimental variation due to differences in phase of growth or state of inoculum.

Some indication of the success of the experimental protocol at obtaining consistency in inoculum, length of lag phase, and growth rate can be extracted from the experimental results. For 54 batch runs varying in volume from 1.0 to 1.8 l, the average initial concentration in the reactor using the
above protocol was $226 \times 10^3$ cells/ml with a standard deviation of $33 \times 10^3$ cells/ml. The time elapsed from the start of the run until the experimental conditions were imposed is a rough estimate of the length of lag phase in the batch culture. This time period is a rough estimate because the experimental conditions were started at a cell concentration anywhere between $400$ and $500 \times 10^3$ cell/ml. The variation in cell concentration at the start of experimental conditions adds some random scatter to the length of lag phase data. For the 54 runs mentioned above, the average lag phase was $28.9$ hours with a standard deviation of $5.8$ hours. Thus, by using the experimental protocol given above, the length of lag phase in virtually all of the reactor runs was similar to that in the 1% and 2% dextran runs shown in Fig. 3.1, which were approximately 25 hours. The lengthy lag phases seen in the 1% and 10% FBS runs of Fig. 3.1 (45 hours) were virtually eliminated by the use of the aforementioned experimental protocol. Mean and standard deviation data on the length of lag phase for different reactor cultures are not readily available in the literature, so it is difficult to determine whether the inoculum and lag phase consistency achieved here is successful in comparison with that obtained by other researchers. Fig. 3.5 shows the elapsed time before experimental conditions were imposed (lag phase) versus the initial concentration in the reactor. As can be seen, no correlation exists between the two variables, which indicates
that the range of initial concentrations that were used were not in a range that would effect the length of lag phase. The variation in cell concentration at the onset of experimental conditions adds some scatter to the data, however, and would tend to mask any correlation that might exist between the two variables.

3.1.5 Typical run using the experimental protocol

The important aspects of the experimental protocol are demonstrated in the typical reactor run shown in Fig. 3.6. Two reactors were inoculated in parallel, one containing no dextran and one containing 2% dextran. The initial cell concentrations in the two reactors were 240 and $220 \times 10^3$ cells/ml, within the range specified by the experimental protocol. The reactors were agitated at a rate of 60 rpm up to the point indicated by the arrow, when the cell concentrations in the two reactors were 430 and $440 \times 10^3$ cells/ml. At that point, the agitation speed was increased to 200 rpm, which resulted in a drop in cell concentration in both reactors. In the control culture, the cells recovered and grew with an apparent growth rate equal to $0.0232 \pm 0.0011$ h$^{-1}$, while in the 2%-dextran culture the cell concentration continued to decline at an apparent growth rate of $-0.100 \pm 0.034$ h$^{-1}$.

At 60 rpm the two parallel cultures containing 0% and 2% dextran grew with very similar growth rates ($0.0328 \pm 0.0116$ h$^{-1}$).
and 0.0347 ± 0.0151 h⁻¹). Thus, the internal controls indicate no effect of dextran at 60 rpm and that both cultures grow with normal growth rates before the experimental conditions are imposed. However, because only 3 points were used to calculate them, the 95% confidence limits for the 60 rpm growth rates are large. The 0%-dextran, external control indicates that cells can grow at 200 rpm, but that the presence of dextran at 200 rpm is detrimental to cell growth.

3.2 Assessment of Experimental Results

In this research, the growth rate, rather than the maximum cell concentration in batch growth, has been chosen as the important growth parameter for correlation with reactor conditions. The growth rate is an intrinsic factor, and is not dependent on the total amount of nutrients available to the cell during batch culture. Therefore, growth rates from different modes of operation, such as batch or continuous culture, can be compared. Likewise, different media, which may have different total amounts of nutrients, can be compared in terms of their ability to allow cell growth. To successfully demonstrate any correlation between growth rate and the reactor environment, several methods of analysis have been developed.

The growth rate measured in this research are the apparent growth rates in the various reactor environments.
The apparent growth rate, $\mu_{\text{app}}$, is determined from the changing cell concentration in the reactor and is not, in general, equal to the actual growth rate of individual cells in the reactor. One general method for correlating these two parameters (the apparent growth rate and the "true" growth rate) has been to define a death rate, $k$, which is the difference between $\mu_{\text{app}}$, which changes with different reactor conditions, and a fixed growth rate, $\mu_0$ ($\mu_{\text{app}} = \mu_0 - k$). This method has been used successfully by researchers in both surface-attached [Croughan et al., 1989; Cherry and Papoutsakis, 1989] and freely-suspended [Tramper et al., 1988] cell systems and is used in the present research. Some subjectivity does exist, though, in the choice of the fixed growth rate used to determine the death rate. In the present research, the highest apparent growth rate routinely obtained in stationary T-flasks or in gently-stirred spinner flasks has been used as the fixed growth rate used to calculate death rates. This choice of $\mu_0$ may differ from the highest growth rate routinely obtained in the bioreactors under conditions of gentle agitation.

The use of the experimental protocol developed here allows different methods for normalizing the apparent growth rate data. First, the apparent growth rate, $\mu_{\text{app}}$, can be normalized using the growth rate at 60 rpm in the reactor, $\mu_{60}$, found before the experimental conditions in the reactor are imposed. However, due to the few points used to
determine the growth rate at 60 rpm, the uncertainty in apparent growth rates normalized in this fashion are somewhat greater than the uncertainty in the non-normalized growth rates. This greater uncertainty makes quantitative determinations of the individual normalized growth rates inexact. Taken together, though, normalized values of growth rates for several batch cultures indicate trends in data and allow qualitative conclusions to be drawn. For this reason, normalization with $\mu_{60}$ is useful as an analytical tool in assessing effects of hydrodynamic damage in the reactor. Furthermore, regardless of the quantitative utility of growth rates at 60 rpm, they are useful in demonstrating successful growth in the reactor before the experimental conditions are imposed.

Second, $\mu_{\text{app}}$ can be normalized using the intrinsic growth rate, $\mu_0$, used in calculating the death rate. However, since $\mu_0$ does not change, no additional information is gained through the use of this normalization technique.

In addition to the use of apparent growth rate, another measurement, unique to this experimental protocol, can be used to gauge the effect of the conditions within the reactor. As shown in the sample reactor run in Fig. 3.6, the cell concentration in the reactors dropped immediately after the increased agitation was imposed, even in the control culture. This initial drop in cell concentration may be used as a measure of the severity of the experimental conditions.
imposed in the reactor. However, the length of time between the imposition of increased agitation and the first sample after the increase was not the same in all experiments, which would add some scatter to the measure of the initial drop in cell concentration.

Finally, the cell yields on substrates and metabolic products are used as measures of the effect of the experimental conditions on cell growth and metabolism. In particular, the cell yields may help to determine the validity of methods used for growth-rate analysis and the mechanisms of the effects of the experimental conditions.

In chapters 4 and 5, the apparent growth rates, death rates, normalized growth rates, and initial cell declines are used to measure the effects of agitation and media composition. In chapter 6, the cell yields are used to explain and clarify these effects.
Table 3.1  Metabolic parameters for cultures shown in Fig. 3.1. Error estimates are the 95% confidence intervals for a regression parameter.

<table>
<thead>
<tr>
<th>Media</th>
<th>FBS 1%</th>
<th>FBS 10%</th>
<th>Dextran 1%</th>
<th>Dextran 2%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Growth Rate</strong> (h⁻¹)</td>
<td>0.0275 ± 0.0036</td>
<td>0.0386 ± 0.0029</td>
<td>0.0339 ± 0.0031</td>
<td>0.0455 ± 0.0077</td>
</tr>
<tr>
<td><strong>Yields</strong> (10⁶ cells/µmole)</td>
<td>glucose</td>
<td>lactate</td>
<td>glutamine</td>
<td>ammonia</td>
</tr>
<tr>
<td>glucose</td>
<td>0.0410 ± 0.0062</td>
<td>0.0267 ± 0.0064</td>
<td>0.249 ± 0.066</td>
<td>0.458 ± 0.24</td>
</tr>
<tr>
<td>lactate</td>
<td>0.0827 ± 0.0159</td>
<td>0.0529 ± 0.0164</td>
<td>0.533 ± 0.169</td>
<td>0.673 ± 0.321</td>
</tr>
<tr>
<td>glutamine</td>
<td>0.0940 ± 0.0226</td>
<td>0.0722 ± 0.0196</td>
<td>0.499 ± 0.103</td>
<td>0.673 ± 0.111</td>
</tr>
<tr>
<td>ammonia</td>
<td>0.0839 ± 0.0164</td>
<td>0.0626 ± 0.0308</td>
<td>0.595 ± 0.167</td>
<td>0.699 ± 0.075</td>
</tr>
<tr>
<td><strong>Metabolite Ratios</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lactate/glucose</td>
<td>1.53 ± 0.60</td>
<td>1.56 ± 0.79</td>
<td>1.30 ± 0.67</td>
<td>1.34 ± 1.29</td>
</tr>
<tr>
<td>ammonia/glutamine</td>
<td>0.54 ± 0.24</td>
<td>0.59 ± 0.39</td>
<td>0.74 ± 0.28</td>
<td>0.85 ± 0.24</td>
</tr>
</tbody>
</table>
Figure 3.1 Growth curves in bioreactors of 1.0 l liquid volume at 80 rpm containing different growth media.
Figure 3.2  Monoclonal antibody concentration for the 1.0 l bioreactor cultures shown in Fig. 2.1.
Figure 3.3 Cell concentration versus time for a 1.0 l bioreactor culture containing 1% serum. Reactor was operated in batch mode until the point indicated by the first arrow, when the reactor was switched to continuous operation, and fresh medium was added at a dilution rate of 0.018 h⁻¹. At the second arrow, the serum concentration was raised to 5%, then allowed to wash out of the reactor.
Figure 3.4  Serum concentration for continuous run shown in Fig. 3.3. At hour 285, 40 ml of serum was added to the reactor, then allowed to wash out at a dilution rate of 0.018 h⁻¹.
Figure 3.5 Initial cell concentration in each batch run versus the hour at which the agitation speed in the reactor was increased. The time before the agitation increase is roughly equal to the length of the lag phase.
Figure 3.6 Typical reactor run. The two reactors contained 1.0 l of media and were inoculated and operated in parallel. They were agitated at a speed of 60 rpm until the point indicated by the arrow, when the agitation speed was increased to 200 rpm. Filled symbols represent the exponential-growth portion of the curve used to calculate the apparent growth rates.
Using the previously described experimental protocol to perform experiments under different reactor conditions, the fluid-mechanical damage mechanisms to suspended hybridoma cells in the agitated bioreactor were studied. The medium used in the experiments reported in the chapter contained 1% FBS, 1% Nutridoma, and added glutamine and antibiotics as mentioned in Chapter 2. First, the cell response to various reactor conditions was examined. Second, characterization of the hydrodynamic environment under various reactor conditions was attempted. By comparing the cell response to the reactor fluid-mechanical conditions, the mechanisms by which cells are damaged can be better understood.

4.1 Cell Response to Various Reactor Configurations

When operated with a liquid volume of less than 2.0 l, the bioreactors used in this investigation contained both a gas phase and a liquid phase containing medium and cells. The form of the gas/liquid interface separating these phases depends upon the agitation rate and reactor volume. When operated with a volume of 2.0 l, the reactors were completely filled with liquid and had no gas headspace, although some
air could be entrained into the liquid during sampling or through leaks in the silicone tubing used to oxygenate the medium.

4.1.1 Cell growth at low agitation rates in the presence of a gas headspace

Using data from reactor runs containing a volume of 1.0 or 1.2 l, Table 4.1 lists $\mu_{\text{app}}$, $\mu_60$, and the normalized apparent growth rate ($\mu_{\text{app}}/\mu_60$). From this table, the larger uncertainty intervals in $\mu_{60}$ than in $\mu_{\text{app}}$ are evident. For the reactor runs containing a volume of 1.0 or 1.2 l, the apparent growth rate of the cells as a function of agitation rate is shown in Fig. 4.1. The positive growth rates are shown on an expanded scale, which is different than the scale used for the negative growth rates. The error bars represent the 95% confidence limit for a regression parameter. The results from the experiments with the 1.0 and 1.2 l reactor volumes have been grouped together because the macroscopic fluid-mechanical characteristics of the reactor appear visually similar at these volumes. In addition, the response of the cells in these two volumes appears similar at the agitation rates shown in Fig. 4.1.

Up to approximately 190 rpm, $\mu_{\text{app}}$ appears to decrease slowly with increasing agitation speed, as shown on the expanded scale for the positive growth rates. At 190-220 rpm, $\mu_{\text{app}}$ drops dramatically to well below zero. Net positive
growth in the 1.0 or 1.2 l reactor does not occur at agitation rates greater than 220 rpm. In contrast to agitation speeds below 190 rpm, above 190-220 rpm cell damage increases much more rapidly that the cells' ability to reproduce, as demonstrated by the large negative $\mu_{\text{app}}$ values. The large scatter in the apparent growth rates seen around 190-220 rpm suggests a delicate balance between cell growth and cell death due to fluid mechanical forces at these agitation speeds. Since 190-220 rpm marks the onset of greatly reduced $\mu_{\text{app}}$, a slight difference in the agitation speed or the physiological condition of the cells may result in a different response at agitation speeds around 190-220 rpm. Because the differences in growth rates at this speed range are greater than the error bars of the data points, the different growth rates seen at 190-220 are not merely scatter in growth rate data at a given speed due to measurement uncertainties.

Shown without error bars, the apparent growth rate and growth rate at 60 rpm are compared in Fig. 4.2. For runs at 60 rpm throughout the batch culture, the values of $\mu_{60}$ and $\mu_{\text{app}}$ are, by definition, identical. Also, above 60 rpm and below 190-200 rpm, $\mu_{60}$ and $\mu_{\text{app}}$ seem to show the same trend, despite the uncertainty in the $\mu_{60}$ values. When hydrodynamic damage to the cells becomes severe, in the 190-220 rpm range, no correlation between the $\mu_{\text{app}}$ data and the $\mu_{60}$ data appear evident. However, the $\mu_{60}$ data in the 190-220 agitation range
do confirm that the cells were actively growing before the higher agitation speeds were imposed, and, thus, act as an "internal" control.

Fig. 4.3 shows the normalized growth rates, which are equal to $\mu_{\text{app}}$ divided by $\mu_{60}$, for each batch run in the 1.0 and 1.2 l reactors. Fig. 4.3 also shows the apparent growth rates, which have been re-scaled by dividing by a fixed growth-rate value of 0.055 h$^{-1}$, for each batch run to allow direct comparison with the normalized growth rates. The normalized growth rates ($\mu_{\text{app}}/\mu_{60}$) appear to remain constant, close to a value of 1.0, up to an agitation speed of 190-220 and do not show the slight decline seen in the apparent growth rates. If a simple model is used in which a death rate is defined such that $\mu_{\text{app}}$ is the difference between a fixed growth rate, $\mu_0$, and the death rate, $k$, ($\mu_{\text{app}} = \mu_0 - k$) the results shown in Figs. 4.2 and 4.3 indicate that, over a range of agitation speeds in which $\mu_{\text{app}}$ varies from positive values to greatly negative values, changes in $\mu_{\text{app}}$ can be attributed more to changes in $k$ than to any changes that might occur in $\mu_0$. In a non-damaging hydrodynamic environment where $k$ is small, any differences in $\mu_{\text{app}}$ may be due to actual changes in the intrinsic growth rates, since these differences were factored out when $\mu_{\text{app}}$ was normalized using $\mu_{60}$. Therefore, $\mu_{60}$ might be a more representative measure of the intrinsic growth rate of the cells in different batch cultures than the fixed growth rate used in the
aforementioned simple model. However, more sensitive measurements of $\mu_{60}$ are needed to confirm this tentative conclusion.

Since the power input to the reactor is a function of reactor volume, and since the amount of gas entrainment is also a function of reactor volume, the growth of cells in reactors containing different volumes of media was investigated. The relationship of power input to reactor volume is shown in Eq. (1.3), where the power input per mass of fluid is inversely proportional to the volume available for energy dissipation, $V_d$, which may be taken as the reactor volume. In the next section, the amount of bubble entrainment is shown to be inversely proportional to the liquid height above the impeller, which increases with increasing volume for a given reactor. Data from reactor runs at 200 and 220 rpm are presented in Table 4.2. Some values in Table 4.2 represent the average growth rate from several batch runs at the same volume and agitation speed. The error estimates represent the 95% confidence interval for a regression parameter or the average confidence interval, as explained in section 2.8. As can be seen from Table 4.2, the apparent growth rate is negative at agitation speeds of 200 and 220 rpm for both 1.0 and 1.2 l volumes, but increasing the volume of the reactor above 1.2 l results in positive apparent growth rates at speeds of both 200 and 220 rpm.
4.1.2 Cell growth at high agitation rates in the absence of a gas headspace

To separate the effects on cell growth due to forces in the bulk of the liquid phase from effects due to interactions with gas/liquid interfaces, several experiments were performed in reactors operated without a gas headspace. In this case, the volume in the reactor was increased to 2.0 l, which completely filled the reactor and brought the fluid level in contact with the reactor headplate. Without any gas headspace, a vortex could not form regardless of the agitation rate. As mentioned previously, each reactor was equipped with 6 m of thin-walled silicone tubing wrapped around a stainless steel support cage to provide oxygenation and pH control. Because the support cage held the tubing within 1 cm of the reactor wall, the fluid region around the impeller remained largely unchanged. Since the flow in the vicinity of the impeller was unchanged, the hydrodynamic forces due to agitation are probably similar in reactors with and without the silicone tubing.

Although the filled reactors eliminated the formation of a vortex, some air was introduced into the reactors either through a leak in the silicone tubing or as samples were removed from an opening in the reactor headplate. At the high agitation speeds used in the filled reactors, any air which entered the reactor was quickly entrained into the liquid in the form of very small bubbles. To eliminate the
entrainment of bubbles, the reactors were further modified
with a sampling system that allowed the removal of sample
without opening a port in the headplate. In addition to the
sampler, a reservoir was attached so that fresh medium could
flow into the reactor as sample medium was removed. Thus,
sample could be removed without allowing the entrance of any
air into the reactor. Since the amount of fresh medium that
was added to the reactor with each sample was small, about 5
ml, the effect of culture dilution due to the addition of
fresh medium was assumed to be negligible. Cell growth in
the filled reactors was studied in the presence of entrained
gas in the form of small bubbles as well as in the absence of
any gas phase.

Figure 4.4 shows the results of experiments using the
filled reactors in which many fine bubbles were formed.
Arrows indicate the point at which the agitation was
increased to the indicated speed. The points indicated by
the filled symbols were taken to be the exponential growth
phase and were used to calculate the apparent growth rate.
To find the range of agitation rates which would result in
slower apparent growth, the agitation rate was increased
several times during each batch run. After an increase in
agitation, 2 or 3 points were taken to determine if the cell
concentration was increasing or decreasing. The agitation
rate was then increased again. Thus, several agitation
conditions could be tested during each batch run, although
the measurement of the growth rate at each agitation rate was somewhat less accurate. However, accurate growth rate measurements were obtained at select agitation rates using additional experiments at a single speed. In Fig. 4.4a, the agitation rate was increased up to 450 rpm with cell growth staying constant at a rate of $0.0511 \pm 0.0042 \, h^{-1}$. Cell growth at a rate of $0.0417 \pm 0.0035 \, h^{-1}$ was unaffected in another run, shown in Fig. 4.4b, by agitation speeds from 450 up to 600 rpm. These growth rates were calculated using points from several speeds, since the increase in agitation speeds did not affect the apparent exponential growth of the cells. The growth rates at speeds of up to 600 rpm were not significantly different than the rates under mild agitation conditions shown in Fig. 4.1, even though 600 rpm is significantly higher than other speeds reported in the literature as damaging to the growth of animal cells. Not until the agitation rate was increased to 800 rpm did a decrease in apparent growth of the cells occur, as shown in Fig. 4.4c. The apparent growth rates of cells at 800 and 900 rpm were calculated from data taken at a single speed only, as shown in Figs. 4.4c and 4.4d, and were found to be $0.0015 \pm 0.0047 \, h^{-1}$ and $-0.0197 \pm 0.0084 \, h^{-1}$. In all runs containing the silicone tubing aeration system, a large jump in cell concentration occurred immediately following the initial increase in agitation from 60 rpm. This jump is the result of the release into the bulk fluid of cells growing loosely
attached in the interstices between the rows of silicone tubing. In summary, cell growth was unaffected by speeds up to 700 rpm in reactors with no gas headspace and where no vortex was formed, even though many small bubbles were entrained and kept in suspension.

Figure 4.5 shows the result of a batch reactor run at 800 rpm in which no bubbles were present in the reactor medium. The cells grew with an apparent growth rate of 0.0155 ± 0.0055 h⁻¹ compared to the much lower rate of 0.0015 ± 0.0047 h⁻¹ obtained in the presence of entrained bubbles at 800 rpm as shown in Fig. 4.4c. The growth rate at 800 rpm without entrained gas was significantly lower than typical growth rates under conditions of no agitation or gentle agitation which averaged approximately 0.050 h⁻¹. The intermediate growth rate suggests that, at agitation rates above 700 rpm, some cell damage is due to hydrodynamic forces within the turbulent liquid in addition to forces associated with the motion, coalescence, and breakup of gas bubbles within the liquid. The results of Fig. 4.5 demonstrate that cells can grow at agitation speeds up to 800 rpm, as long as a vortex and bubble entrainment can be eliminated. Such agitation rates are more typical of microbial bioreactors and have not been previously used in cell culture reactors.
4.2 Hydrodynamic Environment in Various Reactor Configurations

To determine the cause of the different cell responses under the different fluid-mechanical regimes discussed in Section 4.1, the hydrodynamic environment within the bioreactor was investigated in more detail. As in the preceding section concerning the cell response, the reactor fluid-mechanical environments have been divided into two groups, those operated at a volume less than 2 l with a gas headspace and those operated at a volume of 2 l with no gas headspace. The effect of changing volume and changing agitation rates on the vortex formation and bubble entrainment within reactors of less than 2 l was examined macroscopically. Similarly, the completely filled reactors were examined, both in the presence and absence of any entrained gas bubbles. Specifically, the size of the liquid vortex, and the size, concentration, and motion of entrained gas bubbles in the reactors were investigated.

4.2.1 Hydrodynamic environment at low agitation rates in the presence of a gas headspace

For reactor volumes of less than 2 l, damage to the cells occurred at agitation speeds of 190-220 rpm as opposed to damage in the completely filled reactors which occurred at over 700 rpm. Since the main difference in these reactor configurations is the interaction of gas bubbles with a free
gas/liquid interface at the surface of the reactor liquid, an examination of this surface was carried out. A suspension of cells in liquid media at the concentrations used in this investigation was not sufficiently transparent to allow direct visual observation of the vortex and bubble entrainment. Therefore, visual observations were made using cell-free media at volumes of 1.0 and 1.5 l and agitation rates up to 220 rpm. Any vortex or entrained bubbles that were present were rapidly moving and fluctuating, making accurate observation by eye difficult. High speed photographs were taken of the reactors using a method developed by Handa et al. [Handa, 1988]. To distinctly visualize the bubbles and vortex, the reactor was lit from the sides with a dark background in place behind the reactor. Using a 35-mm camera and T-MAX P3200 film (Eastman Kodak, Rochester, NY), pictures were taken to visualize the vortex shape and depth as well as the position and size of any entrained bubbles.

Photographs of the reactor containing 1.0 and 1.5 l of cell culture medium at several agitation rates are shown in Figs. 4.6 and 4.7. Even at the lowest agitation rate photographed, a vortex is present along the impeller shaft. At all speeds, the vortex is irregular in shape with several "lobes" present at the lower tip of the vortex. Even up to 220 rpm, though, the vortex does not come into contact with the blades of the impeller. The tip of the vortex was
unstable and rapidly fluctuating with bubbles detaching at the lower tip of the vortex, especially at higher speeds.

As seen in the photographs for the 1.0 l reactor volume, at 150 rpm no bubbles detach from the vortex and become entrained into the culture medium. Starting at 180 rpm, some bubbles are seen close to the vortex tip and only a few bubbles exist outside of the impeller region at any given time. From the photograph at 200 rpm, the pattern by which bubbles are entrained into the reactor can be seen. Bubbles detach from the irregular lobes of the bottom portion of the vortex, spiral down along the impeller shaft until they reach the disk-shaped paddle support, and are swept radially out into the culture medium outside the vicinity of the impeller. At 220 rpm, an even greater number of bubbles are present along the impeller shaft and along the upper surface of the paddle support, as well as throughout the culture medium. Because an accumulation of bubbles at the lower portion of the vortex is present, bubbles probably detach and re-attach at the vortex tip. Any bubbles swept outside of the impeller region probably rise and burst at the gas/liquid interface at the surface of the reactor liquid.

Due to the presence of probes within the reactor, an accurate estimation of the quantity of bubbles in the reactor at a given time is difficult. At 150 rpm probably fewer than 20 bubbles are entrained, but at 220 rpm several hundred bubbles appear to be present. Although the culture medium
contains dissolved proteins, no foam formation or accumulation of bubbles was seen with time. Therefore, the formation and bursting of bubbles is assumed to be at steady-state within the reactor. By simply using a ruler to measure the size of the bubbles in the photograph, the size of bubbles was found to be approximately 0.5 to 3 mm in diameter. In addition, not all of the bubbles appeared to be perfectly spherical. The hydrodynamic forces within the reactor distort some of the bubbles into oblong shapes.

In the reactors containing 1.5 l volume, a few bubbles are seen close to the irregular vortex tip starting at a speed of 150 rpm. These bubbles were formed when medium was added to the reactors prior to agitation and are present even in photographs of the quiescent reactor. Not until a speed of 220 rpm is reached are a few bubbles drawn down along the impeller shaft into the reactor liquid. The size of bubbles appears the same as in the 1.0 l reactor. However, the vortex in the 1.5 l volume is small and has a depth only about half that of the vortex in the 1.0 l volume.

The onset of gas entrainment seen in the photographs above agrees well with the other researcher's calculations of the agitation speed necessary for "sucking in" gas bubbles at the bottom of a vortex. In general, it has been shown [van Dierendonck et al., 1971] that the gas hold-up in stirred tank reactors without a gas distributor increases as a function of agitation speed in the manner shown in Fig. 4.8.
Once a characteristic agitation speed for sucking in gas bubbles, \( n_0^* \), has been reached, the gas hold-up increases linearly with agitation rate. However, some gas bubbles begin to be entrained at a speed lower than \( n_0^* \). By investigating systems with varying liquid properties of density, viscosity, and surface tension as well as varying reactor dimensions, van Dierendonck and co-workers [1971] found the following correlation for the characteristic agitation speed necessary for sucking in gas bubbles:

\[
\left( \frac{\mu_f n_0^* d_i^2}{D \sigma} \right) \left( \frac{\rho_f g^3}{\mu_f^4} \right)^{1/4} = 2.0(h/D)^{1/2}
\]

(4.1).

Here \( d_i \) is the impeller diameter; \( h \) is the liquid height above the stirrer; \( D \) is the tank diameter; \( \sigma \) is the surface tension; \( g \) is the acceleration due to gravity; \( \mu_f \) is the liquid viscosity; and \( \rho_f \) is the liquid density. In the following calculations, the surface tension and density of water was used as the fluid surface tension and density. In the dimensionless groups paired together in Eq. (4.1), viscosity cancels out and was not found by van Dierendonck et al. [1971] to significantly effect \( n_0^* \). Equation 4.1 was applied to the bioreactors used in this investigation in the following calculations. At a volume of 1.0 l of medium, \( h \) was equal to 0.022 m and the characteristic stirrer speed was calculated to be 204 rpm. In a reactor filled with 1.5 l of medium, \( h \) was equal to 0.059 m and the characteristic stirrer speed was calculated to be 333 rpm. Van Dierendonck and co-
workers found that the minimum stirrer speed necessary to suck in gas bubbles, $n_{\text{min}}$, was 78% of the characteristic speed. Thus, $n_{\text{min}}$ was 159 rpm for the 1.0 l reactor and 260 for the 1.5 l reactor used in this investigation. Although the correlation predicts some bubble entrainment at the minimum stirrer speed, $n_{\text{min}}$, a significant amount of gas entrainment does not occur until the characteristic speed, $n_0^*$, is reached, as shown in Fig. 4.8.

The calculated values for the 1.0 l reactor agree quite well with the experimental results as seen in the photographs, although some bubbles were entrained at 150 rpm, which was a little lower than the predicted $n_{\text{min}}$. The onset of significant cell damage in the reactor, as previously presented, occurs near the characteristic speed for bubble entrainment. For the 1.5 l reactor, agitation speeds above 220 rpm were not investigated. However, a few bubbles were entrained at a rate of 220 rpm, somewhat lower than the predicted $n_{\text{min}}$.

### 4.2.2 Hydrodynamic environment at high agitation rates in the absence of a gas headspace

The use of completely filled reactors eliminated the formation of a vortex, but in some runs gas was incidentally introduced into the reactor. Small amounts of gas were introduced as samples were periodically removed and also, possibly, through a leak in the silicone oxygenation system.
At the high agitation speeds used in the filled reactors, any air within the reactor was quickly entrained into the liquid to form very small bubbles. As the run progressed, and more samples were taken, the concentration of bubbles within the reactor increased. In the batch reactor runs shown in Fig. 4.4, the rapidly-moving, very small bubbles could not be individually seen in the agitated reactor, and gave the reactor medium the appearance of an opaque emulsion.

Several techniques were used to characterize the size and concentration of entrained bubbles in the filled reactors. By pipetting culture medium from the agitated reactor using a standard 5 ml pipet, a representative sample of medium with entrained bubbles could be obtained. Bubbles within the sample rose to the surface of the liquid within the pipet and, due to their small size, formed a stable foam. The volume of the foam layer as well as the total sample volume could then be measured in the pipet to calculate the percentage of volume occupied by the bubbles. Using this technique, the bubbles were estimated to account for approximately 2% of the fluid volume during a typical run.

To get an estimate of the bubble size, the number of bubbles aligned linearly between divisions of the pipet were counted. The distance between divisions divided by the number of bubbles gave a rough estimate of bubble diameter. Using this technique, the average bubble diameter was found to be approximately 150 μm. A more accurate measurement of
bubble size was obtained by placing a sample of bubbles from the reactor in a hemacytometer and observing them microscopically. Bubbles in the hemacytometer remained stable for more than 10 min. Since the gap in the hemacytometer was 0.1 mm, only bubbles with diameters less than 0.1 mm remained spherical within the chamber. Therefore, the reported bubble diameters greater than 0.1 mm using the hemacytometer are only rough estimates. Using the hemacytometer, a range of bubble sizes was seen from 50 μm to approximately 300 μm. If a volume fraction of bubbles of 2 percent and an average bubble size of 150 μm are taken, the concentration of bubbles can be estimated to be on the order of 1500 bubbles/ml. The bubble concentration would range from 500 to 5000 bubbles/ml for average bubble sizes of 200 to 100 μm. According to these values, the batch reactor cultures represented in Fig. 4.4 contained millions of small, stable, suspended bubbles.

The 800 rpm culture shown in Fig. 4.5, on the other hand, contained no entrained bubbles and appeared visually similar to gently agitated cultures, which was quite different than the emulsion-like appearance of the reactors which contained bubbles agitated at high speed. By using the previously-described sampler in conjunction with a reservoir to automatically add fresh medium to replace the sample volume, bubble entrainment was completely avoided.
Since some reduction in cell growth was seen in the reactor even without entrained bubbles, some calculations were carried out to characterize the bioreactor turbulence in order to provide further insight into the cell damage mechanism. Equations (1.1) and (1.3) were used to calculate the characteristic size of the smallest turbulent eddies (Kolmogorov eddies) within the bioreactor. However, two factors make the estimation of the smallest eddies somewhat ambiguous. First, the reactor volume in which energy is dissipated may be taken as the entire reactor volume or merely the volume in the vicinity of the impeller, roughly the impeller diameter cubed [Cherry and Papoutsakis, 1988]. Second, the impeller power number is found using correlations such as those described by Nagata [Nagata, 1975]. The impellers used in this research are not standard marine propellers or flat-bladed, Rushton turbines, thus making the estimation of an impeller power number from existing correlations ambiguous. Therefore, the eddy size versus agitation rate is shown in Fig. 4.9 for a range of power numbers and two different reactor volumes. As can be seen from this figure, an estimate of the eddy size at the agitation rate of 800 rpm may range from 12 to 25, depending upon the assumptions made. The lower end of this size range, however, is equivalent to the size of the suspended cells, which are approximately 12-15 μm in diameter.
4.3 Discussion of Damage Mechanism

In the 1.0 and 1.2 l reactors at 190-220 rpm, bubbles rapidly detach from the bottom of a fluctuating vortex, are entrained, swirl throughout the liquid volume, then rise and disengage at the liquid surface. These conditions result in a rapid decline in cell concentration as a result of irreparable cell damage. By increasing the reactor volume and decreasing the size of the vortex, cell damage can be controlled even at very high agitation rates. For the volume increases used in the present work, it is not valid to argue that the reduced cell damage may be attributed to a larger volume available for energy dissipation, $V_d$--that is, to a lower power input per unit volume, $\varepsilon$, and larger Kolmogorov-scale eddy size, $\eta$. From Eq. (1.3), it can be seen that $\varepsilon$ is inversely proportional to $V_d$ while it increases with the third power of the agitation rate, $n$. Thus, even if one assumes that the agitation power is dissipated in the entire liquid volume, $V_d$ increases by not more than 100 percent, from 1.0 or 1.2 to 2.0 l. On the other hand, $n$ was increased by at least 300 percent, from 200 to 600-700, in the completely-filled reactor without observing significant cell damage. Therefore, although an increase in volume in the completely-filled reactor may have reduced $\varepsilon$ by half, a concurrent increase in agitation speed increased $\varepsilon$ 27 times without any decrease in cell growth.
Thus, the results show that cell damage in the agitated bioreactor in the agitation speed range of 190-220 rpm is associated with the presence of a gas/liquid interface within the reactor. In the absence of a vortex, the presence of a gas/liquid interface in the form of entrained bubbles down to 50 \( \mu m \) in size does not necessarily cause cell damage, as was observed in the completely-filled reactors at agitation speeds below 700 rpm. Only when entrained bubbles interact with a freely moving gas/liquid interface, such as that present between the culture medium and gas headspace, does significant cell damage occur. Neither the small size nor the large concentration of bubbles appear to be by themselves detrimental to cell growth. On the contrary, at lower agitation rates, much fewer and much larger bubbles cause a large amount of cell damage when a gas phase above the medium is present.

The onset of cell damage corresponded quite well with the agitation speed necessary for entrainment of bubbles as presented by van Dierendonck and co-workers [van Dierendonck et al., 1971]. In reactors containing 1.0 and 1.2 l volumes, damage to cells did not occur until the minimum speed necessary for bubble entrainment, \( n_{\text{min}} \), had been surpassed. Significant cell damage, as indicated by greatly negative apparent growth rates, occurred over a range of agitation speeds (190-220 rpm) around the characteristic speed necessary for bubble entrainment, \( n^*_c \) (203 rpm). Therefore,
n_{min} may be useful as a conservative predictor of the maximum speed obtainable in stirred reactors without causing significant cell damage. More experiments investigating the effect of impeller geometry, vessel geometry, and liquid volume on bubble entrainment and cell damage are needed to confirm the utility of the n_{min} correlation. In reactors containing 1.5 to 1.8 l volumes of culture medium, negative apparent growth rates similar to those found in the 1.0 and 1.2 l reactors at 190-220 rpm were not observed at the agitation speeds investigated. Since the correlation of van Dierendonck et al. predicted a minimum speed of 260 and a characteristic speed of 333 rpm for bubble entrainment in the 1.5 l reactor, further experiments at higher agitation speeds are necessary to show any relationship of n_{min} or n_0* to cell damage at the higher volumes.

Although a change in the viscosity of the media would be expected to change the shape of the vortex in the agitated reactor [Bird et al., 1960], Eq. 4.1 does not indicate a correlation with viscosity. The viscosity term cancels out when the two dimensionless groups used by van Dierendonck are combined. Equation 4.1 can be re-cast using more conventional dimensionless groups as follows:

\[(W_{em} \cdot Fr_m)^{1/4} = 2.0(h/D)^{1/2}\]  

(4.2).
Here, \( W_{em} \) and \( Fr_m \) are the Weber and Froude numbers which have been modified by using the term \( d^2/D \) as the characteristic length:

\[
W_{em} = \frac{n^2(d_i^2/D)^3}{\sigma} Fr_m = \frac{n^2(d_i^2/D)}{g}
\]  

The value of \( n \) found using equation 4.2 is equal to \( n_0^* \), the characteristic agitation speed for bubble entrainment. Since the viscosity term cancelled out of the equation, the Reynolds number is not an important dimensionless group in the original correlation. Apparently, the dependence of characteristic speed for bubble entrainment on the viscosity was too small to be included in the correlation. At the speeds necessary for bubble entrainment, flow within the reactor is in the turbulent range, where the power number, \( N_p \), does not change significantly with an increase in the Reynolds number.

On the other hand, the presence of the Weber number indicates that the surface tension plays an important role in determining when significant bubble entrainment, and, hence, cell damage, may occur. Likewise, the presence of the Froude number indicates that gravitational effects are important in predicting cell damage. In addition, \( n_0^* \) depends on the surface tension to only the \( 1/4 \) power. Therefore, a large
change in surface tension would be necessary to counteract any change in reactor configuration or impeller speed.

At 800 rpm, which corresponds to a Kolmogorov eddy size comparable to the cell size of 12-15 μm, cells grow at a very low apparent growth rate when large amounts of small bubbles are entrained in the reactor liquid. In the absence of any entrained gas, the apparent growth rate at 800 rpm is positive but lower than growth under gentle agitation conditions. Therefore, at agitation rates above 600-700 rpm, some cell damage can be attributed to hydrodynamic forces in the turbulent liquid even the absence of a gas phase. In the presence of large numbers of small, entrained bubbles, and at agitation rates above 600-700 rpm, cell damage is apparently due to both bulk-liquid forces and the presence of bubbles. As with other systems of suspended cell "particles"—which include 150-180 μm microcarrier beads [Croughan et al., 1988; Cherry and Papoutsakis, 1989] and 80 μm protozoa cells [Midler and Finn, 1966]—damage of the present 12-15 μm hybridoma cells due to interactions with the turbulent bulk liquid correlates with a Kolmogorov eddy size, η, similar to the "particle" size. Although the ratio of η to cell size may be used as a predictor of cell damage, it provides no details as to how cells are damaged by their interaction with these eddies, or even a proof that a direct cell to eddy interaction indeed exists.
The mechanism by which the interaction of bubbles with a free gas/liquid interface damage cells has not been determined. The area of disengagement of bubbles at the gas/liquid surface has been suggested to be the region of hybridoma cell damage in bubble column reactors [Handa et al., 1987]. Two damage mechanisms were proposed—rapid oscillations due to bursting bubbles and shear forces in draining films in foams. For insect cells grown in a bubble column reactor, it has been suggested that cell damage occurs at both the area of gas injection (sparger) and the area of surface disengagement of the bubbles [Tramper et al., 1988]. Damage was not caused by shear forces resulting from rising bubbles. These mechanisms of cell damage are consistent with the results of the research presented here. In this thesis, cell damage in the presence of a gas/liquid interface only occurred when bubbles could interact with the interface. This damage occurred even in the absence of a foam layer and, hence, the absence of draining liquid films. Also, damage occurred without gas injection through a sparger directly into the liquid medium. These findings in combination with the aforementioned research of other investigators suggest that cells are damaged as bubbles burst at the liquid surface, possibly due to large shear stresses or pressure fluctuations close to the bursting bubble. These forces are distinct from those produced in the liquid media as a direct consequence of power input via the impeller.
Another mechanism of cell damage, which is consistent with the experimental results of the present research, may be possible. In the 1.0 or 1.2 l reactors, the entrained bubbles at agitation speeds of 190-220 rpm are elongated and fluctuating, as are the lobes present at the vortex tip. At lower speeds where no significant vortexing occurs, a fluctuating gas/liquid interface is not present. Likewise, in the completely-filled reactors, entrained bubbles are very small, and, due to their small size, the hydrodynamic forces may not cause significant fluctuation of the gas/liquid interface of bubbles. As explained below, the interface of entrained bubbles is more likely to be rigid for smaller bubbles than for larger bubbles for a given system.

A complex mixture of proteins, salts, lipids, and other components, such as is found in cell culture media, can cause a surface tension gradient to exist across the surface of a moving bubble. This surface tension gradient is due to the change in conformation of media components, especially proteins, at the bubble surface at a rate slower than the changes in equilibrium of the bubble interface. The equilibrium of the bubble interface changes as the bubble expands and contracts due to hydrodynamic forces in the turbulent reactor fluid. For a bubble to maintain a rigid surface, the surface tension difference from front to back across the bubble interface must be greater than the shear force exerted over the bubble surface [Prins and van't Riet,
Therefore, for a given reactor system, larger bubbles are more likely to have a mobile surface than small bubbles. Thus, in the two conditions in which cell damage around a gas liquid interface does not occur—very low speeds in the presence of a gas headspace and high speeds with no headspace but very small bubbles—the gas/liquid interface that is present is less likely to be mobile than under conditions where cell damage was found to occur.
Table 4.1 Data from Figs. 4.1, 4.2, and 4.3 for cultures of 1.0 and 1.2 l liquid volume at various agitation speeds. Error estimates represent the 95% confidence interval for a regression parameter.

<table>
<thead>
<tr>
<th>rpm</th>
<th>Vol. (l)</th>
<th>Apparent Growth Rate (μapp) (1/h)</th>
<th>Growth Rate at 60 rpm (μ60) (1/h)</th>
<th>Normalized Growth Rate (μapp/μ60)</th>
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</thead>
<tbody>
<tr>
<td>0</td>
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<td>0.0442 ± 0.0021</td>
<td>1.00</td>
</tr>
<tr>
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<td>0.0436 ± 0.0015</td>
<td>1.00</td>
</tr>
<tr>
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<td>1</td>
<td>0.0389 ± 0.0050</td>
<td>0.0364 ± 0.0011</td>
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</tr>
<tr>
<td>60</td>
<td>1</td>
<td>0.0442 ± 0.0021</td>
<td>0.0436 ± 0.0015</td>
<td>1.00</td>
</tr>
<tr>
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<td>1</td>
<td>0.0436 ± 0.0015</td>
<td>0.0364 ± 0.0011</td>
<td>0.99</td>
</tr>
<tr>
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<td>0.0302 ± 0.0011</td>
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</tr>
<tr>
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<td>0.71</td>
</tr>
<tr>
<td>200</td>
<td>1</td>
<td>0.0232 ± 0.0012</td>
<td>0.0400 ± 0.0000</td>
<td>-6.06</td>
</tr>
<tr>
<td>225</td>
<td>1</td>
<td>-0.1998 ± 0.0311</td>
<td>0.0380 ± 0.0016</td>
<td>-5.26</td>
</tr>
<tr>
<td>250</td>
<td>1</td>
<td>-0.3696 ± 0.1540</td>
<td>0.0500 ± 0.0036</td>
<td>-7.39</td>
</tr>
<tr>
<td>180</td>
<td>1.2</td>
<td>0.0425 ± 0.0037</td>
<td>0.0303 ± 0.0029</td>
<td>0.63</td>
</tr>
<tr>
<td>190</td>
<td>1.2</td>
<td>-0.0564 ± 0.0076</td>
<td>0.0347 ± 0.0017</td>
<td>-0.74</td>
</tr>
<tr>
<td>190</td>
<td>1.2</td>
<td>-0.0354 ± 0.0199</td>
<td>0.0478 ± 0.0014</td>
<td>-0.74</td>
</tr>
<tr>
<td>200</td>
<td>1.2</td>
<td>-0.0575 ± 0.0085</td>
<td>0.0468 ± 0.0021</td>
<td>-1.23</td>
</tr>
<tr>
<td>220</td>
<td>1.2</td>
<td>0.0272 ± 0.0050</td>
<td>0.0412 ± 0.0011</td>
<td>0.66</td>
</tr>
<tr>
<td>220</td>
<td>1.2</td>
<td>0.0020 ± 0.0036</td>
<td>0.0414 ± 0.0008</td>
<td>0.05</td>
</tr>
<tr>
<td>220</td>
<td>1.2</td>
<td>-0.1789 ± 0.0013</td>
<td>0.0446 ± 0.0010</td>
<td>-4.01</td>
</tr>
<tr>
<td>220</td>
<td>1.2</td>
<td>-0.1521 ± 0.0488</td>
<td>0.0516 ± 0.0021</td>
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</tr>
<tr>
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<td>1.2</td>
<td>-0.1022 ± 0.0036</td>
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</tr>
<tr>
<td>220</td>
<td>1.2</td>
<td>-0.0729 ± 0.0032</td>
<td>0.0469 ± 0.0155</td>
<td>-1.55</td>
</tr>
</tbody>
</table>
Table 4.2  Effect of liquid volume in the reactor on the apparent growth rate for agitation speeds of 200 and 220 rpm. Error estimates represent the 95% confidence interval for a regression parameter.

<table>
<thead>
<tr>
<th>Volume (l)</th>
<th>Apparent Growth Rate (h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>-0.0243 ± 0.0173*</td>
</tr>
<tr>
<td>1.2</td>
<td>-0.0575 ± 0.0085</td>
</tr>
<tr>
<td>1.7</td>
<td>0.0469 ± 0.0019</td>
</tr>
<tr>
<td>1.2</td>
<td>-0.0795 ± 0.0082**</td>
</tr>
<tr>
<td>1.5</td>
<td>0.0280 ± 0.0034</td>
</tr>
<tr>
<td>1.8</td>
<td>0.0424 ± 0.0025</td>
</tr>
</tbody>
</table>

* mean rate from 5 batch runs
** mean rate from 6 batch runs
Figure 4.1  Apparent growth rate as a function of agitation speed in 1.0 l (open symbols) and 1.2 l (closed symbols) reactors. Positive apparent growth rates are shown on an expanded scale. Error bars represent the 95% confidence interval for a regression parameter. The hand-drawn curve is shown to emphasize the trend in apparent growth rate.
Figure 4.2 Apparent growth rate (o) and growth rate at 60 rpm prior to the agitation increase (+) as a function of agitation speed for 1.0 and 1.2 l reactor cultures. Positive growth rates are shown on an expanded scale.
Figure 4.3 Normalized apparent growth rate (o) and re-scaled apparent growth rate (+) as a function of agitation speed for 1.0 and 1.2 l reactors. Dashed line indicates a normalized growth rate equal to unity.
Figure 4.4  Four growth curves (a–d) for the completely filled reactors containing a large number of small, suspended bubbles. Arrows indicate the points at which the agitation speed was increased to the given rpm values. Solid symbols represent the points used to calculate the apparent growth rates.
Figure 4.5  Growth curve for the completely filled reactor in the absence of any entrained bubbles. The arrow indicates the point at which agitation was increased from 60 to 800 rpm. Solid symbols represent the points used to calculate the apparent growth rate.
Figure 4.6 High-speed photographs of reactors containing 1.0 l of sterile medium at 150 (a), 180 (b), 200 (c), and 220 (d) rpm. The reactor was in front of a dark background and was lit from both sides.
Figure 4.7 High-speed photographs of reactors containing 1.5 l of sterile medium at 150 (a), 180 (b), 200 (c), and 220 (d) rpm. The reactor was in front of a dark background and was lit from both sides.
Figure 4.8 Representative plot of gas hold-up in a stirred tank as a function of agitation speed. The minimum speed necessary for bubble entrainment, $n_{\text{min}}$, occurs at the point where the solid line meets the abscissa. The characteristic speed necessary for bubble entrainment, $n_o^*$, occurs at the point on the abscissa indicated by the dashed line, which is an extension of the linear portion of the curve. [Adapted from van Dierendonck, 1971: Fig. 4].
Figure 4.9 Kolmogorov eddy size as a function of agitation speed in the completely filled reactor, depending upon the volume available for energy dissipation, $V_d$, and the value of the power number, $N_p$. The top three curves are for $V_d$ equal to the reactor volume, $V$, and the bottom three curves are for $V_d$ equal to the area around the impeller, $d_i^3$. For each volume, the top curve is for $N_p$ equal to 1; the middle curve is for $N_p$ equal to 2; and the bottom curve is for $N_p$ equal to 3.
CHAPTER 5

Effect of Media Additives on Cell Growth and Implications Concerning the Damage Mechanism

The effect of several media additives on the apparent cell growth in the agitated bioreactor environment was investigated. The media additives used were several that have been reported in the literature as having some protective effect against agitation damage, including fetal bovine serum (FBS) and dextran. By examining the effect of these additives on cell growth and metabolism in the fluid environment described in the previous chapter, information can be gained concerning the protective effect of the media additives as well as a better understanding of the cell damage mechanism.

5.1 Effect of Dextran on Cell Growth and the Reactor Environment

Dextran is commonly used in cell-culture research as an inert, non-toxic additive for increasing the viscosity of media. In the investigation of shear-stress effects on cell growth in viscometers and capillary tubes, an increase in the medium viscosity by dextran addition increased the range of shear stresses which could be investigated in the laminar
flow regime [McQueen and Bailey, 1989; Abu-Resheh and Kargi]. Dextran has also been used in microcarrier cultures to analyze the effects of changing viscosity on the eddy size and corresponding cell damage [Cherry and Papoutsakis, 1989; Croughan et al., 1989]. In the present investigation, dextran is used to examine the effect of increased viscosity on the damage of freely suspended cells in agitated bioreactors.

5.1.1 Cell growth with dextran versus without dextran

As in the previous chapter, results from several experiments performed using a 1.0 or 1.2 l liquid volume in the reactor are grouped together in Fig. 5.1, which shows the effect of agitation speed on apparent cell growth in the presence of dextran in concentrations of 1, 2 and 3% w/v. Dextran-free control cultures from reactors operated in parallel with the dextran-containing cultures are included in Fig. 5.1 for comparison. The apparent growth rates from these dextran-free cultures were also shown in Fig. 4.1. In addition, Table 5.1 lists all of the growth rates from Fig. 5.1 as pairs of cultures grown in parallel. As with Fig. 4.1, the positive growth rates in Fig. 5.1 are shown on an expanded scale, and the error bars represent the 95% confidence limit for a regression parameter. The response of cells to agitation was also similar to the response shown in Fig. 4.1. Up to approximately 190-220 rpm, the apparent
growth rate decreases slowly with agitation speed, at least for cultures containing 2% dextran. At 190-220 rpm, though, the apparent growth decreases dramatically, dropping well below zero. At a concentration of 2%, dextran had no effect on the apparent growth rate of cells cultured without agitation in T-flasks. Figure 5.1 indicates that the precipitous drop in apparent cell growth occurs at a lower agitation speed for the 3% concentration of dextran than for the 2% concentration, although the control cultures with no dextran also show variation in the speed required to produce negative $\mu_{\text{app}}$. For the two cultures containing 1% dextran, the cell response was not significantly different than the 2% dextran cultures or the cultures shown in Fig 4.1.

Figure 5.2 shows $\mu_{\text{app}}$ and $\mu_{60}$ for cultures in 1.0 and 1.2 l volumes containing 1, 2, or 3% dextran. Growth rates of 0%-dextran control cultures are not included in this figure, since they are shown in Fig. 4.2. The growth rate values from Fig. 5.2 are listed in Table 5.2, along with the 95% confidence intervals. As with the 0%-dextran cultures shown in Fig. 4.2, the growth rate measured before the agitation was increased, $\mu_{60}$, varied in a similar fashion as the apparent growth rate measured after the agitation increase, $\mu_{\text{app}}$, for agitation speeds below 190-220 rpm. Therefore, the normalized growth rates ($\mu_{\text{app}}/\mu_{60}$) shown in Fig 5.3 are closer to a value of unity than the apparent growth rates shown scaled by a constant intrinsic growth rate ($\mu_{\text{app}}/\mu_{0}$). At
cultures with and without added dextran difficult to observe. To point out the differences between the cultures with different dextran concentrations, reactor runs performed in parallel must be compared. For runs performed in parallel, the state and concentration of inoculum was identical, and cultures with and without dextran were started at the same time. With this comparison only those effects due to the presence of a medium additive (dextran) are shown, assuming that all other effects due to variable inoculum and culture conditions are subtracted out.

The growth curves for all of the reactor runs included in Fig. 5.1 are shown in Figs. 5.5a-5.5q. The growth curves are grouped in pairs, each pair representing runs performed in parallel. In each pair of reactor runs shown in Fig. 5.5, the culture containing dextran grew with the same or lower apparent growth rate than the dextran-free culture. This observation is not evident when looking at the growth rates as shown in Fig. 5.1. To show more clearly the dependence of $\mu_{app}$ on the presence of dextran, Fig. 5.6 shows the difference between apparent growth rates in parallel reactor runs, that is, the apparent growth rate of cells in a control culture grown without dextran minus the growth rate of a parallel culture grown in the presence of dextran. Values near zero indicate no effect of dextran on growth, while positive values indicate slower apparent growth in the presence of dextran compared to cultures without dextran.
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From Fig. 5.6, it can be seen that the presence of dextran had no effect on cell growth up to around 180 rpm. At approximately 180 rpm, the negative effect of dextran on cell growth is apparent, as significantly positive growth-rate differences were obtained. In addition, cultures containing 3% dextran show a detrimental effect on growth at slightly lower agitation speeds than cultures containing 2% dextran. However, the parallel control cultures corresponding to the 3% dextran runs also show a detrimental effect on growth at a slightly lower agitation speed than the control cultures corresponding to the 2% dextran runs. This variation in control culture response indicates the uncertainty in the speed required to cause significant cell damage, and not necessarily an effect of dextran on that required speed. No significant effect of 1% dextran was seen at the rate of 200 rpm, the only rate at which 1% dextran was tested. In all parallel cultures studied, the apparent growth rate of cells with dextran was not greater than the apparent rate without dextran, as indicated by the absence of significantly negative values in Fig. 5.6.

All of the cultures described above were carried out with a 1.0 or 1.2 l volume. Thus, the detrimental effects on cell growth rates are due to interactions of entrained bubbles with gas/liquid interfaces. The effect of 2% dextran in the completely-filled, 2.0 l reactor without any bubbles at 800 rpm is shown in Fig. 5.7. The two growth curves
included in Fig. 5.7 do not represent experiments run in parallel but are, rather, consecutive experiments using the same reactor. The growth curves appear similar, and, indeed, the apparent growth rates for 0% and 2% dextran are approximately the same—0.0155 ± 0.0055 hr⁻¹ and 0.0170 ± 0.0032 hr⁻¹. So, in the absence of any entrained bubbles, cells are capable of growing in medium containing 2% dextran at the same apparent growth rate as cells in dextran-free medium at an agitation speed of 800 rpm. This is, however, a tentative conclusion that must be verified by additional experiments.

5.1.2 Effect of dextran on the reactor hydrodynamic environment

Dextran was used as a medium additive to increase the viscosity of the medium assuming that it does not significantly alter other medium properties. The fact that cell growth is unchanged in quiescent cultures containing dextran is evidence that dextran does not affect the nutritional environment of the cells. The effect of dextran on the physical properties of the medium, including osmolality, surface tension, and viscosity, was investigated. In addition, the effect of enhanced viscosity on the hydrodynamic reactor environment, including the effect on turbulence structure and bubble entrainment, was examined.
A relatively narrow range of medium osmolalities, between 260 and 320 mmol/kg, is recommended for animal cell culture. ["Osmolality...", 1987]. Dextran concentrations of up to 3% w/v (30 g/l) were needed to substantially increase the medium viscosity. Because the dextran used had a high molecular weight (129,000), addition of 30 g/l only increased the molarity of the medium by 0.023 mM. Since osmolality is generally a function of the total molarity of a solution, the addition of even 30 g/l of dextran was not expected to significantly change the osmolality. However, in culture media, the interaction between amino acids, proteins, salts, sugars, lipids, and other components makes prediction of the effect of added long-chain polymer on the osmolality uncertain. Therefore, an automatic osmometer (Osmette A, Precision Systems, Inc.) was used to check for any change in osmolality due to added dextran. The addition of dextran did not change the osmolality of the culture medium, which was found to be 341-344 mmol/kg.

The surface tension of the medium with added dextran was measured using a Wilhelmy balance. The apparatus and procedure for making this measurement using chromatography paper as a "Wilhelmy plate" have been detailed by Gaver [1988] and are repeated here. To make the surface tension measurement, a short strip of 2.54 cm wide chromatography paper was suspended from a balance (Mettler PM460, Mettler Instrument Co., Hightown, NJ) and allowed to contact the test
liquid and become saturated. The test fluid was held in a petri dish on a platform which could be precisely raised and lowered. While the Wilhelmy plate is in contact with the test liquid, the force measured by the balance was the sum of the weight of the saturated paper and the force on the paper due to the interfacial tension of the liquid. The force on the "Wilhelmy plate" due to interfacial tension is equal to $2\sigma L \cos \theta$, where $\sigma$ is the liquid surface tension, $2L$ is the wetted perimeter of the Wilhelmy plate (5.08 cm), and $\theta$ is the contact angle of the fluid on the plate. As the platform is lowered, the force on the chromatography paper (Wilhelmy plate) increases--due to decreased buoyancy of the paper in the liquid and decreased contact angle of the liquid with the plate--until the platform is lowered so much that the paper loses adhesion to the surface of the liquid, causing a sudden drop in the force on the balance. As the platform was lowered, the force on the chromatography paper could be measured in increments of 0.001 g. The difference between the maximum force on the chromatography paper before contact with the surface is broken and the weight of the saturated paper, measured after the paper is raised out of the liquid, is the force due to the interfacial tension. Just prior to the loss of adhesion of the paper to the liquid, the contact angle of the liquid with the paper is very close to zero, so the surface tension can be estimated as $\sigma = \frac{F}{2L}$, where $F$ is
the maximum force measured prior to the loss of adhesion [Gaver, 1988].

Using this technique, the average surface tension of distilled water from 9 measurements was found to be 74.5 ± 1.7 dynes/cm (95% confidence limit), which gives an indication of the reproducibility of the measurement. This measured value is somewhat higher than the published value of 72.0 dynes/cm, which may be due to underestimating the length of the Wilhelmy plate, L, since edge effects on the plate were neglected. The surface tension of media with 0%, 1%, and 2% dextran were found to be 53.6, 55.5, and 55.7 dynes/cm, respectively, which are not considered to be significantly different from each other. These values agree with the reported surface-tension value of 50 dynes/cm found for RPMI culture medium with 5% serum [Handa et al., 1987].

The viscosity of media containing dextran was measured using a Fluids Rheometer (Rheometrics, Inc.) set up with a cone-and-plate measuring probe. Because the rheometer measures viscosity over a range of shear rates, non-Newtonian behavior can be detected and measured. Fig. 5.8 shows the results of measurements made with the rheometer for several media. Aside from some scatter at low shear rates, media containing dextran exhibit Newtonian behavior for the range of shear rates studied. The viscosity of standard, 1%-serum medium without additives was similar to that of water, about 0.009 poise. Added dextran increased the viscosity
linearly—to 0.013 poise for 1% dextran and 0.017 poise for 2% dextran. Measurements of medium with 3% dextran were not made using the rheometer, but, based on the linear behavior of media with 1% and 2% dextran, the viscosity of 3%-dextran medium would be approximately 0.021 poise. Measurements made using a capillary tube viscometer confirmed this value as the viscosity of medium with 3% added dextran [Lakhotia, 1988]. Fig 5.8 also shows the viscosity of media with added serum, which will be discussed in the following section.

In addition to measurements of the physical properties of media with added dextran, observations of the hydrodynamic effects of the increased-viscosity medium were made. As explained in the previous chapter, high speed photographs were taken of the reactor vessels containing sterile medium at several agitation speeds. Photographs of the reactor containing 1.0 l of medium with 2.5% added dextran at several agitation speeds are shown in Fig. 5.9. The appearance of the vortex and bubble entrainment at the different agitation speeds is not noticeably different than in the photos in Fig. 4.6, which are for the medium without added dextran. Again, depth of vortex and number of entrained bubbles increased with agitation rate. The bottom portion of the vortex had an irregular shape and was the point of detachment of bubbles which were swept out from the impeller region at the higher agitation rates. The concentration and size of bubbles does not appear to have increased with the addition of dextran.
In addition, no accumulation of foam or bubbles was seen with time, indicating a steady-state behavior of bubble formation and bursting.

5.1.3 Implications of dextran results concerning the damage to cells within the reactor

Similar to the results seen without dextran, damage in the 1.0 and 1.2 l reactors in the presence of dextran occurred as bubbles detached from the bottom of the vortex, swirled through the liquid volume, then rose and disengaged at the liquid surface. Damage to cells in cultures containing dextran became severe when agitation speeds reach 190-200 rpm, the same agitation speed range as in cultures without dextran. Since net cell growth in medium containing 2% dextran was possible at agitation speeds of at least 800 rpm when bubble entrainment was eliminated in the completely-filled reactor, the damage seen at agitation speeds of 190-220 rpm can be attributed to the presence entrained bubbles from a gas/liquid interface. In addition, any influence of dextran on the hydrodynamic environment was not due to a change in osmolality or surface tension but, rather, to an increase in the medium viscosity.

Dextran did not affect the agitation speed necessary for large reduction of the apparent growth rate. Rather, once the agitation speed necessary for significant cell damage was reached, greater reduction of $\mu_{\text{app}}$ was seen in the presence of
dextran than in the absence of dextran. However, the speed necessary for significant cell damage varied from 190-220 rpm even in the absence of dextran, so any influence of dextran on this speed might not be easily observable. The lack of influence of dextran on the agitation speed necessary to cause significantly reduced apparent growth rates is not unexpected in view of the lack influence of viscosity on \( n_0^\ast \) or \( n_{\text{min}} \), the characteristic and minimum speed necessary for bubble entrainment.

The use of control cultures grown in parallel with dextran-containing cultures revealed the dependence of apparent growth rate on the presence of dextran in the range of agitation speeds that cause significant cell damage. These results point out the importance of the use of consistent inoculum, such as in the case of parallel cultures, in the investigation of cell growth rates, especially when relatively small changes in cell growth are to be observed.

The effects of dextran observed herein are in contrast to effects that have been previously reported in the literature. Dextran, among other polymers, was found to improve cell growth in some cases when used to partially substitute for serum in stirred cultures of freely-suspended hematopoietic cells [Mizrahi, 1970]. In Mizrahi's work, dextran was used in a low concentration (0.2%) and its effect was attributed to interactions with the cell membrane as
opposed to an influence on the medium viscosity. Dextran has also been used by researchers to affect the hydrodynamic environment in cell culture by changing the medium viscosity. Surface-attached cells grown on microcarrier beads grew better in the presence of dextran [Cherry and Papoutsakis, 1989; Croughan et al., 1989]. This effect was attributed to an increase of the Kolmogorov eddies to a length larger than the diameter of the microcarrier beads. On the other hand, dextran added to suspension cultures of mammalian cells had no effect on cell lysis in capillary flow devices [McQueen and Bailey, 1989].

In the present work, the influence of dextran on cell damage occurs at agitation speeds of 190-220 rpm in the presence of a gas-liquid interface. Under these conditions, the change in the Kolmogorov eddy size due to increased viscosity should have no effect on the damage mechanism, since the eddy length does not approach the cell size at these agitation speeds. One explanation for the harmful effect of dextran may be that enhanced viscosity somehow alters the structure of bulk turbulence or the fluid properties in the microenvironment of entraining or bursting bubbles. If the cell damage occurs due to pressure fluctuations and shear stresses within the immediate vicinity (thin films) of the bursting bubble, then the increased liquid viscosity may increase cell damage. However, the fluid-mechanical properties in the microenvironment of
bursting bubbles and the effect of dextran on that environment are not known.

In the completely-filled reactors, with no vortex or bubble entrainment, the apparent growth of cells in dextran-free medium was indistinguishable from the growth in medium containing 2% dextran. In both cultures, the apparent growth rate was lower than was typical for gently agitated cultures. As mentioned in the previous chapter, a lower growth rate may be attributed to turbulence characterized by Kolmogorov-scale eddies of a size similar to the cell diameter. In Eq. (1.1), the Kolmogorov eddy size is a function of kinetic viscosity to the three-fourths power. Addition of 2% dextran would raise the smallest characteristic eddy size by a factor of \((0.017/0.009)^{3/4}\), an increase of 60%. Due to uncertainties in estimating the eddy size, the scale of the Kolmogorov eddy size with or without dextran is comparable to the cell size, and is consistent with the proposed mechanism for cell damage.

5.2 Effect of Serum on Cell Growth in the Agitated Reactor Environment

One goal in animal cell-culture research is the elimination of serum in cell culture media. Despite its relatively high cost, fetal bovine serum (FBS) is the type of serum most commonly used due to the low concentration of antibodies in the fetal blood and a decreased chance of viral
contamination in the fetus. Several problems with serum make its elimination from cell culture media desirable. First, the composition of serum is variable, which contributes variability of cell growth from culture to culture. Second, the complex and undefined mixture of proteins, hormones, and other components that make up serum render separation of the desired product, typically a protein or hormone, more difficult.

Several "serum-free" media are now commercially available, but, as was mentioned in the introduction, they are usually cell-line specific and may not adequately support growth of a range of cell types. In addition, serum-free media often do not allow growth in agitated cultures or growth over extended periods of time. Here, serum in suspension-cell culture is examined for its ability to protect cells from agitation damage.

5.2.1 Cell growth in the bioreactor as a function of serum concentration in the presence of a gas headspace

Initially, the CRL-8018 hybridoma cells used in this investigation were grown in medium containing 20% FBS. As part of this investigation, the cells were adapted to grow with 1% Nutridoma medium additive and only 1% added serum. Nutridoma is a proprietary, serum-free media supplement composed of insulin, transferrin, bovine serum albumin (BSA), low density lipoproteins (LDL) from bovine plasma, vitamins,
amino acids, and other small organic molecules. It contains no growth factors, mitogens, hormones, or sterols. The protein concentration of media with 1% Nutridoma and no serum is 100-150 µg/ml. Adaptation was accomplished by subculturing the cells into medium containing 1% Nutridoma and progressively lower levels of FBS until it was found that serum at less than a 1% concentration resulted in decreased cell growth. After adaptation, the cells were routinely cultured in medium containing only 1% added serum. With Nutridoma and 1% FBS, the protein concentration of media is 430-560 µg/ml depending upon the source of serum.

Since all of the experiments used media which contained 1% Nutridoma, experiments were performed to determine if the addition of up to 10% serum in addition to the components in Nutridoma had any effects on cellular growth in cultures at low agitation with no entrained bubbles. These experiments were performed in 100 ml spinner flasks which were stirred at a gentle agitation speed of 120 rpm. An agitation rate of 120 rpm in the spinner flask provides much less power input to the culture volume than an agitation rate of 120 rpm in the bioreactors, due to the smaller impeller size. At this agitation speed, no significant vortex was formed, and no bubbles were entrained into the culture media. Cells were grown in the presence of 1% Nutridoma plus 1, 3, 6, or 10% serum in duplicate flasks for each serum concentration. The growth rate as a function of serum concentration is
summarized in Fig. 5.10. In the presence of 1% Nutridoma, the addition of up to 10% FBS had no significant effect on the apparent growth rate at a low agitation rate in spinner flasks. The growth rates in the duplicate flasks were very close and well within the statistical 95% confidence limits shown in Fig. 5.10. In addition, the apparent growth rates in the spinner flasks, even the 1% serum rates, averaged around 0.058 h⁻¹, which were higher than those found for the bioreactor cultures grown in 1% serum.

To gauge the effect of serum on apparent growth in the presence of a hydrodynamically unfavorable environment, cultures were grown in bioreactors containing a 1.2 l volume. An agitation speed of 220 rpm was used in these experiments because it was the minimum speed necessary to cause a significant reduction in the apparent growth rate. Since this speed marked the borderline between cell growth and death, any positive effect of the addition serum ought to be observable. To eliminate any delay of the effect of serum in the bioreactors, the spinner-flask cultures used as inoculum for the reactors contained the same FBS concentration as the reactor. The growth curves from 5 pairs of parallel reactor runs containing varying concentrations of FBS are shown in Figs. 5.11a-5.11e. In three pairs of experiments, a culture containing 1% serum was grown in parallel with 3%, 6%, or 10% cultures (Figs. 5.11a-c). The remaining two parallel
cultures contained 2% and 8% serum and 3.5 and 5% serum (Figs. 5.11d,e).

In three of the pairs of batch runs, samples were drawn from the reactors approximately 8 h into the experiments and grown in T-flasks to serve as non-agitated control cultures. Ten identical flasks containing 10 ml of culture were started from each reactor. For these runs, each time the reactor was sampled, one T-flask was harvested and treated in the same manner as the reactor sample. The apparent growth rates in the T-flasks and in the bioreactors are shown in Fig. 5.12.

In addition to the control T-flasks, the growth rate at 60 rpm prior to the agitation increase, $\mu_{60}$, can be used as a control value of the growth rate for each batch run. Fig. 5.13 shows that $\mu_{60}$ was constant in comparison to the apparent growth rate at 220 rpm over the range of serum concentrations investigated. Table 5.3 lists the values of $\mu_{60}$, $\mu_{app}$ at 220 rpm, and the growth rate in the T-flasks.

As can be seen in Figs. 5.12 and 5.13, serum had no effect on the growth of cells in the control T-flasks or in the reactors at 60 rpm but had a dramatic effect in the agitated bioreactors at 220 rpm. Below approximately 3-4% serum, no net cell growth was observed in the reactors. Even at a 10% serum concentration, the apparent growth was not as high as in the quiescent T-flasks or growth at lower agitation rates as shown in the previous chapter. It is significant to note that, for the values below zero, the
apparent growth rate decreased more rapidly as the serum concentration approached 1%. Overall, the effect of serum displayed a saturation-type behavior, that is, the apparent growth rate slowly approached a maximum value with increasing serum concentration.

Since the presence of 10% FBS protected the cells from bubble entrainment and vortex damage at 220 rpm, an additional experiment was performed to determine if serum would protect cells at even higher agitation speeds. In the batch run shown in Fig. 5.14, the agitation speed was increased from 60 to 260 rpm, several points were taken, and the agitation was increased again to 280 rpm. At 260 rpm, cells grew at an apparent rate of $0.0243 \pm 0.0045 \text{ h}^{-1}$, and at 280 rpm, cells grew at an apparent rate of $0.0071 \pm 0.0071 \text{ h}^{-1}$. Thus 10% FBS protects cells at agitation speeds significantly higher, up to approximately 280 rpm, than speeds which cause death in the 1%-serum medium.

5.2.2 Protective effect of serum as a function of length of exposure in culture

In order to investigate whether the protective effect of FBS against cell damage is physical or nutritional in nature, experiments were performed in which FBS was added shortly before or shortly after the agitation was increased from 60 to 220 rpm in batch bioreactor experiments. A nutritional effect is one in which serum components are taken up and
utilized in some fashion by the cell, a process which is assumed to require a length of time of the order of hours to occur. A physical effect is one in which the serum acts to change the hydrodynamic environment of the bioreactor, either macroscopically or in the immediate vicinity of the cell, and is assumed to take time of the order of minutes or less to occur after serum is added. Fig. 5.15 shows the effect of serum on the growth of cells when added shortly before or somewhat after an increase in agitation from 60 to 220 rpm. Fig 5.15a shows two cultures grown in parallel and started from the same inoculum. In one culture, 110 ml of serum was added to the reactor, which increased the serum concentration from 1% to 10%. In the other culture, 110 ml of fresh medium containing 1% serum was added to the reactor to act as a control. The agitation was increased from 60 to 220 rpm 5 minutes following the addition of serum or control medium. As can be seen, serum acted as a protective agent even when added only 5 minutes before the onset of vortex formation and bubble entrainment associated with an agitation rate of 220 rpm. With only 1% serum, the apparent growth rate was 

\[ -0.0729 \pm 0.0032, \] 

but an apparent growth rate of 

\[ 0.0432 \pm 0.0044 \] 

was possible in the presence of 10% serum.

In Fig. 5.15b, which shows the result of a batch run without a parallel control culture, cells were grown in medium containing 1% FBS. An agitation increase from 60 to 250 rpm resulted in a rapid decline in cell concentration,
even though 110 ml of fresh, 1%-serum medium was added 5 min. prior to the agitation increase. Two hours after the onset of higher agitation, 110 ml of FBS was added to the reactor, but the agitation speed remained at 250 rpm. Upon addition of the serum, the apparent cell growth rebounded to a positive rate of \(0.0316 \pm 0.0107\). The immediate decrease in cell concentration that occurred following the addition of FBS can be accounted for by the dilution of the culture.

From the two experiments shown in Fig. 5.15, it is apparent that serum acts to protect the cells by a rather fast-acting mechanism. The rapid action of serum suggests the effect on cells and their interaction with the reactor environment is, at least in part, a physical one.

5.2.3 Modeling of the effect of serum on apparent growth rate

In the previous chapter, in response to variations in agitation speed, and in this chapter, in response to variations in serum concentration, the apparent growth rate of the hybridoma cells has been shown to vary considerably, from a maximum rate of approximately 0.055 h\(^{-1}\) to very large negative rates. To represent this behavior, a simple model is proposed in which the apparent growth rate, \(\mu_{\text{app}}\), is taken to be the sum of an unchanging "intrinsic" growth rate, \(\mu_0\), and a death rate, \(k\), which varies with different reactor conditions:
The model assumes that the only way in which the apparent growth rate varies is by a change in the rate at which cells die. In other words, any change in the cells' metabolism which might cause a reduced apparent growth rate is negligible compared to the reduction in apparent growth rate as a result of cell death. First, the fact that the apparent growth rate is often largely negative indicates that actual elimination of whole cells within the reactor is occurring, which supports the notion of decreased $\mu_{app}$ due to cell death. Second, without detailed and accurate metabolic information, the proposed model is the simplest one which can account for the experimental results.

A growth rate of 0.055 h$^{-1}$ was used as the value of $\mu_o$ to calculate the death rate for each bioreactor run included in Fig. 5.12. This value was chosen because it is the average growth rate in the quiescent T-flask cultures, which would be the maximum possible growth rate that could be expected in the bioreactor. Furthermore, this value of $\mu_o$ was constant over a range of serum concentrations from 1-10%. This value of the "intrinsic" growth rate does not apply for serum concentrations below 1%, however. In general, a different value of $\mu_o$ would apply for different media formulations and, of course, different cell types. Although the growth rate at 60 rpm in the reactor prior to the agitation increase, $\mu_{60}$, might be a better estimate of the "intrinsic" growth rate in
the reactor than $\mu_o$, it has not been used due to the large
uncertainties in its measurement.

A plot of the death rate versus serum concentration is
shown in Fig. 5.16, along with the previously shown apparent
growth rates for comparison. The death rates merely mirror
the apparent growth rates and appear to approach zero with
increasing serum concentration. The death rate is still
significantly positive at 10% serum concentration, however,
reflecting the fact that the cells grew better in
non-agitated cultures than at 220 rpm in the presence of 10% serum.

After noting the saturation-type behavior of serum on
$\mu_{app}$ and $k$, the data were fitted with the following equation:

$$k = k_{max} - \frac{k^*s}{s + a_1} \quad (5.2).$$

By using the relationship in Eq (5.1) to substitute for $k$,
Eq. (5.2) can be re-written for the apparent growth rate data
as follows:

$$\mu_{app} = \frac{a_2 + a_3s}{s + a_1} \quad (5.3)$$

where

$$a_2 = (\mu_o - k_{max})a_1 \quad (5.4)$$

and

$$a_3 = \mu_o - k_{max} + k^* \quad (5.5).$$

In this model, $s$ is the percent FBS concentration
(dimensionless); $a_1$ is also dimensionless; and $k_{max}$, $k^*$, $a_2$, $a_3$. 
and $a_3$ have units of hr$^{-1}$. Because $\mu_0$ used in this model is only valid for serum concentrations from 1-10%, and because the data to be fitted lie within this range, the results only apply for serum concentrations in the range from 1-10%.

However, the model does display logical asymptotic behavior for serum concentrations approaching zero, that is for $s=0$, $k = k_{\text{max}}$ and $\mu_{\text{app}} = \mu_0 - k_{\text{max}} = a_2/a_1$. However, as $s$ becomes large, $k$ approaches $k_{\text{max}} - k^*$, so another constraint must be applied:

$$k_{\text{max}} - k^* \geq 0$$

(5.6)

since $k$ cannot be less than zero. Practically, the hybridoma cells used in this research require media components aside from serum, and will not grow in 100% serum. As mentioned, these cells were originally routinely maintained in media containing 20% serum, however. Thus the detrimental effects of too high a concentration of serum, which require concentrations of more than 20%, are not predicted by the model.

This model was then fitted to the data shown in Fig. 5.16 to obtain values for the model parameters. Since the equations are non-linear, a non-linear regression analysis software program (SYSTAT software package, Evanston, IL) was used to calculate the parameter values. To assure accuracy, the form of the model given by Eq. (5.2) was applied to the death rate data, and, independently, the form of the model given by Eq. (5.3) was applied to the apparent growth rate
data. In addition, two different convergence methods were used to obtain the model parameters. Equations (5.4) and (5.5) could then be used to check the consistency of the model parameters. In each case, the software package found consistent values for the model parameters. When applied using the model, the data indicated a value of $k^*$ greater than $k_{\text{max}}$. In other words, extrapolation of the curvature of the data points using this model indicated that the death rate would drop below zero for high serum concentrations. Therefore, the constraint of equation 5.8 was applied to the model, meaning that $k^* = k_{\text{max}}$. The model did not dictate that $k$ must drop to zero with increasing serum concentration, but the data indicated that $k$ would indeed become zero with high enough serum concentrations.

After substituting $k^* = k_{\text{max}}$, the model reduces to:

$$k = \frac{k_{\text{max}} a_1}{s + a_1} \quad (5.9)$$

or,

$$\mu_{\text{app}} = \frac{a_2 + \mu_0 s}{s + a_1} \quad (5.10).$$

The data in Fig. 5.16 were then re-fitted to the simplified form of the model as before using the SYSTAT package. Again the different convergence techniques produced the same parameter values, which were consistent between the two model forms and data sets. The parameter values were found to be $k_{\text{max}} = 1.27 \text{ h}^{-1}$, $a_1 = 0.187$, and $a_2 = -0.228$. 
Using these values, the model prediction is plotted in Fig. 5.16 with both the death rate and apparent growth rate data. As can be seen, the model fits the data closely. The maximum value of $k$ indicated by $k_{\text{max}}$ is not meaningful, since the model is not valid for a serum concentration of zero percent. The maximum value of $k$ which can be confidently predicted using this model is $0.200 \text{ h}^{-1}$ at a serum concentration of 1%. The parameter $a_1$ is a measure of how rapidly the model curve approaches zero, or the "potency" of the effect of serum. For $a_1 = 0.187$, the death rate drops to one half of its maximum value (the value for 1% serum) at a serum concentration of 2.2%. A higher value of $a_1$ would mean that a higher concentration of serum would be necessary to cut the maximum death rate by half.

Thus, the model which has been presented is useful for determining the effect of serum on apparent cell growth for a given agitation speed. One would expect a given FBS concentration to have a quantitatively different effect on $\mu_{\text{app}}$ or $k$ depending on the reactor conditions, and that the model parameters would vary accordingly. The effect of serum on death rate would change depending upon the amount of vortexting and bubble entrainment produced by a given reactor condition. Therefore, the model parameters that describe the effect of serum on death rate, would themselves be functions of the reactor parameters of agitation speed, reactor geometry, impeller geometry, and reactor volume. This
functionality is demonstrated in Fig. 5.14 which shows that 10% FBS allows the cells to grow with different \( \mu_{\text{app}} \) and \( k \) values at 260 and 280 rpm. Further research is needed to determine the functionality of the model parameters on the reactor parameters that influence vortexing and bubble entrainment. This model may be useful in comparing the effect of serum to other media additives or serum substitutes. The model parameters which indicate the maximum effectiveness (\( k_{\text{max}} \)) and the potency (\( a_1 \)) could be compared for different media additives.

5.2.4 Discussion of the protective effect of serum and implications concerning the damage mechanism

How serum components act to protect cells from damage due to bubble entrainment and associated hydrodynamic forces is largely unknown. The protective effect of serum cannot be attributed to its effect of increasing the viscosity of culture medium. As shown in Fig. 5.8, the viscosity of media is only increased approximately 13% by the elevation of serum concentration from 1% to 10%. As previously discussed, an increase in the viscosity of the media of 50% or 100% by the addition of high MW dextran did not result in better cell growth at high agitation speeds of 200-220 rpm. On the contrary, the addition of dextran was detrimental to the apparent growth rate in all cases at these agitation speeds. Therefore, the protective effect of serum cannot be
attributed to its effect of modest increase in media viscosity. In addition, the surface tension of media with different serum concentrations (measured using the previously mentioned Wilhelmy balance) was found to be constant for serum concentrations of 1%, 5%, and 10%. Therefore, serum probably does not act through its influence on the surface properties of bubbles or other gas/liquid interfaces. Serum may, however, influence the surface properties of cells through specific interactions of serum components with cell surface components.

The protective effect of serum demonstrated in this investigation is in agreement with other studies. It has long been known that serum provides some protection against damage due to bubbling [Kilburn and Webb, 1968]. The exact mechanism by which serum acts, however, has not been determined. Some serum components, by themselves, have been shown to provide some protection against sparging or agitation damage. Bovine serum albumin has been shown to be useful in limiting sparging damage to cells in suspension culture [Hülcher and Onken, 1988], which may indicate that serum proteins similarly play a role in cell protection. Higher growth rates were achieved in stirred spinner-flask cultures with the addition of a soybean phospholipid fraction to a serum-free medium [Murakami et al., 1983]. Since serum contains a wide variety of lipids, it may allow cells to form a more stable cell membrane, as compared to
cells grown in serum-free media containing a single lipid source.

Whatever the source of protection in serum, the protective effect was seen less than one hour after addition to the culture medium, suggesting that its protective effect, to some extent, derives from a fast-acting mechanism by one or more of its components. As mentioned earlier, such a fast-acting mechanism is perceived to be a physical rather than a nutritional one. A physical protection mechanism might involve changes in the surface of the cell, such as the immediate incorporation of certain lipids or adsorption of proteins and glycoproteins already present in serum. In addition, a rapid mechanism includes possible effects of serum on the physical environment in the reactor, such as viscosity changes or adsorption of protein or lipids onto gas/liquid interfaces. A nutritional protection mechanism might involve changes of the cell cytoskeleton, membrane, or other cell parts and organelles due to the processing of serum components by the cell.

It is possible that a nutritional effect could occur at a rather rapid pace. Using known metabolic rates for transcription and translation processes, it is possible to estimate the minimum time required for a cell to manufacture a cell component. For example, in mammalian cells, the transcription process has been shown to proceed at a rate of 30 base pairs per second. For bacterial cells, translation
of ribonucleic acid to proteins can occur at a rate of 20 amino acids per second [Alberts et al., 1983]. So, for a typical protein such as hemoglobin, the time from initiation of transcription to the formation of a protein could occur in as little as 1.5 minutes. This time represents ideal conditions and neglects all transport processes. Also, this time is the time for manufacture of a single protein. To manufacture enough cellular components to significantly alter the physical properties of the cell would probably take considerably longer. At the maximum end of the metabolic time spectrum, the time necessary to form a complete cell is approximately equal to the doubling time, which is a minimum of 13 h for these cells. Overall, the immediate effect of serum seen in this investigation is assumed to indicate a physical effect, although as the aforementioned argument indicates, a nutritional mechanism in addition to a physical mechanism of cell protection cannot be ruled out.
Table 5.1  Data from Fig. 5.1 for 1.0 and 1.2 l cultures at various agitation speeds. Test cultures containing 1%, 2%, or 3% dextran were grown in parallel with control cultures containing 0% dextran. Parallel cultures were inoculated at the same time with identical inoculum. Error estimates represent the 95% confidence interval for a regression parameter.

<table>
<thead>
<tr>
<th>Dextran (%)</th>
<th>Speed (rpm)</th>
<th>Volume (l)</th>
<th>Apparent Growth Rate ($\mu_{app}$) (1/ h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test</td>
<td>Control (0% Dextran)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>---------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 200</td>
<td>1 0.0210 ± 0.0020 0.0349 ± 0.0025</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 200</td>
<td>1 0.0254 ± 0.0053 0.0328 ± 0.0031</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 0</td>
<td>1 0.0498 ± 0.0063 0.0452 ± 0.0038</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 0</td>
<td>1 0.0366 ± 0.0052 0.0422 ± 0.0041</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 0</td>
<td>1 0.0404 ± 0.0026 0.0389 ± 0.0050</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 60</td>
<td>1 0.0458 ± 0.0030 0.0442 ± 0.0021</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 60</td>
<td>1 0.0472 ± 0.0034 0.0436 ± 0.0115</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 100</td>
<td>1 0.0252 ± 0.0035 0.0361 ± 0.0035</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 150</td>
<td>1 0.0401 ± 0.0032 0.0354 ± 0.0025</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 150</td>
<td>1 0.0346 ± 0.0044 0.0312 ± 0.0042</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 200</td>
<td>1 0.0307 ± 0.0028 0.0299 ± 0.0149</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 200</td>
<td>1 -0.0996 ± 0.0346 0.0232 ± 0.0012</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 220</td>
<td>1.2 -0.0698 ± 0.0033 0.0272 ± 0.0050</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 220</td>
<td>1.2 -0.0749 ± 0.0059 0.0020 ± 0.0036</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 180</td>
<td>1.2 0.0304 ± 0.0166 0.0425 ± 0.0037</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 190</td>
<td>1.2 -0.2151 ± 0.0555 -0.0354 ± 0.0199</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 190</td>
<td>1.2 -0.0927 ± 0.0113 -0.0564 ± 0.0076</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5.2 Data from Figs. 5.2 and 5.3 for cultures of 1.0 and 1.2 l liquid volume at various agitation speeds containing 1%, 2%, and 3% dextran. Error estimates represent the 95% confidence interval for a regression parameter.

<table>
<thead>
<tr>
<th>Dextran (%)</th>
<th>rpm</th>
<th>Vol. (l)</th>
<th>Apparent Growth Rate ($\mu_{\text{app}}$) (1/h)</th>
<th>Growth Rate at 60 rpm ($\mu_{60}$) (1/h)</th>
<th>Normalized Growth Rate ($\mu_{\text{app}}/\mu_{60}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>200</td>
<td>1</td>
<td>0.0254 ± 0.0053</td>
<td>0.0420 ± 0.0419</td>
<td>0.61</td>
</tr>
<tr>
<td>1</td>
<td>200</td>
<td>1</td>
<td>0.0210 ± 0.0020</td>
<td>0.0212 ± 0.0477</td>
<td>0.99</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0.0498 ± 0.0063</td>
<td>0.0419 ± 0.0477</td>
<td>1.20</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0.0404 ± 0.0026</td>
<td>0.0472 ± 0.0034</td>
<td>1.00</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0.0366 ± 0.0052</td>
<td>0.0472 ± 0.0034</td>
<td>1.00</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>1</td>
<td>0.0472 ± 0.0034</td>
<td>0.0472 ± 0.0034</td>
<td>1.00</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>1</td>
<td>0.0458 ± 0.0030</td>
<td>0.0458 ± 0.0030</td>
<td>1.00</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>1</td>
<td>0.0418 ± 0.0032</td>
<td>0.0348 ± 0.0003</td>
<td>1.20</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>1</td>
<td>0.0252 ± 0.0035</td>
<td>0.0214 ± 0.0053</td>
<td>1.18</td>
</tr>
<tr>
<td>2</td>
<td>150</td>
<td>1</td>
<td>0.0401 ± 0.0032</td>
<td>0.0338 ± 0.0015</td>
<td>1.19</td>
</tr>
<tr>
<td>2</td>
<td>150</td>
<td>1</td>
<td>0.0346 ± 0.0044</td>
<td>0.0337 ± 0.0152</td>
<td>1.03</td>
</tr>
<tr>
<td>2</td>
<td>175</td>
<td>1</td>
<td>0.0364 ± 0.0065</td>
<td>0.0337 ± 0.0194</td>
<td>1.08</td>
</tr>
<tr>
<td>2</td>
<td>200</td>
<td>1</td>
<td>0.0307 ± 0.0028</td>
<td>0.0436 ± 0.0348</td>
<td>0.70</td>
</tr>
<tr>
<td>2</td>
<td>200</td>
<td>1</td>
<td>-0.0996 ± 0.0346</td>
<td>0.0347 ± 0.0151</td>
<td>-2.87</td>
</tr>
<tr>
<td>2</td>
<td>220</td>
<td>1.2</td>
<td>-0.0698 ± 0.0033</td>
<td>0.0316 ± 0.0164</td>
<td>-2.21</td>
</tr>
<tr>
<td>2</td>
<td>220</td>
<td>1.2</td>
<td>-0.0749 ± 0.0059</td>
<td>0.0284 ± 0.0037</td>
<td>-2.63</td>
</tr>
<tr>
<td>3</td>
<td>180</td>
<td>1.2</td>
<td>0.0304 ± 0.0166</td>
<td>0.0562 ± 0.0215</td>
<td>0.54</td>
</tr>
<tr>
<td>3</td>
<td>190</td>
<td>1.2</td>
<td>-0.0927 ± 0.0113</td>
<td>0.0287 ± 0.0102</td>
<td>-1.71</td>
</tr>
<tr>
<td>3</td>
<td>190</td>
<td>1.2</td>
<td>-0.2151 ± 0.0555</td>
<td>0.0381 ± 0.0081</td>
<td>-3.53</td>
</tr>
</tbody>
</table>
Table 5.3 Data from Figs. 5.12 and 5.13 for cultures of 1.2 l liquid volume at 220 rpm containing 1-10% FBS. Error estimates represent the 95% confidence interval for a regression parameter.

<table>
<thead>
<tr>
<th>FBS (%)</th>
<th>Apparent Growth Rate (μapp) (1/h)</th>
<th>Growth Rate at 60 rpm (μ60) (1/h)</th>
<th>Growth Rate in Control T-Flasks (1/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-0.1521 ± 0.0488</td>
<td>0.0516 ± 0.0218</td>
<td>0.0549 ± 0.0089</td>
</tr>
<tr>
<td>1</td>
<td>-0.1022 ± 0.0036</td>
<td>0.0422 ± 0.0038</td>
<td>0.0607 ± 0.0029</td>
</tr>
<tr>
<td>1</td>
<td>-0.1789 ± 0.0013</td>
<td>0.0446 ± 0.0103</td>
<td>0.0502 ± 0.0086</td>
</tr>
<tr>
<td>2</td>
<td>-0.0697 ± 0.0076</td>
<td>0.0481 ± 0.0163</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>-0.0298 ± 0.0021</td>
<td>0.0437 ± 0.0244</td>
<td>0.0538 ± 0.0058</td>
</tr>
<tr>
<td>3.5</td>
<td>0.0101 ± 0.0019</td>
<td>0.0464 ± 0.0693</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.0028 ± 0.0019</td>
<td>0.0448 ± 0.0069</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.0284 ± 0.0107</td>
<td>0.0386 ± 0.0030</td>
<td>0.0569 ± 0.0121</td>
</tr>
<tr>
<td>8</td>
<td>0.0323 ± 0.0016</td>
<td>0.0429 ± 0.0081</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.0362 ± 0.0048</td>
<td>0.0556 ± 0.0798</td>
<td>0.0549 ± 0.0053</td>
</tr>
</tbody>
</table>
Figure 5.1 Apparent growth rate as a function of agitation speed in reactors of 1.0 and 1.2 l liquid volume containing 1%, 2% and 3% dextran. Open symbols represent the cultures containing dextran, and the solid symbols represent the corresponding control cultures containing 0% dextran. Positive apparent growth rates are shown on an expanded scale. Error bars represent the 95% confidence interval for a regression parameter. The hand-drawn curve is shown to emphasize the trend in apparent growth rate.
Figure 5.2 Apparent growth rate, $\mu_{\text{app}}$, and growth rate at 60 rpm prior to the agitation increase, $\mu_{60}$, as a function of agitation speed for reactors of 1.0 and 1.2 l liquid volume containing 1%, 2% and 3% dextran. Positive growth rates are shown on an expanded scale.
Figure 5.3  Normalized apparent growth rate and re-scaled apparent growth rate as a function of agitation speed for 1.0 and 1.2 l reactors containing 1%, 2%, and 3% dextran. Dashed line indicates a normalized growth rate of unity.
Figure 5.4 Initial drop in cell concentration following the agitation increase in the reactors as a function of agitation speed for all of the cultures included in Figs. 4.1 and 5.1. Positive values indicate a drop in cell concentration, and negative numbers indicate a rise in cell concentration following the agitation increase.
Figure 5.5(a-q) Growth curves of control cultures and test cultures grown in parallel 1.0 or 1.2 l reactors. Control cultures contained 0% dextran (o) and test cultures contained either 1%, 2% or 3% dextran (Δ). Arrows indicate the point of increase in agitation speed from 60 rpm to the speed given in the figure legends. Solid symbols represent the portions of the curves used to calculate the apparent growth rates.
Figure 5.5a  0% dextran (o) and 2% dextran (Δ) at 200 rpm in 1.0 l reactors.

Figure 5.5b  0% dextran (o) and 2% dextran (Δ) at 60 rpm in 1.0 l reactors.
Figure 5.5c 0% dextran (○) and 2% dextran (△) at 150 rpm in 1.0 l reactors.

Figure 5.5d 0% dextran (○) and 1% dextran (△) at 200 rpm in 1.0 l reactors.
Figure 5.5e 0% dextran (o) and 2% dextran (Δ) at 100 rpm in 1.0 l reactors.

Figure 5.5f 0% dextran (o) and 2% dextran (Δ) at 60 rpm in 1.0 l reactors.
Figure 5.5g  0% dextran (o) and 2% dextran (∆) at 0 rpm in control T-flasks.

Figure 5.5h  0% dextran (o) and 2% dextran (∆) at 150 rpm in 1.0 l reactors.
Figure 5.5i  0% dextran (o) and 2% dextran (Δ) at 0 rpm in control T-flasks.

Figure 5.5j  0% dextran (o) and 2% dextran (Δ) at 200 rpm in 1.0 l reactors.
Figure 5.5k  0% dextran (o) and 2% dextran (Δ) at 0 rpm in control T-flasks.

Figure 5.5l  0% dextran (o) and 1% dextran (Δ) at 200 rpm in 1.0 l reactors.
**Figure 5.5m** 0% dextran (o) and 2% dextran (Δ) at 220 rpm in 1.2 l reactors.

**Figure 5.5n** 0% dextran (o) and 2% dextran (Δ) at 220 rpm in 1.2 l reactors.
**Figure 5.5o** 0% dextran (o) and 3% dextran (Δ) at 180 rpm in 1.2 l reactors.

**Figure 5.5p** 0% dextran (o) and 3% dextran (Δ) at 190 rpm in 1.2 l reactors.
Figure 5.5g 0% dextran (o) and 3% dextran (Δ) at 190 rpm in 1.2 l reactors.
Figure 5.6 Difference in apparent growth rates of control cultures and cultures containing 1%, 2%, or 3% dextran as a function of agitation speed for reactors of 1.0 and 1.2 l liquid volume. Positive differences indicate better growth in the control culture than in the culture containing dextran. Data can also be read along the right-hand ordinate as a percentage of the unchanging intrinsic growth rate ($\mu_o = 0.055 \text{ h}^{-1}$).
Figure 5.7 Growth curves for the completely filled reactor in the absence of any entrained bubbles for cultures containing 0% (a) and 2% (b) dextran. The arrow indicates the point at which agitation was increased from 60 to 800 rpm. Solid symbols represent the points used to calculate the apparent growth rate.
Figure 5.8  Viscosity of water and various media at 37°C as a function of shear rate, measured using a rheometer with cone and plate geometry.
Figure 5.9 High-speed photographs of reactors containing 1.0 l of sterile medium with 2.5% dextran (μf = 0.019 poise) at 150 (a), 180 (b), 200 (c), and 220 (d) rpm. The reactor was in front of a dark background and was lit from both sides.
Figure 5.10 Apparent growth rates of cultures in 100 ml spinner flasks at an agitation speed of 120 rpm as a function of FBS concentration. Duplicate spinner flasks were grown in parallel. Error bars represent the 95% confidence interval for a regression parameter. The straight line indicates the average apparent growth rate in the flasks.
Figure 5.11a Growth curves of 1.2 l reactor cultures grown in parallel containing 1% (○) and 10% (△) FBS. Arrow indicates the point at which agitation was increased from 60 to 220 rpm.

Figure 5.11b Growth curves of 1.2 l reactor cultures grown in parallel containing 3% (○) and 1% (△) FBS. Arrow indicates the point at which agitation was increased from 60 to 220 rpm.
**Figure 5.11c** Growth curves of 1.2 l reactor cultures grown in parallel containing 1% (o) and 6% (Δ) FBS. Arrow indicates the point at which agitation was increased from 60 to 220 rpm.

**Figure 5.11d** Growth curves of 1.2 l reactor cultures grown in parallel containing 2% (o) and 8% (Δ) FBS. Arrow indicates the point at which agitation was increased from 60 to 220 rpm.
Figure 5.11e Growth curves of 1.2 l reactor cultures grown in parallel containing 5% (o) and 3.5% (Δ) FBS. Arrow indicates the point at which agitation was increased from 60 to 220 rpm.
Figure 5.12  Apparent growth rates of control T-flask cultures and 1.2 l reactor cultures at 220 rpm as a function of FBS concentration. Error bars represent the 95% confidence interval for a regression parameter. The straight line indicates the average apparent growth rate in the stationary T-flask cultures.
Figure 5.13 Apparent growth rate, $\mu_{\text{app}}$, and growth rate at 60 rpm prior to the agitation increase $\mu_{60}$, as a function of FBS concentration for 1.2 l reactor cultures at an agitation speed of 220 rpm.
Figure 5.14 Growth curves for bioreactor cultures containing 1% serum. The serum concentration was increased from 1% to 10% at the point indicated by the first arrow followed 5 min. later by an increase in agitation from 60 to 260 rpm. The agitation rate was increased to 280 rpm at the point demarcated by the second arrow. Points represented by the black symbols were used to calculate the apparent growth rate at 260 rpm, and the points represented by the gray symbols were used to calculate the apparent growth rate at 280 rpm.
Figure 5.15 Growth curves for cultures in 1.0 l reactors. Solid symbols denote the portions of the curves used to calculate the apparent growth rate during exponential phase. 

a: The arrow indicates an increase in agitation from 60 to 220 rpm. 110 ml of FBS (circles) or 110 ml of fresh medium containing 1% FBS (squares) was added to each reactor prior to the agitation increase. 

b: The first arrow indicates the point at which 110 ml of fresh medium (containing 1% FBS) was added to the reactor followed 5 min. later by an increase in agitation from 60 to 250 rpm. The second arrow indicates the point at which 110 ml of FBS was added to the reactor, which brought the FBS concentration to 10%.
Figure 5.16 Apparent growth rate (filled symbols) and death rate (open symbols) versus FBS concentration for 1.2 l reactor cultures at 220 rpm. Error bars represent the 95% confidence interval for a regression parameter. Solid lines are model predictions.
CHAPTER 6

Cell Yields and Antibody Production in Various Reactor Environments

By investigating the changes in cell yields and antibody production in response to the various reactor agitation intensities and media compositions, information can be gained not only about the cell metabolism but also about the mechanism of cell damage in the reactor environment. In this chapter, the growth of batch cultures, including consumption of key metabolites, production of cellular metabolic products, and production of monoclonal antibody, are presented.

6.1 Batch Growth Characterization

Fig. 6.1 shows the growth of the CRL-8018 hybridoma cells in the bioreactor with a volume of 1.0 l and an agitation speed of 50 rpm. Because this reactor run was performed prior to the implementation of the standard protocol, relatively few data points were taken during the course of the batch culture. The total cell concentrations presented in Fig. 6.1 were determined using a hemacytometer and may not be as accurate as those counted using a coulter counter, due to the relatively few total cells counted in each measurement. As was typical of all batch runs
inoculated with healthy, exponentially-growing cells, the lag phase lasted somewhere between 12 and 36 hours. Exact determination of the start of exponential growth and the end of the lag phase is difficult, as is the determination of the end of exponential growth, due to the length of time between data points. From the exponential portion of the growth curve, shown as solid symbols, the apparent growth rate, $\mu_{\text{app}}$, is calculated to be $0.0365 \pm 0.0038$. The error range represents the 95% confidence limit for a regression parameter.

In Fig. 6.2, the concentrations of glucose, glutamine, lactate, and ammonia are shown as a function of time during the batch run. The depletion of glucose and accumulation of lactate ceased at hour 84, corresponding to the end of exponential growth of the cells. However, the depletion of glutamine and accumulation of ammonia continued even after the cells had stopped growing, until approximately hour 150. Likewise, as shown in Fig. 6.3, the production of monoclonal antibody continued well after the end of the exponential phase. At the time of these antibody determinations, an IgM antibody standard was not available. Therefore, the concentration of antibody is presented simply as an absorbance reading. Approximately 40% of the antibody produced during the batch run was produced after the cells had ceased growing and entered the stationary and death phases. The accumulation of antibody after the cells had
stopped growing exponentially agrees with other cell lines, as reported in the literature [Backer et al., 1988; Reuveny et al., 1986; Birch et al., 1985].

The few points taken during exponential growth, along with the difficulty in determining the length of the exponential growth phase, make estimation of the cellular yields of metabolites and products somewhat uncertain. In Fig. 6.4, the concentration of metabolites and products are plotted versus the cell concentration. Only data taken during the exponential growth phase have been included. From the slopes of these plots, the apparent cell yields, in units of $10^6 \text{ cells/µmole}$, are calculated as follows: glucose, $0.077 \pm 0.017$; lactate, $0.045 \pm 0.017$; glutamine, $1.07 \pm 0.41$; and ammonia, $1.32 \pm 1.04$. Again, error ranges represent the 95% confidence limit for a regression parameter. In this investigation, yields are defined as the number of cells produced per amount of glucose or glutamine consumed or the number of cells produced per amount of lactate or ammonia produced. Thus, all cell yields are reported as positive numbers.

Since lactate is mostly a direct product of glucose metabolism via glycolysis, the ratio of lactate produced to glucose consumed can give an indication of the efficiency of energy utilization by the cells. In this case, 1.7 moles of lactate were produced per mole of glucose consumed. A ratio of 2 would be expected if all of the glucose was converted to
lactate via glycolysis. Likewise, the amount of ammonia produced per mole of glutamine consumed can give an indication of the amount of glutamine that is de-aminated to glutamate and incorporated by the cell as an energy source [Reitzer, et al., 1979]. Here, 0.8 moles of ammonia were produced for each mole of glutamine consumed.

Aside from information about the metabolism of the hybridoma cells, some points concerning the accuracy of determining the various metabolic parameters can be made. As seen from the error estimates, and also from the scatter in the data on the graphs, the calculation of glucose and lactate yields are more accurate than those of glutamine and ammonia. Two reasons may account for this discrepancy. First, the assays for glucose and lactate using the YSI analyzer are more reliable and reproducible than the colorimetric assays used to determine glutamine and ammonia concentrations. Second, the utilization of glucose and subsequent production of lactate were closely growth-associated. Cessation of cell growth corresponded with the depletion of glucose. Therefore, calculating the yields based on data taken only during the phase of exponential growth is appropriate for glucose and lactate. However, glutamine utilization and the production of ammonia were not associated merely with the growth of cells, since utilization of glutamine continued during the decline phase of batch growth. So, correlating the increase of cell concentration
with glutamine consumption or ammonia production only during exponential phase is probably an additional source of uncertainty in the yield calculation, since the equations used to calculate the cell yields assume that substrate is only utilized for cell growth, and not for maintenance or product formation.

Since the production of antibody is not associated only with cell growth, calculation of antibody yields was not routinely done. In addition, the scatter in the ELISA measurements is greater than in the colorimetric assays used to determine glutamine and ammonia, probably due to the large number of steps in the ELISA procedure. Due to these sources of uncertainty, the calculation of the antibody yields is of little practical significance to the objectives of this work, which focus on fluid-mechanical cell damage of exponentially growing animal cells.

6.2 Cell Yields as a Function of Growth Rate

Using the equations explained in the materials and methods chapter, the cell yields on glucose, lactate, and glutamine have been determined for most of the batch runs presented in chapters 4 and 5. Because the yields are determined from the actual concentrations of cells and substrates and metabolites within the reactor, they are the apparent cell yields and they do not necessarily reflect the actual moles of substrate necessary to produce a given number
of cells, or moles of metabolite produced by a given number of cells. For example, if the cell concentration is declining in the reactor (which occurs with a negative apparent growth rate) at the same time as glucose is being depleted, the apparent cell yield on glucose is negative.

Figs. 6.5, 6.6, and 6.7 show representative plots of glucose, lactate, and glutamine concentrations versus cell concentration for three different reactor environments. The slope of each plot is equal to the cell yield. The data shown in Figs. 6.5 and 6.6 were taken from two 1.0 l batch reactors grown in parallel at 220 rpm: a control culture with no added dextran and a culture with 2% added dextran. (These representative cultures are the same as those shown in Fig. 3.6.) The data shown in Fig. 6.7 is from a 1.2 l culture with 8% added FBS at 220 rpm. In all cases, the metabolite and product concentrations vary linearly with cell concentration, which implies that the cellular yields are constant during exponential growth. In addition, Fig. 6.6 shows the negative yields associated with a negative growth rate. For the reasons mentioned previously, the data scatter in the glutamine plots are noticeably larger than in the glucose or lactate plots. The greater scatter is evident in the 95% confidence intervals calculated for the cell yields on glutamine.

In this section, the cell-yield data are broken up into groups that correspond to the sections of previous chapters:
low agitation speeds (0-250 rpm) with 0-3% added dextran, low agitation speeds with added serum, and high agitation speeds (>270 rpm).

6.2.1 Cell yields at low agitation speeds in the presence of dextran

Table 6.1 lists the apparent growth rates and apparent cell yields for 1.0 and 1.2 l batch reactor cultures with medium containing 1% FBS, 1% Nutridoma, and 0, 1, or 2% added dextran. These data correspond to the growth rate data shown in Figs. 4.1 and 5.1, although the cell yields were not determined for every batch run shown in those figures. Fig. 6.8 shows the glucose yields from Table 6.1 as a function of the growth rate. The positive growth rates and yields are shown in an expanded-scale portion of the graph. Likewise, Figs. 6.9 and 6.10 show the lactate and glutamine cell yields as a function of growth rate. In the glucose and lactate plots, the cell yields appear to increase linearly with increasing growth rate, especially when the negative yields and growth rates are included. In the expanded scale for the positive glucose and lactate yields versus growth rates, the data appear as a "cloud" in which it is difficult to see any clear trends. The presence of dextran made no apparent difference in the relationship between cell yield and growth rate. The wide scatter in the data in the plot of glutamine
yields (Fig. 6.6) make any trends with growth rate difficult to detect.

6.2.2 Cell yields at low agitation speeds with added serum

The cell yields corresponding to the data shown in Fig. 5.7 (batch runs in 1.0 and 1.2 l reactors at 220 rpm with 1–10% added serum) are listed in Table 6.2. Figs. 6.11, 6.12, and 6.13 show the apparent cell yields of glucose, lactate, and glutamine from Table 6.2 plotted versus the apparent growth rate. Again, the glucose and lactate apparent cell yields (Figs. 6.11 and 6.12) increase linearly with increasing apparent growth rate. The large scatter in the glutamine yields makes any clear trend difficult to observe.

Overall, the trend of increasing apparent cell yield with increasing apparent growth rate is valid for all the reactor conditions in which cell damage is caused by the presence of a vortex, entrained bubbles, and a free gas/liquid interface. The linear relationship exists regardless of the means by which cells are protected, either through decreased agitation speed or increased serum concentration.

6.2.3 Cell yields at high agitation speeds

The apparent cell yields calculated for the completely filled reactors operated without a gas headspace (2.0 l volume) have been grouped together in Table 6.3. The first
four entries are for the cultures in which large quantities of small bubbles were entrained. The following two entries are for cultures containing no added dextran and 2% added dextran in which entrained bubbles were completely absent. As before, the general trend in these batch cultures is for a larger apparent cell yield with larger apparent growth rates. A decisive correlation is difficult to show due to the small number of data, particularly for the cultures without any entrained bubbles.

6.3 Modeling of Apparent Cell Yields

In Eq. (5.1) of the previous chapter, a model was proposed in which the apparent growth rate, $\mu_{app}$, was defined as the sum of an unchanging "true" growth rate, $\mu_0$, and a death rate, $k$, which changed with reactor conditions ($\mu_{app} = \mu_0 - k$). By this model, the rate of formation of new cells, $r_p$, is calculated from the defining equation for $\mu_0$:

$$r_p = \mu_0 C$$

whereas the apparent growth rate is calculated from the actual change in the cell concentration in the reactor:

$$\frac{dC}{dt} = \mu_{app} C$$

As mentioned, $C$ is the concentration of cells within the reactor. Likewise, a "corrected" cell yield, $Y^{*}_{C/S}$, is defined as:
\[
\frac{Y_{C/S}^*}{Y_{C/S}} = \frac{\mu_o}{\mu_{app}} = \frac{\frac{dC/dt}{dS/dt}}{Y_{C/S}^*}
\]

(6.3)

In contrast to the apparent cell yield:

\[
Y_{C/S} = \frac{dC/dt}{dS/dt}
\]

(6.4).

Combining Eq. (5.1) and Eqs. (6.1)-(6.4) gives:

In the model, \( \mu_o \) is the "true" growth rate of the cells in the reactor and does not change with reactor conditions. Instead, cells that are formed die at a certain rate, causing an apparent growth rate which is different from the true growth rate. Eq. (6.5) predicts that the slope of a plot of apparent cell yield versus apparent growth rate is equal to \( Y_{C/S}^*/\mu_o \), and that a curve through the data intercepts the axes at the origin. In addition, if \( \mu_c \) is constant over a range of reactor conditions and the plot of \( \mu_{app} \) versus \( Y_{C/S} \) is linear, then the "corrected" cell yield, \( Y_{C/S}^* \), must also be constant over the range of reactor conditions. The graphs of Figs. 6.8 and 6.9 and, in particular, of Figs. 6.11 and 6.12 indicate that the relationship between \( \mu_{app} \) and \( Y_{C/S} \) is linear, so the "corrected" cell yields on glucose and lactate are constant over a range of reactor conditions. Because of the wide scatter, the glutamine data do not support or contradict the proposed model. Thus, a model in which the growth rate
and cell yields change only because of a changing death rate adequately explains the metabolic behavior seen in batch cultures over the range of reactor conditions studied.

The plots of growth rate versus cell yields shown in Figs. 6.8-6.13 were fitted with a straight line using a least-squares technique. The slopes and intercepts of these lines, along with the correlation coefficient for each least squares fit, are shown in Table 6.4. For glucose utilization and for lactate production, these slopes, which represent $Y_{C/S}^{*}/\mu_{o}$, were nearly the same despite the different causes of reduced growth rate represented in each figure. For glutamine utilization, no correlation was seen due to the wide scatter of the data, as is evident by a correlation coefficient near zero. In Figs. 6.8, 6.9, and 6.10, the different growth rates were caused by differences in agitation speed and dextran concentration. In Figs. 6.11, 6.12, and 6.13, the different growth rates were caused by differences in serum concentration. Overall, the dependence of the glucose and lactate cell yields on growth rate was the same, regardless of the causes of reduced growth rate. When the negative growth rates are included, the model given in Eq. 5.1, which contributes any differences in apparent growth rate to differences in death rate, is adequate to explain the overall trends. Upon closer examination of the positive growth rates, however, the linear relationship between apparent growth rate and cell yield is not so evident, which
suggests that the intrinsic growth rate, $\mu_0$, (Eq. 5.1) may not be constant in all cases. The differences seen in $\mu_{60}$, which seem to correlate with $\mu_{app}$ when the death rate is not large, further support the notion of a $\mu_0$ that is not constant. However, any changes in $\mu_0$ caused by differences in inoculum conditions are small in comparison with the potential changes in the death rate, $k$, caused by increased agitation, lower serum concentrations, or added dextran.

Using the data from Table 6.4, the overall cell yields on glucose and lactate can be determined. For the cell yield on glucose, using a value of $2.0 \times 10^6$ cells/$\mu$ mole)/h$^{-1}$ for $Y_{c/s}/\mu_0$ (an average of the values from Table 6.5) and a value of $0.055$ h$^{-1}$ for $\mu_0$ gives an overall "corrected" yield of $1.1 \times 10^8$ cells/mmole glucose. Likewise, for the cell yield on lactate, an average value of $1.1 \times 10^6$ cells/$\mu$ mole)/h$^{-1}$ for $Y_{c/s}/\mu_0$ and a value of $0.055$ h$^{-1}$ for $\mu_0$ gives an overall "corrected" yield of $0.61 \times 10^8$ cells/mmole lactate. Finally, combining these values gives an overall ratio of 1.8 moles of lactate produced per mole of glucose consumed.

The overall yield on glucose found in this investigation is somewhat lower than that found by other researchers. For human leukemia cells and human hematopoietic cells, glucose yields were found to be $2.7 \times 10^8$ cells/mmole and $4.3 \times 10^8$ cells/mmole [Taya et al., 1986]. Closer to the value found in the present research, a glucose yield of $2 \times 10^8$ was found
for hybridoma cells grown in stirred bioreactors similar to the ones used here [Miller et al., 1988].
Table 6.1  Growth rate and corresponding metabolite yields for cultures of 1.0 and 1.2 l liquid volume at various agitation speeds containing 0%, 1%, and 2% dextran. Error estimates represent the 95% confidence interval for a regression parameter.

<table>
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<tr>
<th>Dextran (%)</th>
<th>rpm</th>
<th>Vol. (l)</th>
<th>Apparent Growth Rate (μapp) (1/h)</th>
<th>Apparent Cell Yields (millions cells/µmole)</th>
<th>Glucose</th>
<th>Lactate</th>
<th>Glutamine</th>
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<th>Dextran (%)</th>
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<th>Apparent Cell Yields (millions cells/μmole)</th>
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</tr>
<tr>
<td>2</td>
<td>150</td>
<td>1</td>
<td>0.0401 ± 0.0032</td>
<td>0.0815 ± 0.0071</td>
</tr>
<tr>
<td>2</td>
<td>150</td>
<td>1</td>
<td>0.0346 ± 0.0044</td>
<td>0.0637 ± 0.0103</td>
</tr>
<tr>
<td>2</td>
<td>175</td>
<td>1</td>
<td>0.0364 ± 0.0065</td>
<td>0.0908 ± 0.0240</td>
</tr>
<tr>
<td>2</td>
<td>200</td>
<td>1</td>
<td>0.0307 ± 0.0028</td>
<td>0.1043 ± 0.0128</td>
</tr>
<tr>
<td>2</td>
<td>200</td>
<td>1</td>
<td>-0.0996 ± 0.0346</td>
<td>-0.281 ± 0.0686</td>
</tr>
<tr>
<td>2</td>
<td>220</td>
<td>1.2</td>
<td>-0.0698 ± 0.0033</td>
<td>-0.164 ± 0.0124</td>
</tr>
<tr>
<td>2</td>
<td>220</td>
<td>1.2</td>
<td>-0.0749 ± 0.0059</td>
<td>-1.837 ± 9.516</td>
</tr>
</tbody>
</table>
**Table 6.2** Growth rate and corresponding metabolite yields for cultures of 1.2 l liquid volume at 220 rpm containing 1-10% serum. Error estimates represent the 95% confidence interval for a regression parameter.

<table>
<thead>
<tr>
<th>FBS %</th>
<th>rpm</th>
<th>Vol. (l)</th>
<th>Apparent Growth Rate (µapp) (1/h)</th>
<th>Apparent Cell Yields (millions cells/µmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Glucose</td>
<td>Lactate</td>
</tr>
<tr>
<td>1</td>
<td>220</td>
<td>1.2</td>
<td>-0.1789 ± 0.0013</td>
<td>-0.291 ± 0.0694</td>
</tr>
<tr>
<td>1</td>
<td>220</td>
<td>1.2</td>
<td>-0.1521 ± 0.0488</td>
<td>-0.260 ± 0.0624</td>
</tr>
<tr>
<td>1</td>
<td>220</td>
<td>1.2</td>
<td>-0.1022 ± 0.0036</td>
<td>-0.227 ± 0.0443</td>
</tr>
<tr>
<td>2</td>
<td>220</td>
<td>1.2</td>
<td>-0.0697 ± 0.0076</td>
<td>-0.122 ± 0.1204</td>
</tr>
<tr>
<td>3</td>
<td>220</td>
<td>1.2</td>
<td>-0.0298 ± 0.0021</td>
<td>-0.0608 ± 0.0068</td>
</tr>
<tr>
<td>3.5</td>
<td>220</td>
<td>1.2</td>
<td>0.0101 ± 0.0019</td>
<td>0.0198 ± 0.0024</td>
</tr>
<tr>
<td>5</td>
<td>220</td>
<td>1.2</td>
<td>0.0228 ± 0.0019</td>
<td>0.0060 ± 0.0042</td>
</tr>
<tr>
<td>6</td>
<td>220</td>
<td>1.2</td>
<td>0.0284 ± 0.0107</td>
<td>0.0625 ± 0.0114</td>
</tr>
<tr>
<td>8</td>
<td>220</td>
<td>1.2</td>
<td>0.0323 ± 0.0016</td>
<td>0.1005 ± 0.0111</td>
</tr>
<tr>
<td>10</td>
<td>220</td>
<td>1.2</td>
<td>0.0362 ± 0.0048</td>
<td>0.0740 ± 0.0058</td>
</tr>
</tbody>
</table>
Table 6.3  Growth rate and corresponding metabolite yields for cultures in the completely filled reactors at various agitation speeds. Data is for cultures that contained many small entrained bubbles; one culture that was bubble-free and contained no dextran; and one culture that was bubble-free and contained 2% dextran. Error estimates represent the 95% confidence interval for a regression parameter.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>rpm</th>
<th>Vol. (l)</th>
<th>Apparent Growth Rate (μapp) (1/h)</th>
<th>Apparent Cell Yields (millions cells/μmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td>Small bubbles</td>
<td>300-450</td>
<td>2</td>
<td>0.05114 ± 0.00416</td>
<td>0.0972 ± 0.0100</td>
</tr>
<tr>
<td>Small bubbles</td>
<td>450-600</td>
<td>2</td>
<td>0.04167 ± 0.0035</td>
<td>0.0686 ± 0.0039</td>
</tr>
<tr>
<td>Small bubbles</td>
<td>800</td>
<td>2</td>
<td>0.0015 ± 0.0047</td>
<td>0.0664 ± 0.0260</td>
</tr>
<tr>
<td>Small bubbles</td>
<td>900</td>
<td>2</td>
<td>-0.0187 ± 0.00838</td>
<td>-0.0751 ± 0.0433</td>
</tr>
<tr>
<td>No bubbles</td>
<td>800</td>
<td>2</td>
<td>0.01547 ± 0.00547</td>
<td>0.0508 ± 0.0137</td>
</tr>
<tr>
<td>No bubbles, 2% Dex</td>
<td>800</td>
<td>2</td>
<td>0.0170 ± 0.00326</td>
<td>0.0752 ± 0.0242</td>
</tr>
</tbody>
</table>
Table 6.4 Results of least-squares regression of the data shown in Figs. 6.8-6.13.

<table>
<thead>
<tr>
<th></th>
<th>slope ( \left( \frac{Y^c_s}{\mu_0} \right) )</th>
<th>intercept</th>
<th>correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Various agitation speeds and dextran concentrations (Figs. 6.8, 6.9, and 6.10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>glucose</td>
<td>1.83</td>
<td>7.44 x 10^{-3}</td>
<td>0.976</td>
</tr>
<tr>
<td>lactate</td>
<td>1.06</td>
<td>-1.06 x 10^{-4}</td>
<td>0.976</td>
</tr>
<tr>
<td>glutamine</td>
<td>-124</td>
<td>1.92</td>
<td>0.307</td>
</tr>
<tr>
<td>Various serum concentrations (Figs. 6.11, 6.12, and 6.13)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>glucose</td>
<td>2.13</td>
<td>2.91 x 10^{-3}</td>
<td>0.966</td>
</tr>
<tr>
<td>lactate</td>
<td>1.16</td>
<td>1.27 x 10^{-2}</td>
<td>0.947</td>
</tr>
<tr>
<td>glutamine</td>
<td>-35.3</td>
<td>2.99</td>
<td>0.095</td>
</tr>
</tbody>
</table>
Figure 6.1 Growth curve in a bioreactor of 1.0 l liquid volume at an agitation speed of 50 rpm.
Figure 6.2 Concentrations of glucose and lactate (a) and glutamine and ammonia (b) during the course of the batch culture shown in Fig 6.1.
Figure 6.3  Monoclonal antibody concentration for the reactor culture shown in Fig. 6.1. Concentration of antibody is given as an absorbance reading at 405 nm.
Figure 6.4 Concentrations of glucose and lactate (a) and glutamine and ammonia (b) as a function of cell concentration for the reactor culture shown in Fig. 6.1.
Figure 6.5 Glucose, lactate, and glutamine concentration versus cell concentration for the 1.0 l reactor culture at 200 rpm containing 0% dextran and 1% FBS. Straight lines are a least-squares fit of the data points. Slopes of these lines are equal to the cell yields.
Figure 6.6 Glucose, lactate, and glutamine concentration versus cell concentration for the 1.0 l reactor culture at 200 rpm containing 2% dextran and 1% FBS. Straight lines are a least-squares fit of the data points. Slopes of these lines are equal to the cell yields.
Figure 6.7 Glucose, lactate, and glutamine concentration versus cell concentration for the 1.2 l reactor culture at 220 rpm containing 0% dextran and 8% FBS. Straight lines are a least-squares fit of the data points. Slopes of these lines are equal to the cell yields.
Figure 6.8 Apparent cell yield on glucose as a function of growth rate for 1.0 and 1.2 L liquid volume reactor cultures containing 0%, 1%, and 2% dextran at agitation speeds from 0-250 rpm. The positive portion of the curve is shown on an expanded scale in the lower figure. Straight line is a least-squares fit of all of the data points.
Figure 6.9 Apparent cell yield on lactate as a function of growth rate for 1.0 and 1.2 l liquid volume reactor cultures containing 0%, 1%, and 2% dextran at agitation speeds from 0-250 rpm. The positive portion of the curve is shown on an expanded scale in the lower figure. Straight line is a least-squares fit of all of the data points.
Figure 6.10 Apparent cell yield on glutamine as a function of growth rate for 1.0 and 1.2 l liquid volume reactor cultures containing 0%, 1%, and 2% dextran at agitation speeds from 0-250 rpm. The positive portion of the curve is shown on an expanded scale in the lower figure. Straight line is a least-squares fit of all of the data points.
Figure 6.11 Apparent cell yield on glucose as a function of growth rate for 1.0 and 1.2 l liquid volume reactor cultures containing 0-10% FBS at an agitation speed of 220 rpm. Straight line is a least-squares fit of all of the data points.
Figure 6.12  Apparent cell yield on lactate as a function of growth rate for 1.0 and 1.2 l liquid volume reactor cultures containing 0-10% FBS at an agitation speed of 220 rpm. Straight line is a least-squares fit of all of the data points.
Figure 6.13 Apparent cell yield on glutamine as a function of growth rate for 1.0 and 1.2 l liquid volume reactor cultures containing 0-10% FBS at an agitation speed of 220 rpm. Straight line is a least-squares fit of all of the data points.
CHAPTER 7

Conclusions and Recommendations

7.1 Conclusions from This Work

The major goal of this study was to investigate the causes of hydrodynamic damage to suspended animal cells in stirred reactors. To accomplish this goal, it was necessary to first develop an experimental protocol that allowed reproducible growth of cells in the stirred bioreactor. It was also necessary to develop an appropriate technique for assessing the effects of the stirred reactor environment on cell growth. Specifically, damage to cells in reactors containing entrained bubbles and in bubble-free reactors was explored. Also, the mechanism by which certain media components or media additives alter the cell response to hydrodynamic damage was investigated. The investigation included analysis of the changes in metabolism of cells in response to hydrodynamic forces.

7.1.1 Important aspects of the experimental protocol

To successfully test the response of cells to different reactor conditions, reproducible cultures at the same stage of cell growth must be available. Because animal cells are sensitive to a great number of variables, attaining consistent cultures is not trivial. The following elements
are identified as being important to the protocol used for obtaining reproducible batch growth in the bioreactor.

1. Consistent inoculum should be used for each batch culture, since the characteristics of the inoculum can affect the growth rate of the cells throughout the batch culture. Three characteristics of the inoculum have been found to be especially important:

   A. Cell concentration
   B. Phase of batch growth at the time of inoculation
   C. History of growth conditions

2. The experimental conditions, such as increased agitation speed, should be imposed at a consistent cell concentration, since the response of cells may vary with the phase of batch culture.

3. Enough data points should be taken during the exponential phase of cell growth to assure statistically meaningful estimation of the growth rate and other growth parameters.

7.1.2 Damage mechanism in the agitated bioreactor

To provide sufficient mass transfer, batch cultures of animal cells are subjected to various hydrodynamic forces,
which cause damage to the cells and may also have an impact on the cell growth and metabolism. Several causes of cell damage in stirred reactors have been identified.

At low agitation speeds, below 250 rpm, cell damage is caused when relatively large (1-3 mm) bubbles are entrained at the vortex tip, move through the bulk liquid in the reactor, and burst at the gas/liquid interface at the liquid surface. Two causes of cell damage at these speeds have been ruled out. First, damage is not caused merely by the presence of entrained bubbles, since growth was possible in the presence of small (50-300 μm) bubbles at agitation speeds up to 700 rpm when no gas/liquid interface was present at the liquid surface. Second, damage is not caused by interactions with bulk-liquid turbulence produced by power input via the impeller, since cell growth is possible at agitation speeds up to 800 rpm when entrained bubbles and a gas/liquid interface are not present in the reactor.

Two mechanisms for cell damage are proposed which are consistent with the conditions found to cause cell damage at low agitation speeds. First, cell damage may be caused by the hydrodynamic forces generated in the vicinity of bursting bubbles. Second, cell damage may be caused by interactions with a mobile rather than rigid gas/liquid interface. In addition, a model used for predicting the onset of bubble entrainment in the stirred reactor may be useful in predicting the onset of cell damage in the reactor.
When bubble entrainment and the presence of a gas/liquid interface are eliminated, reduced cell growth is found at an agitation speed of 800 rpm. This speed corresponds with a Kolmogorov-scale eddy size in the reactor similar to the cell size. These results are consistent with a model that uses the Kolmogorov eddy size as a predictor for the onset of turbulent damage in stirred reactors.

7.1.3 Influence of media additives on the cell damage mechanism

When cell damage is caused by vortexing and bubble entrainment at low agitation speeds, the presence of dextran at a concentration of 2-3% causes increased cell damage but does not influence the agitation speed at which damage occurs. Two mechanisms are proposed for this finding. First, dextran may adversely influence the hydrodynamic environment in the vicinity of bursting bubbles or at gas/liquid interfaces. Second, dextran may cause cells to adhere more tightly to each other and be more severely damaged when torn apart. When cell damage occurs at high speeds in the absence of both a gas/liquid interface and entrained bubbles, addition of dextran was found to have no effect on cell damage, although the viscosity and, hence, the eddy size were substantially increased.

On the other hand, when cell damage is caused by vortexing and bubble entrainment at low agitation speeds,
increasing the serum concentration decreases cell damage. Since serum was effective in reducing cell damage almost immediately upon addition, its mechanism of action is perceived to be, at least partly, physical in nature. The beneficial effect of serum is not due to changes in the viscosity or surface tension of the medium. Greater changes in viscosity due to dextran addition than that due to serum addition did not protect cells, and addition up to 10% serum did not change the surface tension of the medium. Data relating apparent growth rate, $\mu_{\text{app}}$, and serum concentration, $s$, in the reactor was successfully fitted to the following saturation-style model:

$$\mu_{\text{app}} = \mu_0 - k = \mu_0 - \frac{k_{\max}a_1}{s + a_2}$$

where $\mu_0$ is an unchanging intrinsic growth rate and $k$ is a death rate which varies with serum concentration. In this model, $k_{\max}$ and $a_1$ are empirical parameters.

7.1.4 Assessment of cell damage

When cell damage is caused by vortexing and bubble entrainment at low agitation speeds, the simple model of apparent growth rate as a sum of an unchanging intrinsic growth rate and a changing death rate ($\mu_{\text{app}} = \mu_0 - k$) can be used to model the reduction of apparent growth rate regardless of the cause of cell damage—increased agitation, increased dextran concentration, or reduced serum.
concentration. When the apparent growth rate is examined over a wide range, from positive values to greatly negative values, the death rate is the determining factor in the magnitude of $\mu_{\text{app}}$. In fluid-mechanical regimes where cell damage is not severe, $\mu_{\text{app}}$ may vary more due to a change in $\mu_c$, the intrinsic growth rate of the cells, than to a change in the death rate of the cells. By determining the growth rate of the cells in batch culture at 60 rpm, before the experimental conditions are imposed, an estimate of the intrinsic growth rate can be obtained and may used to normalize the apparent growth rate.

7.1.5 Cell Yields and Antibody Production

In batch culture, the monoclonal antibody production of the hybridoma cells studied is only partially growth-associated, since a significant fraction of antibody is produced after the cells have ceased exponential growth. Likewise, utilization of glutamine and production of ammonia continues past the exponential growth phase. On the other hand, utilization of glucose and production of lactate are closely growth associated. During exponential growth phase of batch culture, the apparent cell yields on glucose and lactate, as well as the apparent growth rate, remain constant. Over the entire range of experimental conditions, the ratio of the apparent cell yield on glucose and on
lactate to the apparent growth rate remained constant within
the ability to measure the metabolic yields and growth rates.

7.2 Recommendations for Future Work

In any research, each conclusion raises new questions, and this research is no exception. From the work on development of the experimental protocol, several recommendations for improvement of future experiments can be made. In addition, several directions for future research on the causes of damage to animal cells in reactors can be envisioned.

7.2.1 Improvements in the experimental procedure

The importance of consistent inoculum as a requirement for reproducible cell growth in the reactor has been emphasized. Although the initial cell concentration in each reactor run has been carefully documented, no information about other conditions of the inoculum are readily available from the present research. In future experiments, it is recommended that the cell concentration in the inoculum cultures be carefully tracked and recorded. From this information, the growth rate of cells prior to inoculation can be determined. Also, the effects of growth phase of the inoculum on subsequent growth in the reactor can be gauged.
Characterization of the hydrodynamic environment in the reactor was impeded by the use of a non-standard impeller type. It is recommended that a simple flat-bladed or Rushton impeller be used in future experiments when existing correlations from experiments using these types of impellers are to be applied to characterize eddy size, bubble entrainment, or other hydrodynamic conditions.

Since the growth rate at 60 rpm was found to be a potentially useful tool in normalizing the apparent growth rate after the experimental conditions were imposed, it is recommended that care be taken to assure a statistically meaningful determination of the growth rate at 60 rpm. The cell concentration should be carefully monitored at the start of the batch culture to help reduce the subjectiveness in determining the end of the lag phase and the start of exponential growth. Also, 5-6 data points should be taken at 60 rpm once exponential growth has started, to allow statistically meaningful calculation of the growth rate.

Since a large amount of data is needed during the exponential portion of batch growth, both before and after the experimental conditions are imposed, some method of continuously monitoring the cell concentration in the reactor would be useful. Glucose utilization has been found to be closely growth associated over a wide range of experimental
conditions, so a method for continuously monitoring glucose or some other metabolite may be a convenient indirect method for monitoring the cell concentration.

Although glucose and lactate could be measured precisely, accurately, and conveniently, the measurement of glutamine, ammonia, and monoclonal antibody are in need of improvement. High performance liquid chromatography may be useful in measuring glutamine (as well as most other amino acids) and antibody concentrations.

7.2.2 Future research directions

Three broad approaches to future research are presented. First, the information gained from this research may be applied to the design of novel bioreactors which, through design improvements, reduce the amount of cell damage due to vortexing and bubble entrainment while still allowing sufficient mass transfer in the reactor. Since damage was found to take place only when bubbles could interact with a free gas/liquid interface, perhaps a reactor could be designed which allows bubbles to contact cells in the bulk fluid but isolates the cells from the region of bubble disengagement at the gas/liquid interface. Furthermore, turbulence has been found to be non-damaging for Kolmogorov eddy sizes almost as small as the cell size. This information may be useful in designing impellers that do not
induce vortexing or bubble entrainment but are still capable of providing considerable mass transfer within the bulk fluid.

Second, the information gained in this research may be extended by additional research in the identification of important correlators and predictors of cell damage in mixed reactors. By completely filling the reactor and providing aeration via silicone tubing, the effects of bubble entrainment and vortexing have been uncoupled in this research from the effects of bulk-liquid turbulence on the cell growth. In this environment, results were found which were consistent with a model that used the Kolmogorov eddy size as a predictor for cell damage. Further research is needed to independently examine the effects of different impeller geometry, agitation speed, and viscosity on cell growth in the absence of a gas phase to confirm any correlation of cell damage to the eddy size.

A model used to determine the onset of bubble entrainment was useful in predicting the onset of cell damage in the reactor for a volume around 1.0 liter. Further research is necessary to correlate the effect of liquid height above the impeller, reactor diameter, and surface tension with cell damage to confirm the utility of the bubble entrainment model.
In addition, this phenomenological approach may be used to develop other models of the reactor environment that provide a useful correlation with cell damage.

Third, information gained in this research may be used to design other research equipment, aside from the mixed bioreactor, that duplicate in a controlled manner the specific portions of the hydrodynamic environment that have been shown to cause cell damage, similar in fashion to the way flow chambers or rotating viscometers have been used to specifically investigate shear stresses independently from the environment in which the shear stresses are produced. Since bursting bubbles and gas/liquid interfaces have been shown to be important in causing cell damage, equipment that allows visualization of cells in the vicinity of bursting bubbles or measures the microenvironment around a bursting bubble would be valuable in further determining the mechanism of cell damage due to sparging or bubble entrainment. In addition, a device which allowed controlled contact of cells with rigid or mobile gas/liquid interfaces would allow further elucidation of the damage mechanism of cells in mixed reactors.

Finally, the investigation of cell response in the reactor environment in this research has been limited to measurements of the growth rate and cell yields of a few key
substrates and metabolites. A more detailed and subtle investigation of changes in cell metabolism is recommended as a further research goal. Cytoskeletal structure, membrane composition, energy utilization, protein excretion, and the growth phase, among many others, may be important physiological parameters to investigate.
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APPENDIX A

Freezing and Thawing Cells

[from Zola and Brooks, 1982].

Freezing Cells:

1. Centrifuge at low speed healthy hybridoma cells taken from exponential phase of cell growth, then re-suspend at 6 x 10^6 cells/ml in fresh culture medium containing 50% FBS.

2. To this cell suspension, add an equal volume of fresh medium containing 20% dimethy sulfoxide (DMSO) dropwise with continuous gentle mixing.

3. After thorough but gentle mixing, transfer 1.5 ml of cell suspension to freezing ampules.

4. Seal ampules tightly and freeze using a programmed freezing device so that cells are cooled at a rate of 1-2 °C/min down to -25 °C. From this point to -100 °C, cells can be cooled at a rate of 5-10 °C/min.

5. Once cells have reached -100 °C, transfer ampules to the liquid nitrogen dewar.

Thawing Cells:

Day 1:

Thaw cells rapidly in 37 °C water bath. Add contents of ampule to a 15 ml centrifuge tube. Dropwise over several minutes, add an equal volume of complete, fresh medium (1.5 ml) Let stand undisturbed for 15 min. Slowly add medium to make 10 ml total (7 ml). Let stand undisturbed for 15 min. Centrifuge at low speed, remove supernatant. Re-suspend in 10 ml fresh medium. Place in 25 cm^2 T-flask in incubator.

Day 2:

Centrifuge cells, remove medium, and re-suspend in 10 ml fresh medium. Add 5 ml each to two 25 cm^2 T-flasks. Add 5 more ml of fresh medium to each flask and incubate.
Glutamine Assay -- Microplate Modification

Adapted from Boehringer Mannheim (B-M) Kit #139-092

The following volumes are for 32 samples/standards in triplicate microplate wells (one plate):

20 ml ASSAY SOLUTION (solutions from B-M kit #139-092)
- 4 ml solution 1
- 1.4 ml solution 2
- 1.4 ml solution 3
- 0.2 ml solution 4
- 13.5 ml ddH₂O

1 ml GLNase SOLUTION
- 0.4 ml Glutaminase (B-M #102-903)
- 0.6 ml Phosphate Buffered Saline (PBS)

1. Prepare glutamine standards from frozen 200 mM glutamine solutions (0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 mM standards).
2. Prepare 1/10 dilution of samples in PBS.
3. Add 200 µl/well of ASSAY SOLUTION to a 96 well microplate (Nunc) plus 10 µl/well of the standards or 1/10 dilution samples in triplicate.
4. Wait ~20 min, then read A₄₉₀ nm on a microplate reader.
5. Add 10 µl/well GLNase SOLUTION to each well.
6. Wait ~20 min (or until the color is fully developed), then read A₄₉₀ nm on the microplate reader.
7. The glutamine concentrations of the samples can then be read from a standard curve of absorbance difference before and after GLNase addition.
APPENDIX C

Ammonia Assay

Adapted from Sigma Kit #170-A

The following volumes are for 100 samples/standards:

150 ml ASSAY SOLUTION

- 0.0504 g a-ketoglutarate Sigma #K-1875
- 0.0128 g NADH Sigma #N-8129
- 2.178 g Tris buffer Sigma #T-1503
- 150 ml ddH$_2$O

1.5 ml GLDH SOLUTION Boehringer Mannheim #127-710

1. Prepare ammonia standards of NH$_3$Cl in ddH$_2$O (0, 20, 40, 60, 80, 100 µg NH$_3$/ml standards, not µg NH$_3$Cl).

2. Add 1.5 ml ASSAY SOLUTION plus 25 µl samples/standards to each cuvette (polycarbonate cuvettes for low ultraviolet absorbance).

3. Wait ~5 min, then read $A_{340}^{nm}$ on spectrophotometer.

4. Add 15 µl GLDH SOLUTION to each cuvette and mix by gentle inversion.

5. Wait ~5 min, then read $A_{340}^{nm}$ on spectrophotometer as rapidly as possible.
APPENDIX D

IgM Monoclonal Antibody Assay

The following volumes are for 32 samples/standards in triplicate microplate wells (one plate):

CB (Carbonate Buffer):

100 ml  $H_2O$
0.84 g  $\text{NaHCO}_3$  0.1 M  MW 84
pH 9.5

PBS (Phosphate Buffered Saline):

600 ml  $H_2O$
5.26 g  $\text{NaCl}$  0.15 M  MW 58.4
0.828 g  $\text{NaH}_2\text{PO}_4$  0.01 M  MW 138
pH 7.4

BLOCKER:

20 ml  PBS
0.6 g  BSA (Bovine Serum Albumin)

WASH:

800 ml  PBS
0.4 ml  TWEEN-20

DILUTANT:

10 ml  WASH
0.3 g  BSA

PBS-BSA:

10 ml  PBS
0.1 g  BSA

SUBSTRATE:

20 ml  $H_2O$
0.01 g  $p$-Nitrophenyl phosphate
0.084 g  $\text{NaHCO}_3$  0.05 M
0.0041 g  $\text{MgCl}$  1 mM
pH 10
Store in a light-tight bottle

1. Prepare fresh reagents before starting procedure. Prepare samples by diluting 1/100 in the appropriate media.

2. Prepare a serial dilution of IgM standard (ICN Immunobiologicals #64-339) (0.1, 0.2, 0.5, 1.0, 2.0 μg/ml standards).

3. Dilute anti-mouse IgM (ICN Immunobiologicals #64-365) 1/100 in CB (20 ml/plate). Add 200 μl/well and incubate overnight on a Nutator.

4. Empty microplate wells by shaking the contents into the sink, then patting onto a lab towel. Avoid cross-contamination of the wells. Add 200 μl/well of BLOCKER and incubate for 1 hour.

5. Empty the wells and wash 3 times with WASH.

6. Add 100 μl/well of DILUTANT, then add 10 μl/well of the samples, each in triplicate. Include appropriate blanks (i. fresh media; ii. culture supernant from some IgG producing cells). Incubate for 3 hours.

7. Empty the wells and wash 3 times with WASH.

8. Dilute phosphatase conjugate (Sigma #A-7784) 1/1000 in PBS-BSA and add 100 μl/well. Incubate for 3 hours.

9. Empty the wells and wash 3 times with WASH.

10. Add 200 μl/well SUBSTRATE.

11. Immediately load the microtiter plate in the plate reader and take a reading every five minutes at 405 nm.

12. Stop the readings when the assay has run to completion (when the absorbance readings are past the linear range).

13. Use the absorbance reading from the reading having a linear standard curve.