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With Contributions by
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# Process Development for Hybridoma Cells

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This review attempts to cover those factors that would need to be considered for the optimisation of process control and development strategies for the production of monoclonal antibodies from hybridoma cell lines. The currently available experimental data for cell growth and antibody production of monoclonal antibodies is reviewed against the theoretical background developed mainly from microbial systems. The various parameters which affect the kinetics of hybridoma cell activities are then described in detail, concentrating on shear effects and oxygen requirements which are two of the most important scale-dependent effectors of cell culture growth and productivity. Finally an attempt is made to consider how all the various requirements of hybridoma cells could be incorporated into a process optimisation strategy, with particular reference to bioreactor design and mode of culture operation.

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## 1 Introduction and Scope of Review

Mammalian cells are being increasingly employed for the production of various diagnostic and therapeutic biologicals. In particular the generation of monoclonal antibodies from hybridoma cells has attracted much attention since these reagents are now widely used in veterinary and human diagnostic assay kits, in blood grouping and in immunopurification procedures. There is also great interest in their application as immunotherapeutic agents for the treatment of infectious diseases, tumour destruction and the removal of circulating toxins and pathogens via extracorporeal shunts.

Large-scale processes are being developed in order to meet both the current and future demands for monoclonal antibodies. The current commercial requirement is being fulfilled by a number of biotechnology companies who have adopted several different strategies for bulk production.

This review will attempt to describe the kind of knowledge that is needed to establish an effective process for monoclonal antibody production and will concentrate on the requirements for a rational process development and control system with the understanding that the primary aim of the overall process is to maximise cell productivity while minimising production costs. In Sect. 2, the pertinent areas of knowledge required to achieve this aim are outlined while the current state of knowledge with regard to growth and production kinetics of animal cell culture systems, particularly monoclonal antibody production from hybridoma cells, is presented in Sect. 3.

The data is reviewed against the background of the theory of growth kinetics which has been developed to explain the growth processes of microorganisms and their product formation.

In Sect. 4 we describe the major parameters which have been identified as having significant effects on the kinetics outlined in Sect. 3. For effective process development it is important to both monitor and control the parameters, and the type of equipment which is available to do that is discussed in Sect. 5. Finally, in Sect. 5, we have attempted to describe how these various considerations can be integrated into an overall process design strategy. Two aspects are considered: 1) the type of culture method or mode of culture (i.e. batch, fed-batch, perfusion and continuous) which would achieve optimum antibody yields; 2) the techniques of cultivation or more particularly bioreactor designs which have been developed to meet the cells' biological, chemical and physical requirements. In this section, extensive reference is made to microbial growth systems (and in particular yeast growth and metabolism). This is undertaken to draw comparison and analogy with other life systems. Yeast metabolism, with its major metabolic characteristics of fermentation and respiration and the control thereof, provides an excellent analogy to the growth and metabolism of other eukaryotic cells.

## 2 Integrated Approach to Process Development

In cell cultures the sum of biological activities, which we see as cell growth and product formation and which involves mass transformations and kinetics, is governed by a large number of intracellular and extracellular parameters. Although the net-

work and interdependence of the factors involved in the generation of animal cells and their products is highly complex the important parameters need to be identified. These parameters are physical, chemical and biological in nature and are not, necessarily easy to measure. It is also important to determine whether a measured variable is a parameter which actually governs the process or is a variable suitable for indicating the state of the culture. Figure 1 illustrates the major steps required for an integrated approach to product optimisation using hybridoma cell lines. Firstly, there is process simplification whereby the major metabolic variables characterising the growth of hybridoma cell lines and production of the desired product (antibody) are identified. These are then translated into a model which may be anything from something as

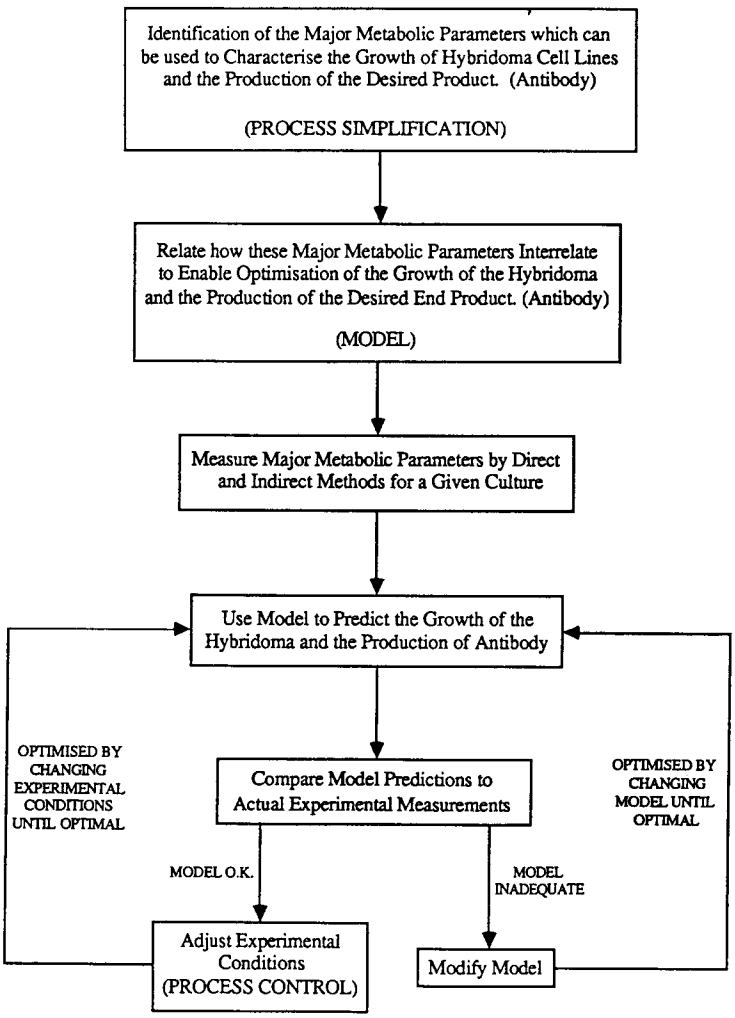


**Fig. 1.** Integrated approach for product optimisation using hybridoma cell lines

simple as a mass balance relating these variables to a much more sophisticated model. The decision as to what level to model the system is a complex one — a balance between simplicity, ability to measure experimentally major components of the model and the accuracy and speed of control are required amongst other things. Comparison of experimental measurements to model predictions is made (if this is possible) and an iterative process for product optimisation is commenced. This may involve model modification or process variable manipulation (or a combination of both). This process has been discussed in detail with respect to microbial growth systems [1].

Implicit from Fig. 1 is the fact that the successful optimisation of any animal cell culture system, as with microbial production systems, requires detailed knowledge of the following: 1) the pathways, kinetics and thermodynamics of cell growth and product formation; 2) the relationships of these factors to the overall cell metabolism including specific uptake rates of essential nutrients; and 3) the relationships between the cells' physiology and product formation and their external environment, i.e. the effectors of cell behaviour such as oxygen availability, concentration of essential substrates in the medium, the temperature, pH, shear stress effects and reactor design.

This kind of approach has been widely and successfully applied to many large-scale processes involving both prokaryotic and eukaryotic organisms. Computer techniques, in addition to the traditional empirical approach, are being increasingly used to construct mass and energy balances for the growth, energetics and product distribution in microbial growth processes [2]. The use of computer simulations has not yet been widely used in animal cell culture systems. There are a number of reasons for this which may be identified with respect to the integrated approach illustrated in Fig. 1. The first two steps of the process, process simplification and model formation, have not been attempted to any significant extent in hybridoma cell growth. In bacterial and eukaryotic growth, often the process may be simply reduced to a model consisting of a detailed mass balance with only a limited number of process variables required for an accurate prediction. This is often the result of homofermentative growth (only one or two major end products of anaerobic metabolism) or completely respiratory metabolism (again characterised by a limited number of end products; e.g. $CO_2$ and $H_2O$). In addition to this, the detailed pathways are well understood with respect to their stoichiometry and hence can be easily combined into a simple model (in this case, merely a mathematical form relating variables via a mass balance). Hybridoma cells have a much more complex metabolism both with respect to the range of substrates and nutrients utilised and the metabolic pathways (and hence, possible products) available. Insufficient experimental work has been undertaken to date to allow a mass balance with any degree of accuracy. Hence even the simplest model for the system is not available. The complexity of the product (antibody), where contributions to its structure are made by an extensive range of catabolic and anabolic pathways, only exacerbates this situation.

In bacterial and eukaryotic systems, simple substrate and product regimes have led to the use of a range of on-line measurement devices (notably oxygen uptake analysis, carbon dioxide production rate and ethanol excretion rate) where a direct comparison between experimental and model predictions may be made and changes to either the process variables under control or the model used to predict these variables

may be made (see Fig. 1). More recently, more sophisticated on-line measuring devices (e.g. mass spectrometer and HPLC) have been used. Iterative techniques may then be used to approach product optimisation. By contrast the product spectrum of hybridoma cells does not allow on-line determination in general. Consequently while such approaches have been used extensively in microbial growth studies [3], notably in the baker's yeast industry, they have received very little attention in the field of hybridoma growth. A further consideration when making the comparison of microbial to animal cell cultivation and control is that of the time-scale of the control process. Microbial processes are generally much faster and require more rapid evaluation, comparison and control action than does animal cell cultivation. Consequently, what by necessity would be on-line in a microbial cultivation may not necessarily be so in an animal cell cultivation and the control strategy may then be considerably different.

# 3 Kinetics of Cell Growth and Antibody Production

## 3.1 Theoretical Considerations

### 3.1.1 Cell Growth

The growth of hybridoma cells in batch cultures (see Fig. 2) follows the classical profile observed for most microorganisms. Thus mammalian cell populations exhibit a series of growth phases:

1) lag phase — zero net growth (specific growth rate $\mu = 0$),

2) accelerating phase,

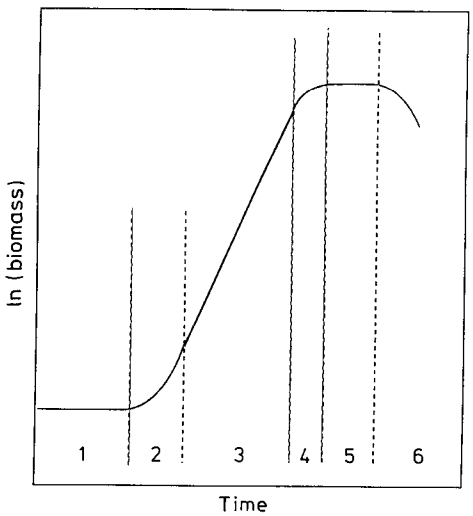


**Fig. 2.** Growth of hybridoma cells in batch culture

3) exponential growth phase ($\mu = \mu_{max}$),

4) decelerating phase,

5) stationary phase ($\mu = 0$),

6) decreasing phase and death.

In batch cultures environmental conditions are constantly changing; essential nutrients become depleted and metabolites and cell products accumulate. Cell mass enters the decelerating phase when either a) essential nutrients become limiting or b) inhibitors accumulate above toxic thresholds, or c) both a) and b). If the culture conditions were such that each cell was able to grow under favourable conditions, such an excess of all nutrients and the cell concentrations can be modelled by:

$$\frac{dx}{dt} = \mu_{max} x \tag{1}$$

where,

x is the biomass concentration
$x_0$ is the biomass concentration at zero time
t is time

$\mu_{max}$ is the maximum specific growth rate at the given conditions.

The Monod equation is applied if the growth conditions do not permit maximum growth, and where it is assumed that growth is limited by the availability of substrate, S [4],

$$\mu = \mu_{max} \frac{S}{K_s + S} \tag{2}$$

where

S is the concentration of the limiting substrate and
$K_s$ is a saturation constant, the Michaelis-Menten constant.

Monod [5] was the first to demonstrate in 1949 that kinetics of growth, in a bacterial system, closely resembled the Michaelis-Menten equation for enzymatic substrate conversion to product in a buffer system. According to this model, cell growth was limited by the concentration of a single substrate with cells only growing at their maximum in conditions of excess limiting substrate. Since then the model has proved applicable to the growth of eukaryotic cells such as yeasts as well as prokaryotic cells. Studies concerning the continuous culture of mammalian cells have recently been reviewed by Tovey [6]. The first reports of the cultivation of animal cells in a chemically defined medium in chemostat culture were obtained with mouse LS cells growing in a chemically defined, protein-free medium [7-10], under glucose [11] and choline [12] limitation. Most of the subsequent chemostat studies, until the advent of hybridoma cells, involving animal cells have been carried out with mouse leukaemia L1210 cells [13-18] with the successful use of glucose limitation.

Stable steady-state cultures of L1210 were obtained in the chemostat at various dilution rates and the relationship between the steady-state cell concentration and dilution rate was found to be in good agreement with the theoretical curves of cell density and glucose concentration computed from the Monod equations [14, 19]. At high dilution rates however there was substantial deviation from the theoretical curves.

Recently Hu and Wang [20] have commented that these findings may not be surprising considering the complexity of mammalian cell culture media. They point out that the Monod model as expressed in Eq. (2) is for cell growth limited by a single substrate and that these growth-limiting substrates are more easily identified in chemically-defined microbial growth media than in the more complex mammalian cell culture medium containing various amino acids, vitamins, fatty acids and a serum supplement. It is therefore possible that other mathematical models [4] which describe growth independent of substrate concentration, with more than one substrate limiting, or in terms of substrate inhibition may prove more appropriate for hybridoma growth.

In a chemostat the rate of growth is determined by rate of input of fresh medium and the cells are maintained in exponential growth at a constant concentration whereby:

$$\frac{dx}{dt} = \mu x - Dx \tag{3}$$

The specific dilution rate D relates the nutrient feed rate F to the chemostat volume V. Thus $D = F/V = 1/\gamma$, where $\gamma$ is the residence time.

For steady state operation, with respect to x,

$$\frac{dx}{dt} = 0$$

and

$$\mu x = Dx$$

$$\mu = D = F/V \tag{4}$$

Thus the specific growth rate equals the specific dilution rate at steady state. In substrate unlimited cultivation $\mu = \mu_{max}$. In his study with hybridoma cells in continuous culture Fazekas de St. Groth [21] extended the equation to incorporate a term for the significant number of dying cells.

### 3.1.2 Relationships Between Product Formation and Cell Growth

A rational scale-up approach requires knowledge of cellular physiology particularly cell growth and antibody production kinetics. To date there have been few detailed studies concerning antibody production kinetics reported in the literature. There are two major reasons for this:

1) The significant research commitment required to obtain accurate and reliable data.
2) Commercial sensitivity.

Kinetic data may be obtained either in batch or continuous culture (or preferably both, enabling the most complete analysis to be undertaken).

Traditionally, in microbial growth systems, batch kinetic analysis is undertaken by taking samples for biomass and product at sufficient regularity to enable a differential rate analysis to be performed. In this method over a differential time element, differential biomass and production concentrations are calculated. This primary data is then processed into the commonly quoted secondary data viz specific growth rate and specific product formation rate.

In the kinetic analysis of monoclonal antibody producing cell lines, significant differences exist from these traditional approaches in microbial growth systems, although the extent to which these differences are of significance may vary from cell line to cell line. Firstly in analysis of microbial systems, generally both the biomass and product may easily be measured accurately with high frequency. In addition both the biomass viability is high (>95%) and the product formed stable. These two

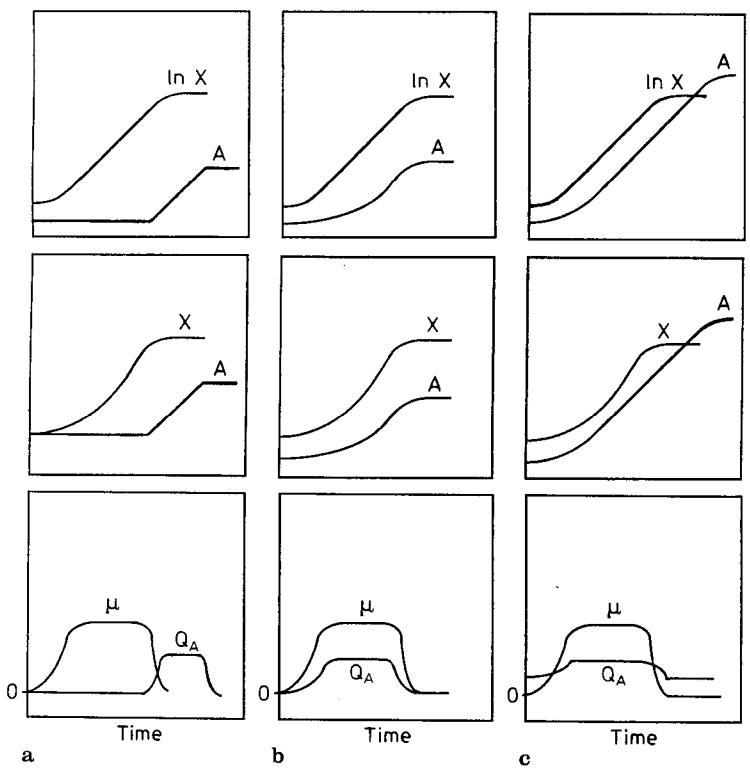


**Fig. 3a–c.** Typical biomass, antibody, specific growth rate and specific antibody formation rates in batch culture

characteristics enable a kinetic analysis to be undertaken with high degree of accuracy and the secondary data derived from primary measurements have a correspondingly high degree of accuracy and reliability.

As a result, in microbial growth systems, typical kinetic responses have been characterised into three major types, viz growth associated product formation (see Fig. 3b), non-growth associated product formation (Fig. 3a) and combined growth and non-growth rate associated product formation (Fig. 3c). Fig. 3 illustrates these major types by taking typical primary data in two forms, namely biomass and product concentrations versus time and log biomass concentration and production concentration versus time, and illustrating their transformation into secondary data, namely specific growth rate and specific product formation rate. An underlying assumption in these profiles is that both specific growth rate and specific product formation rate become balanced within the duration of the batch.

While these major types may also be used to characterise monoclonal antibody producing cell lines, significant differences exist between these kinetic analyses and those of typical microbial growth systems. Firstly, the measurement of antibody concentrations does not have the same degree of accuracy as microbial growth products in general and their analyses are more time-consuming with the net result that they are generally taken less frequently and have less absolute accuracy. In addition the biomass may exhibit less viability than a microbial growth system. It is not clear whether non-viable cells (however measured) produce antibodies although this is unlikely and, further, whether non-viable cells release stored antibodies into the medium. Antibody degradation is also possible leading to a very complicated kinetic analysis. It may be possible to use indirect correlations for some of these estimates, e.g. the use of lactic acid concentration, although no clear evidence for the general use of such correlations exists.

Consequently it should be noted that firstly, the primary data is generally less accurate, reliable and frequent and that secondly, data derived from primary data is subject to conceptual considerations not generally associated with microbial growth systems. For example, it would generally be accepted that if viable ($X_v$) and non-viable ($X_{nv}$) biomass were present then the specific growth rate ($\mu$) would be defined as:

$$\mu = \frac{1}{X_v}\frac{dX_v}{dt} \tag{5}$$

since, by definition, non-viable biomas is defind as biomass unable to grow and divide. However the calculation of specific antibody production rate requires the decision as to whether only viable or both viable and non-viable biomass produce antibodies. Hence if an antibody degradation rate ($K_D(k^{-1})$) is defined then the specific product formation rate may be represented in a number of forms. This basic form may typically be given as follows:

$$Q_A = \frac{1}{X_v}\frac{dA}{dt} - \frac{K_D A}{X_v} \tag{6}$$

where antibody is produced only by viable biomass

(that is $\frac{dA}{dt} = Q_A^v X_v$ = specific antibody production by viable biomass);

or

$$Q_A = \frac{1}{X_{TOT}} \frac{dA}{dt} - \frac{K_D A}{X_{TOT}} \tag{7}$$

where

$$X_{TOT} = X_v + X_{nv}$$

and antibody is produced by both viable and non-viable biomass (that is $\frac{dA}{dt} = Q_A^v \cdot X_v + Q_A^{nv} \cdot X_{nv}$ where $Q_A^{nv}$ = specific antibody production by non-viable biomass). It is important to appreciate that these are only two possibilities based on simplistic assumptions. However, without a conceptual basis for antibody production, conversion of primary data to secondary data is not possible.

Within this context the literature on the kinetic analysis of batch data for antibody production may be discussed. Boraston et al. [22] and Velez et al. [23] have followed increases in cell numbers and antibody levels in batch cultures and their findings suggest that a considerable amount of antibody production occurs after cell growth has ceased (Fig. 3c). This data implies that a significant proportion of antibody synthesis is non-growth associated and this would appear to be the general consensus of opinion among workers in the field. There are other reports however, e.g. Lavery et al. [24], which suggest that antibody production is directly related to growth since no increase in antibody levels occurs after the peak of cell growth (Fig. 3b). These differing results may reflect the fact that each hybridoma cell line is unique with its own kinetic properties.

The kinetic characteristics of each cell line has important implications for process development and these are discussed more fully in Sect. 5. Clearly it is difficult to

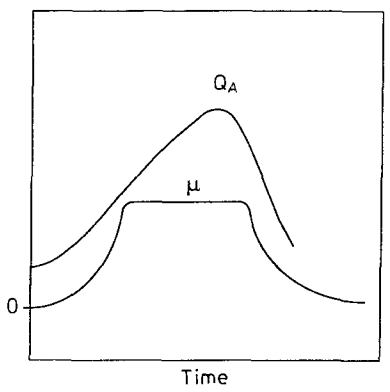


**Fig. 4.** Schematic diagram of experimentally obtained specific growth rate and specific antibody formation rate profiles in batch culture

determine what is happening in batch systems although Low [25] has recently compared the specific antibody production rate with specific growth in batch culture. This is shown schematically in Fig. 4. This data indicates that a proportion of antibody is produced as the specific growth rate decreases and is therefore partly not growth associated. The antibody production towards the end of the culture could be due to release of antibody from cells as they die, the antibody having been synthesised earlier in the culture.

## 3.2 Experimental Data

Definitive studies to determine the relationship between antibody productivity and cell growth require the use of continuous cultures. The report by Fazekas de St. Groth in 1983 [21] was one of the first concerning the continuous culture of hybridoma cells and showed the potential for the automated production of monoclonal antibodies in a cytostat. The work of Birch et al. [26] investigated the complex interaction between antibody production kinetics and cell growth. They studied the growth of one cell line in continuous culture at different growth rates with different limiting substrates, i.e. glucose, glutamine or oxygen. Their data indicated that antibody synthesis was not growth-rate dependent (see Fig. 5c) and this supported their batch culture data which showed that antibody synthesis continued during the decline phase of the culture. Also shown in Fig. 5 are three other possible specific antibody production rate trends with specific growth rate. Two of these, 5a and 5b, are illustrated for comparison purposes with Fig. 3 (namely 3b and 3c) in which growth associated antibody production (Fig. 3b) and both growth and non-growth associated antibody production (Fig. 3c) are shown. Also included (Fig. 5d) is a schematic representation of antibody production for a cell line studied in our laboratory [25]. This study indicated that antibody production was both growth and non-growth associated up to a critical specific growth rate, after which antibody

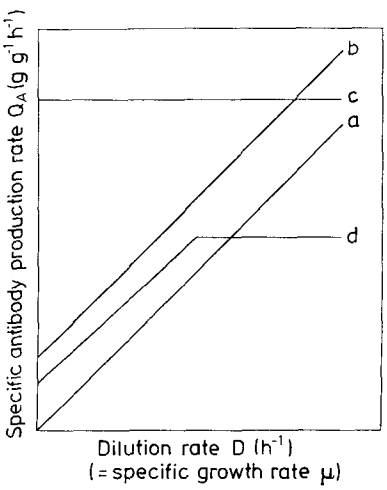


**Fig. 5.** Schematic diagram of typical specific antibody formation rate profiles in continuous culture

production was not growth associated. Such a response may be considered a combination of antibody production responses, illustrated by Fig. 5b and 5c. An investigation of this profile has not been undertaken in detail but the implication is that antibody production places a significant anabolic load on a hybridoma and that this reaches a maximum level at a growth rate (critical growth rate) less than the maximum specific growth rate. Consequently a saturated profile exists past this critical specific growth rate. There is evidence for such profiles in both bacterial and yeast cultivation [27].

It is then clear that significant variability in the kinetics of cell lines exists indicating substantial cell line dependence and that detailed definitive kinetic studies are few in the literature and provide a lack of a rational basis for scale-up.

## 4 Parameters Affecting Cell Growth and Antibody Production

Effective process development requires that the nature of all the parameters which affect the cell culture process be first identified and then measured accurately. This can be achieved by either employing the traditional empirical approach or, by adopting the more recent application of simulation experiments based on mathematical models of kinetics [2]. From the data presented in Sect. 3 the simulation approach would appear to be feasible. However, due to the complexity of mammalian cell culture systems this approach would be much more difficult than with bacterial systems. As yet little work has appeared based on this type of approach and thus most of the data presented in this Section has been obtained from an empirical approach.

In most respects the parameters which affect animal cell culture processes are the same as those identified as being important for microbial systems. However there is one important difference and that is the fact that mammalian cells, in contrast to most microorganisms, possess shear-sensitive cell membranes. This characteristic has directed those involved in the scaling-up of animal cell culture system to develop novel bioreactors which aim to reduce or avoid the use of mechanical mixing and aeration and their associated problems. Although, as discussed later, these novel processes have proved very successful, there was, until very recently, little published data upon which to base a rational system design and scale-up approach, particularly in the area of shear sensitivity.

In this review we concentrate on two of the most important scale-dependent effectors on cell culture growth and productivity, i.e. shear forces and dissolved oxygen levels. However as these problems are resolved more attention will be focused on the need for culture media suitable for the maintenance of high cell numbers. We shall therefore discuss media design as an integral part of process control and development.

### 4.1 Effects of Shear on Mammalian Cells

There is general agreement that the cultivation of animal cells should avoid the vigorous agitation systems employed for microbial systems. Telling and Radlett [28] found that agitation speeds used in vessels stirred by a single turbine impellor in the