DYNAMIC OPERATION OF MAMMALIAN CELL
FED-BATCH BIOREACTORS

by

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M.S. Royal Institute of Technology,
Stockholm, Sweden, 1981

Submitted to The Department of Applied Biological Sciences
in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF SCIENCE

in

BIOCHEMICAL ENGINEERING

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

June, 1992

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(To my mother)

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Abstract
The objective of this research was to improve the performance of mammalian cell fed-batch bioreactors using dynamic operation, which is defined as rapid changes in the culture environment, faster than those occurring during the semi-steady states of a batch culture. The hybridoma CRL 1606 was used as a model cell line. Performance was defined by antibody concentration and quality as well as process productivity. The experiments examined the response by the cultures, after an initial nutrient limitation period, to step and ramp changes (changes) in the concentration of glucose, glutamine, or the combination of glucose and glutamine. A control system was constructed that relies on a feedback estimation of viable cell density, obtained from a program that automatically measures the dynamic oxygen uptake rate of the culture, together with a feed-forward model predictive controller, to maintain a desired nutrient concentration trajectory. The main experimental finding was that dynamic operation can improve antibody production by about 40% over a reference batch culture, to an average of 119±3 mg/L from 86±1 mg/L. Glucose change cultures had 20% higher viable cell number and 50% higher specific antibody productivity, and about 60% higher volumetric productivity compared to a batch culture. Glutamine change cultures had lower viable cell number, but about two times higher specific productivity than a batch culture, resulting in an overall improved production. The volumetric productivity was lower than for a glucose change culture due to a prolonged growth period. There was no difference seen in performance between step and ramp changes, neither was the nutrient concentration change difference during a step important. There appears to be a window in time during which the nutrient concentration change can affect process performance. If the change comes too early or late there is no improved production. Dynamic operation of a reactor has an impact on cell metabolism and cell size distribution. Compared to a batch culture, cells in a dynamically controlled culture have a smaller average size before, and a significantly larger size after the concentration change; a more anaerobic metabolism which causes higher formation rate of ammonia for a glutamine change culture, and both lactate and ammonia for a glucose change culture. The metabolic waste product concentration in a dynamically controlled reactor are therefore higher than in a comparable batch reactor. The stimulation of the culture from dynamic operation appears to outweigh eventual waste product inhibitions. There was no impact seen on antibody quality from dynamic operation of the culture, as measured in western blot and gel electrophoresis assays. The results are examined in runtime as well as in integral viability time (defined as the integral of the total viable cells in the reactor over time); a model was developed that describes productivity of cultures as a function of the integral viability time.

Thesis Supervisor: Charles L. Cooney
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Acknowledgement

One does not go through a place like MIT without help from others, and I have lots of people to thank. First of course I want to express my sincere gratitude to my trusty advisor, Professor Charles Leeland Cooney, who supported me in good times and bad. An outstanding scientist, and perhaps the best science writer there is. Charlie, I learned an awful lot from you. Thanks also goes to my thesis committee, Professors Sinskey, Stephanopoulos, Danny Wang (the sharpest man in the business, who was very patient with me), and Harvey Lodish (who's vast knowledge of cell biology always amazes me).

A number of people worked together with me. I screwed up, and they fixed it. Jean-Louis Romette, who's ability to get MIT's physical plant to do his bidding was nothing short of miraculous. Jamie Piret who know MIT like no one else. And not least Fernando Sa'nches Riera, Tom Chavez, and Bo Persson. And I of course have to especially mention my undergraduate students: Tim Hale, and in particular David Brown and Jean Condon, who both kept coming back for more. They probably thought I could not make it without them, and they where right.

The only reason for anyone to read an acknowledgement is to see if they have been mentioned, so I thought I would simply mention all the other people at MIT who has helped me. Here goes in random order:

Enno/Mike/David/Claus/Raquel/John/Matt/David(he helped me twice)/David(another David)/Shi-Ping/Rahul/Jack/Raju/Greg/Brian/Dan/Gary/Bob/Mary/Doug/Dave/Steve/Gautam/Demetri/Nick/Neil/Erno/Se-Jung/Takahiro/Bernard/Mark/Dawn/Wen/Wen(she also helped me twice)/Gino/Stewart/Chris/Ed/Bruce/Craig/Debra/Audrey/Sonia/
Jean-François/Bob/Lynne/Diana/Karen/Yizu/Anna/Arun/Vijay/Viia/John/............

The list goes on, and there are a number of other people at MIT that have helped me. You may not have been mentioned, but you are not forgotten.

I had the support of a number of Swedish foundations over the years, without which it would have been very difficult to remain at MIT: The Fulbright foundation, the Swedish-American Foundation, the Swedish Board of Technical Development (STU), the Wallenberg Foundation, J O Naucle'rs Foundation (Perstorp AB), Adelskölds Foundation (The Royal Swedish Academy of Science).

Just as important, I also want to acknowledge all the people outside MIT who have helped me. To keep my sanity, I needed to escape the rigors of MIT and party or just hang out. I kept my sanity a lot; one can never be too careful. In random order, thanks to you all: Kent/Yvonne/Anja/Sissi/Anders/Bent-Erik/Susan/Shuichi/Lars/Anders/Roger/Chris/Thomas/Carina/Jocke/Cyn/Urban/Ann/Chris(another Chris)/Dragan/Maria/Martha/Susan(again)/Chris/Chris(there are many Chrisises)/Barbara/Bonita/Gary/Deebee/Rune/Pam/Felip/Håkon/Jens-Petter/Kim/Cecilia/Henrik/Bob/Susan(again, she meant a lot)/Jack/Lorette/Ellen/Tuomas/Susanne..... and all my other friends around the world. After all this listing, if I have missed anyone they are probably going to be real upset.

Lastly, I want to thank my family for being my family, and which the best to my sister who is going to have a baby in a couple of months.

Things got rather hectic towards the end, lots of work and no fun. Now I am going out to party with my friends. Martha and Henrik, thanks for fixing the party; can you fix the hangover too?
1. Introduction

Mammalian cells have a lot going for them. They are able to make a number of functional proteins that at present can not be produced in any other way. These proteins have a several potential uses, perhaps most importantly as therapeutics. There are also a number of serious problems associated with culturing these cells in large production scales that can lead to high production costs. The problems include low productivity, low cell density, fragility of the cells which causes difficulties for oxygenation via sparging, and complex medium requirement. Problems such as these makes mammalian cell cultures difficult to run and control on an industrial manufacturing scale. Effective development of appropriate process configurations and c:isigns is therefore of great importance for the success of a manufacturing operation utilizing this technology.

This thesis describes a novel approach to the development and control of large scale mammalian cell cultures. The objective of this research was to investigate the behavior and productivity of a model fed-batch hybridoma culture in response to step and ramp changes in the concentration of the major nutrients, glucose and glutamine. A secondary objective is to introduce a methodology for process development that e:x:ines cellular response to changes in the culture environment.

The research was motivated by the need for rapid development efficient and robust mammalian cell culture processes. This need stems from the requirements placed on the development efforts by the economic and regulatory forces that affect these manufacturing processes. These drivers for process development are different than those for low value products, such as antibiotic. Manufacturers using mammalian cell cultures to produce high
value products generally puts a premium on getting a product rapidly to market, rather than optimizing the process to obtain a competitive advantage from low operating costs.

1.1 The Regulatory Approval Process for Biologicals

The regulatory requirements for proteins produced from mammalian cell cultures are at present considered to be most rigorous in the U.S., which is also the country where the majority of these substances are manufactured and tested. The U.S. is the largest single market for this type of products, and few companies involved in sales of therapeutic proteins can afford to bypass this market. For reasons like these the U.S. government agency responsible for approval of therapeutic substances, the Food and Drug Administration, tends to set the worldwide standard for manufacturing and use of these products. Only the U.S. regulatory environment will therefore be considered here.

In the U.S., products from mammalian cell cultures that are used as human therapeutics are regulated and sold as biologicals, rather than drugs. The regulatory requirements for biologicals are quite stringent, in part reflecting a lack of knowledge about the influence of protein structure and composition on their in vivo functions. Before a biological is approved by the FDA for sale as a therapeutic substance, it has to be proven to be safe and efficient. To do so, it has to go through three phases of clinical trials: Phase 1, where the biological is first tested a few in healthy humans to ensure its safety. Phase 2, where it is tested in a limited number of patients to determine efficacy. Finally, it is tested in phase 3 trials, where it is administered to a large number of patients to determine its efficacy, safety, and possible side effects. After approval for sale, efficacy and side effects has to be further monitored by the manufacturer, and any serious incidents has to be reported to the FDA.
Little is known about how the manufacturing process for proteins from mammalian cell cultures affect their structure and composition and thus their function. This is at present compounded by the difficulties in routinely determining the exact composition of a protein, especially concerning micro-homogeneity in the product mixture. For these reasons the FDA defines the composition of a biological product by its manufacturing process, rather than by analysis of composition of the final product. The manufacturing process is considered to be an integral part of the final product, and may not be changed significantly without submitting the product to a renewed series of clinical trials. The process may further not be transplanted to another manufacturing site without re-testing the product. Biologicals tested in phase 3 clinical trials and usually also in phase 2 trials, has to be produced in the production facility that is to be used for the final approved product. The manufacturing plant therefore has to be built long before any cash flows from sale of the product can be realized.

1.2 The Marketing Environment for Biologicals

Most products manufactured by mammalian cell culture processes are protected from competition in the market by some form of exclusivity. Usually this takes the form of a patent on the substance, or on the use of the substance. Another form of protection is Orphan drug status, which the FDA may award the manufacturer of a therapeutic with a small target patient population. Orphan drug status means that no other manufacturer will get approval for the same use of the substance. This is intended to make development of drugs with small markets economically viable for a manufacturer, and thus ensure that patients will have an opportunity to benefit from the therapy.

The timeframe for market exclusivity is limited by patent expiration times. For instance, in
the U.S. patent protection is provided for 17 years, and Orphan drug status is awarded for seven years. Thus, after development and clinical trials, perhaps less than ten years are available for marketing a product without competitive pressure. It is imperative to get the product to market quickly in order to reap the benefits of market exclusivity for as long as possible. For many young biotechnology companies it is also important to obtain a cash flow from the sale of the product as soon as possible. In cases where a substance can not be protected by patents and other means, lead time to market is of paramount importance. If a significant lead in development time can be established over potential competitors, a situation comparative to exclusivity in the market may be established for a significant time.

There is a silver lining on these clouds for biologicals. Because a biological is defined by its manufacturing process, any competitor wanting to sell the substance for the same use will have to go through a separate series of clinical trials for its product. This is a marked difference from the situation in the generic drug market, and it creates significant barriers for entrance to the market for competitors.

1.3 Requirements on Mammalian Cell Culture Processes Development
The requirements placed on the development of industrial mammalian cell culture processes are derived from the regulatory and marketing environment outlined above. As the product is defined by its production process, the process becomes essentially locked once the product is in its pivotal clinical trials. After this stage, only minor changes can be made to the process without risking that the FDA will require a new series of clinical trials to re-approve the product. It is therefore vital that the process developed and used in the early stages of a project can be scaled up to produce commercial quantities of the protein. It is important to select an appropriate process early in the project, i.e. to "get it right" from the
start. The selected process should be robust and reliable to ensure product safety, and there is therefore little room for experimentation with exotic process configurations. For reasons like these, most industrial mammalian cell culture processes are very conservatively designed.

A consequence of an exclusive marketing situation is that productivity and optimal operation of the processes is not a major concern. The manufacturer can charge enough for the product to obtain a reasonable return on his investments, and does not have to obtain a competitive advantage from low operating costs. However, time to market for the product is a major concern as outlined above, and rapid process development is important.

The economic and regulatory driving forces described above translates into somewhat different requirements for the development of mammalian cell culture processes, as compared to more traditional bioreactor manufacturing processes such as antibiotic fermentations. The focus of the process development efforts shifts from an optimal process, to rapid development of a robust and reliable process. The most common processes used in industry for mammalian cell cultures are batch and repeated batch cultures in stirred tanks. In a repeated batch culture, the media is drained off at the end of the culture, and a small amount of media and cells left in the reactor is used as the inoculum for the next batch. Potentially more productive but complicated processes, such as continuous cultures, are seldom used. Batch processes fulfill most of the requirements for an industrial mammalian cell culture. They represent a well established technology that is familiar to the regulatory authorities. They are reliable and generally robust, with a homogenous and verifiable culture environment. They are comparatively easy to scale up. It is also usually possible to develop a large scale batch process quite rapidly. Batch processes are not necessarily the ideal manufacturing tool though. They have low
productivity, and do not produce very high product concentrations, which is a major concern for the downstream processing of the product. There are also limited possibilities to control the culture conditions in batch process, and therefore limited possibilities for improving process performance.

1.4 Motivation
The motivation for this work comes from a desire to improve the performance of mammalian cell culture fed-batch bioreactors. Though optimal process performance is not a major concern, it is still desirable to improve process performance. More productive processes require smaller scale equipment and less initial investment for plant construction. Alternatively, better process performance permits increased production levels, for instance to meet increased market demands, without further scaling up or multiplying the process. For reasons such as these there will always be an interest in more productive processes for these products, as long as the process can meet the requirements outlined above.

One process configuration that does meets those requirements is the fed-batch process. Fed-batch cultures have most of the advantages of a batch culture in terms of reliability, homogenous and verifiable culture environment, and scalability. In addition, they have the advantage that culture conditions can be controlled and changed during the course of the culture, which gives an opportunity to improve process performance if a suitable control strategy can be developed. Although though they are not as well established as batch processes, they are similar enough that regulatory approval likely can be obtained without extensive tests and clinical trials to ensure the safety of the product. Also, if an existing process can be switched from a batch to a fed-batch mode of operation the same equipment can be used more productively, without a need for additional investments. For these
reasons, it is likely that fed-batch processes will be used in industry if suitable operating strategies for such cultures can be developed, and if the resulting improvement in performance is substantial.

1.5 Background

Development of operating strategies for bioreactors in general has received considerable attention, although only a small part of this literature has been devoted to strategies specifically for mammalian cell cultures (see literature review). A common approach to develop control strategies for fed-batch bioreactors is to perform a large series of smaller scale batch cultures under varying conditions. The different cultures are included in a standard factorial design, intended to separate the influence of different parameters from each other (see for example Glacken, 1987). From the result of such an investigation a mathematical model of culture behavior can be derived, and this can be used to design an optimal control strategy given a desired control objective.

There are several assumptions implicit in this approach, and some of them may be of particular importance for mammalian cell processes. One assumption is that there are no significant effects on culture behavior resulting from changes in control parameters per se, i.e. only the absolute level of a parameter affects the culture, while its rate and direction of change of has no influence. Another assumption is that the behavior of the culture does not depend on the history of parameter trajectories, or on the history of the culture. Thus, effects resulting from rapid changes in culture conditions will probably not be detected in this approach to process development. Neither is it likely that this type of behavior will be detected in fed-batch cultures developed from such investigations, as these are typically operated in a narrow range of conditions around the desired process trajectory.
1.6 Opportunities for Improving Fed-batch Culture Performance

There are further opportunities to improve culture productivity that the approach described above may miss. Mammalian cells are known to be influenced to a significant degree by changes in culture environment and by the history of the culture (see literature review). By introducing appropriate changes in culture conditions, it may therefore be possible to utilize this behavior to stimulate process productivity. A common approach to investigate the effect of rapid changes in culture conditions is to subject a continuous culture to a series of pulse and step changes in the levels of culture parameters, such as nutrient concentrations. However, a continuous culture is inherently a different process than a batch or fed-batch culture, and it is usually not possible to translate responses from a continuous culture to a fed-batch culture. To investigate such effects in fed-batch cultures, a more appropriate approach may be to introduce such changes directly in fed-batch cultures.

There are other opportunities for improving process performance that can be missed in investigations relying on series of batch cultures. Cells subjected to environmental stress frequently display an enhanced specific production and secretion of proteins (see literature review). This type of behavior also have been demonstrated for commercially interesting cell lines such as hybridomas (Miller, Blanch, and Wilke, 1988; Maiorella 1988; Øyaas et al., 1988). Stress conditions are defined as culture conditions that are outside the normal range for the cell, and that result in death or clearly sub-optimal growth of the culture. All types of cells possesses a network of proteins whose expression is elevated in response to a number of different stress situations. Some of these proteins have been shown to be part of the protein processing and secretion mechanisms of mammalian cells (see literature review). Among conditions that elicit this response are heat shock (Lindquist, 1986; Lindquist and Craig 1988), glucose deprivation (Lee, 1987; Kozutsumi et al., 1988), non-optimal pH (Whelan and Hightower, 1982), hyperosmolarity (Tanaka, Jay, and Isselbacher, 1988;
Øyaas et al., 1988), and low oxygen concentrations (Whelan and Hightower, 1982; Sciandra et al., 1984). The higher specific productivity displayed in such cultures suggest some possible tactics for developing control strategies for commercial processes. However, the non-optimal growth conditions also result in a decreased cell viability and density, and volumetric productivity therefore decreases (Miller, Blanch, and Wilke, 1988). A control strategy utilizing such stimulatory phenomena has to increase overall protein production, and should ideally enhance both cell density as well as specific productivity. To formulate investigative designs that can discover such phenomena can be quite challenging, but some approaches have already been explored, among them temperature changes (Passini and Goochee, 1989; Sureshkumar and Mutharasan, 1991) and nutrient deprivation (Ozturk and Palsson, 1991).

1.7 Objective and Approach

The objective of this work is to investigate the effect of dynamic changes in culture conditions on the behavior, productivity, and product quality of model mammalian cell fed-batch culture, with an aim towards developing improved control strategies. Dynamic changes are defined here as changes in the culture environment that are significantly faster than changes that normally occur during the semi-steady states of a typical batch or fed-batch culture. Although a number of culture parameters influence the behavior of a mammalian cell culture, nutrient levels have perhaps the most significant impact. Glucose and glutamine are the major energy sources for these cells, and this work was therefore focused on effects resulting from dynamic changes in the concentration of glucose, glutamine, and the combination of the two.

A direct approach to evaluate the effect of dynamic nutrient concentration changes in fed-
batch bioreactors is to introduce such changes in a fed-batch culture. There are, however, limitations to such experiments. It is possible to introduce large amounts of nutrients into the reactor without affecting the volume of the medium too much, but it is not possible to dilute the same nutrients to a low concentration without significantly diluting the cells in the culture. Therefore, upwards changes in nutrient concentration are much easier to implement than downward changes. In this work step and ramp changes where chosen as input functions for the nutrient concentrations because they are relatively easy to implement in a fed-batch system, and because they are standard inputs used for system identification purposes. For a step change, the culture is started at a low nutrient concentration, and after a delay time the concentration is raised quickly, and maintained at the higher level, fig 1.1. For a ramp change, the concentration raise is linear with time for the duration of the ramp, and then maintained at the high level. The step change is designed to examine effects from a rapid change in conditions, while the ramp change is designed to examine effects dependent on the time derivative of the input.

A further rationale behind this approach to experimental design is to attempt to utilize a stress related response to increase specific productivity of the culture. During the period of low nutrient concentration the culture will be limited for energy. This may cause an increased in protein production as cells under environmental stress frequently display an enhanced specific productivity (see literature review). At the time of the step or ramp change in concentration, the limitation will be released. The hope is that an increased specific productivity will be maintained for a prolonged period of time, without a reduction in growth rate and cell density; the net result being an improved volumetric productivity.
Fig. 1.1. Example of nutrient concentration trajectories for step and ramp changes.
One assumptions inherent in this approach to process development should be mentioned. Mammalian cell behavior commonly depend on the cell density of the culture (see literature review). Parameters such as oxygen uptake rate and protein production rate can change with cell density. However, the rapid dilution of cells which will occur during the introduction of feed medium for the concentration step, is here assumed not to affect the behavior of these cultures. This is a reasonable assumption for an actively growing culture, and dilution factors below 2-3 times, as will be shown by the results. It should be emphasized, that this approach is intended to complement to basic factorially designed batch experiments. The methodology is aimed at investigating and utilizing possible stimulatory effects from dynamic changes in culture conditions that may be overlooked in a statistically designed experimental series, but it can not replace such an approach. Indeed, the present investigation uses as a starting point for experimental design data gathered on the CLR 1606 hybridoma in a factorially designed experimental series by Glacken (1987).

1.8 Goal

The goal for this work is to increase the final product concentration and the volumetric productivity of the protein of interest. In addition, the influence on product quality will be investigated. Product quality is here defined by the composition and functionality of the protein.

The impact on the whole production process including downstream purification steps have been considered in setting this goal. The performance parameters of a mammalian cell culture that are most important for the efficiency of the overall production process are final product concentration, volumetric productivity, and product quality. Final product concentration influences the effectiveness of the downstream purification of the product, and
thus has a major impact on the overall productivity of the manufacturing process.
Volumetric productivity, which is the product of the cell density and the specific
productivity, is the parameter which determines the effectiveness of the mammalian cell
culture unit operation. In choosing between these parameters, final product concentration
was considered the most important, as it may significantly influence the effectiveness and
cost of the downstream purification steps, which usually dominate the manufacturing costs.
However, for some production processes other performance parameters may be of primary
importance.

1.9 Model system
As a model system for the present work fed-batch cultures of the hybridoma CRL 1606 was
chosen (Schoen, Bentley, and Klebe, 1982). There is a large body of process development
data published for this cell line, by which the present approach can be judged (Adema, 1989;
In particular, Glacken (1987) investigated the behavior of small scale cultures of this cell
line in detail, and much of this data provide a basis for choosing the range in which to
investigate parameters in this investigation. This cell line produces an anti-fibronectin
antibody, which is a chimeric antibody made up of fibronectin antibody chains stemming
from the spleen cell parent, and the of MOPC 21 antibody chains which are constitutively
secreted by the X-63 myeloma used to generate this hybridoma. Although the presence of
chimeric antibody makes investigation of antibody quantity and quality more difficult, it
does not interfere with the basic experimental procedures of this investigation.
1.10 Experimental Equipment and Scale

The experimental objective is to investigate the effects of (upward) step and ramp changes in concentration of the major nutrients, glucose and glutamine or the combination of the two, on the culture behavior, antibody productivity, and antibody quality of CRL 1606 hybridoma fed-batch cultures. To assure that the effects seen on antibody production result from the step and ramp changes only, the nutrient concentration trajectories may not deviate too much from their setpoints. Unfortunately, this rules out performing the experiments in a simple reactor with manual feeding of nutrients. In all likelihood this will result in serious deviations from the desired trajectories, fig. 1.2. In such a case there is a possibility that effects seen result from repeated variations in nutrient concentration, and not from the step and ramp changes. To avoid such uncertainties, computer control of the bioreactor was be used to achieve accurate and reproducible control of the nutrient concentration trajectories. Developing the technology and control programs needed for dynamic bioreactors operation was therefore be an integral part of this work, and considerable attention will be focused on obtaining the desired nutrient trajectories. Experiments was be carried out in a 2 L stirred reactor, that mimics the well mixed environment of a production scale bioreactor.
Fig. 1.2. Concentration trajectories for a step-change in nutrient concentration without accurate control.
2. Literature review

2.1 Overview of Mammalian Cell Culture Process Literature

This survey will cover literature directly related to this research. Subjects of concern for this investigation include: the development of mammalian cell culture processes; the dependence of mammalian cells on culture conditions and media composition; conditions known to influence the specific productivity of proteins in mammalian cells; the stress response of mammalian cells; the synthesis, processing and secretion of proteins in lymphocytes; and approaches to utilize the stress response to enhance culture productivity.

There is a large literature concerning the use of mammalian cell culture as an industrial production tool, and there have been a number of books written on the subject (for instance: Ho and Wang, 1991; Lydersen, 1987; Mizrahi, 1989; Spier and Griffiths, 1987; Spier et al., 1988). In particular, the development of these processes for industrial purposes have been reviewed by Reuveny and Lazar, 1989; Batt and Kompala, 1989; Harbour, Barford, and Low, 1988; Macmillan et al., 1987.

2.2 Process development and design of mammalian cell cultures

That mammalian cells are influenced by the environment and history of the culture is well documented in the literature (see for example Seaver, 1987). A number of authors has systematically investigated the effects of culture conditions on mammalian cells, both in batch (Bree et al., 1988; Glacken, 1987; Wergeland and Wallberg, 1987; Dalili and Ollis, 1989; Miller, Blanch, and Wilke, 1988; Duval et al., 1991), fed-batch (Reuveny, et al., 1986 a, b, and c; Wagner et al. 1988 and 1990; Macmillan 1987; Miller, Blanch and
Wilke, 1988a; Lee, Varma, and Palsson, 1991), and continuous culture (Hiller et al., 1991; Seamans and Hu, 1990; Batt and Kompala, 1989; Miller, Blanch, and Wilke, 1988; Wagner, et al., 1990; Bree et al., 1988) (For a more complete listing see reviews by Reuveny and Lazar, 1989; Batt and Kompala, 1989; Macmillan et al., 1987.)

2.2.1 Response to transients

There has also been a few investigations concerning the response of mammalian cells to transients and rapid changes in culture conditions, mainly in continuous culture. In a series of excellent papers, Miller et al. systematically examined the response of hybridomas to pulses of glucose (Miller, Wilke, and Blanch, 1989a), glutamine (Miller, Wilke, and Blanch, 1989b), pH (1988a), lactate and ammonia (1988b), and varying oxygen concentrations (Miller, Wilke, and Blanch, 1987) in continuous culture. These studies provide a large amounts of valuable information regarding specific uptake and production rates for nutrients and waste products, characteristic delay times for metabolism, and estimation of ATP requirements for growth and maintenance of the cells. Unfortunately, as these investigations focus on the metabolism of the cells, no data is presented for antibody production in response to these pulses. Batt et al. (1989) later developed a structured model for growth and antibody production of hybridomas, and compared this model with published data, primarily from Miller et al. (1988). The model divides the metabolites of the cells into four of different pools, and describes the utilization of substrate, formation of waste products, as well as the formation of cell mass and antibodies. Using this model, the authors were able to predict the metabolic response to glucose and glutamine pulses quite accurately, as compared to the data of Miller. No attempt was made to predict the antibody production of a hybridoma culture in response to nutrient pulses.
Siano and Mutharasan (1991) investigated the effect of glucose and glutamine pulses, as well as of aerobic-anaerobic and anaerobic-aerobic transitions, on the NADH fluorescence of hybridoma cells. The cells where cultivated in continuous culture in a perfusion reactor, in order to obtain large cell densities for a sufficiently high fluorescence signal. After transition to anaerobic conditions, they found a significant increase in both specific lactate production rate (Pasteur effect), as well as an increase in (mitochondrial) NADH fluorescence, which they ascribed to the cessation of electron transport due to lack of available oxygen. For glucose depleted cultures, but not for cultures with higher glucose concentrations, they found a transient in NADH fluorescence after a glucose pulse. For both glucose depleted culture and higher glucose concentration cultures, the specific oxygen uptake rate decreased, and the specific lactate production rate increased, following the pulse. These effects the authors explain by a significant competition for ADP between the mitochondria (oxidative phosphorylation) and the cytosol (glycolysis) during the glucose pulse (Crabtree effect). Unfortunately, no data for antibody production was presented.

A few investigations have utilized fed-batch cultures of mammalian cells directly for process development purposes (Miller, Blanch, and Wilke, 1987; Shaughnessy and Kargi, 1990 a and b). In particular, Truskey at al. (1990) used fed-batch cultures of hybridoma, lymphocyte, and T cell lymphoma cells to develop accurate models for growth rate as a function of culture conditions. The reason to use fed-batch cultures for this purpose was that some of the cell lines used display a significant dependence on cell density during batch growth. This problem was circumvented by diluting the culture during growth to maintain a relative constant cell density and nutrient concentration. Models describing cell growth, nutrient consumption, and waste product formation where developed form the data obtained.

Lastly, there have been a few recent publications regarding the use of changes in culture
conditions for process development for systems other than mammalian cells. O'Niel and Lybertos (1990) pointed out the value of using dynamic step response's for determining the delay times in the development of models for continuous cultures of Saccharomyces cerevisiae. They contended that including delay times in the process model and control was useful for making the process insensitive to disturbances, and also useful to evaluate the merits of various transient operating schemes, including periodic variations in operating conditions. Lastly, Szewczyk (1991) pointed out the limitations inherent in using variations in dilution rates for the dynamic operation of continuous culture processes.

2.3 Waste Product Inhibitions

A large number of investigations have been concerned with detecting and minimizing inhibitions by waste product formation, and much of the earlier literature is reviewed in Reuveny and Lazar (1989). The main waste products are lactate and ammonia, and considerable attention has been focused on measuring the influence of these compounds, and devising strategies to minimize inhibitions. The recent literature on this subject for hybridoma cells has been summarized by Ozturk, Riley, and Palsson (1992). Some of the most recent articles on the subject are McQueen, and Bailey (1990 a and b), Iriassel, Gleave, and Buttler (1991), Omasa et al. (1992), and Doyle and Butler (1990). Many of these investigations found ammonia in particular to be inhibitory to cell growth and also to some extent to product formation. Also lactate displayed some degree on inhibition, but to a lesser extent. The level of inhibitions vary significantly with cell types (Ozturk, Riley, and Palsson, 1992; Hassel, Gleave, and Butler 1990) and culture conditions, but seem to be a general phenomena. For most cell types, ammonia concentrations over 5 mM and lactate concentrations over 25 mM is inhibitory, although some cell lines seem to be able to tolerate up to twice these concentrations. For the cell line used in this investigation, the CRL 1606
hybridoma, Glacken (1987) found ammonia to inhibit growth at low cell densities, and lactate to weakly inhibit antibody production.

2.4 Conditions that Increase Specific Productivity

There are a number of indications in the literature that mammalian cells increase their specific productivity when subjected to stressful culture conditions, such as low nutrient concentrations (Sugiura, 1992; Hayter et al., 1992; Miller, Blanch, and Wilke, 1987 and 1988; Linardos, et al., 1991). Sugiura (1992) and Miller, Blanch, and Wilke (1987) found that the specific antibody production of a hybridoma in continuous culture increased under nutrient limitation. Also Birch at al. (1984) reported that specific antibody production increased in continuous culture when cells where limited for either glucose, glutamine, or oxygen. Increased temperature or heat shocks have also been shown to increase the specific antibody productivity of hybridomas (Sureshkumar and Mutharasan, 1991). In addition, hyperosmolaric conditions (Öyaas et al., 1988; Ozturk and Palsson, 1991a) as well as low pH (Miller, Blanch, and Wilke, 1988; Ozturk and Palsson, 1992) have been demonstrated to cause an increase in specific antibody productivity.

A number of authors have noted that protein production is increased in these cells under conditions of stress (Miller, Blanch, and Wilke, 1988; Sureshkumar and Mutharasan, 1991), and some of them have also suggested a connection with the protein processing and secretion machinery of the cells (Miller, Blanch, and Wilke, 1988). It has been suggested that this phenomena can be utilized in control strategies to increase production (Miller, Blanch, and Wilke, 1988; Passini and Goochee, 1989; Sureshkumar and Mutharasan, 1991; Ozturk and Palsson, 1991a). However, a common drawback to such strategies is that while detrimental culture conditions may increase specific protein production it also impairs cell
growth, and the volumetric productivity of the culture may therefore decline. Among strategies suggested to circumvent the problem with non-optimal growth and lower cell densities are cell recycle systems and cell immobilization systems, to maintain large number of cells under adverse conditions (Miller, Blanch, and Wilke, 1988; Ozturk and Palsson, 1991). Lin et al. (1989) has investigated the response of hybridomas to glucose deprivation and recovery, and to heat shocks, although the main focus of their effort seems to be to utilize the promoters of the GRP and hsp genes for regulation of genetically engineered proteins. A temperature shift strategy where the cells are grown at optimal temperature for growth up to the maximum cell density, and then shifted to optimal temperature for antibody production was tested by Sureshkumar and Mutharasan (1991), with mixed results. Also the effect of medium osmolarity on the behavior and protein production of mammalian cells have been investigated. The normal osmolarity range for optimal growth of hybridomas is 290 - 350 mOsmol, and is quite flat (Waymouth (1973); Schlaege, and Schupmp (1988)). Increasing osmolarity to 480 mOsmol completely inhibits cell growth, while specific antibody production increases (Øyaas et al. (1988)).

The investigations reviewed above have focused on the potential of using the stress response of mammalian cells to increase protein production. There is in addition a literature on the general stress response of mammalian cells, for conditions such as heat shock (Lindquist, 1986; Lindquist and Craig 1988), glucose deprivation (Lee, 1987; Kozutsumi et al., 1988), non-optimal pH (Whelan and Hightower, 1982), hyperosmolarity (Tanaka, Jay, and Isselbacher, 1988; Øyaas et al., 1988), and low oxygen concentrations (Whelan and Hightower, 1982; Sciandra et al.,1984). There is a common thread to all these investigations of the stress response. All types of cells possesses a network of proteins whose expression is elevated in response to a number of different stress situations. Some of these proteins have been shown to be part of the protein processing and secretion mechanisms of
mammalian cells (see below). To further highlight the connection between the stress response of mammalian cells and the protein processing network, some recent literature on this part of the cellular machinery is reviewed below.

2.5 Mammalian cell protein synthesis, processing and secretion

Mammalian cell protein secretion is believed to take place by two different pathways: the "regulated" pathway, and the constitutional pathway (Kelly, 1985; Gebhart and Rudden, 1986; Rose and Dorns, 1988; Lodish, 1988; Pelham, 1989; de Silva, Balch, and Helenius, 1990). In both pathways, proteins undergo post translational processing in the endoplasmic reticulum (ER) and the Golgi complex. In the "regulated" pathway, proteins are stored in secretory vesicles just under the plasma membrane, and are released upon stimulation of the cell by an external stimuli, such as a hormone. In the constitutive pathway, proteins are not stored, but are secreted at the rate with which they are produced and processed.

Immunoglobulins (Ig) are secreted via the constitutive pathway, and no storage of immunoglobulin is believed to take place in plasma cells, or in hybridomas. However, Ig light chains are often synthesized in excess, but they are retained in the cell, and are eventually degraded (Baumal, Potter, and Scharff, 1971; Hendershot et al., 1987; Hendershot 1990).

2.5.1 The post-translational processing of proteins

Research on protein processing and secretion by mammalian cells is still far from complete, but parts of the picture are now emerging. Post translational protein processing appears to
be conducted by a specific and highly organized proteinaceous network, that ensures the proper folding, assembly, and quality control of polypeptides. Processing is believed to take place mainly in the ER, where folding, disulfide bond formation, and subunit assembly takes place. Also the first sugar units are attached to glycoproteins here. Proteins that are not properly folded or assembled are retained in the ER, usually as aggregates, and are eventually degraded. Correctly processed proteins are transferred to the Golgi stacks, where attachment of additional hydrocarbon units, and trimming of oligomeric hydrocarbon chains, takes place. Some proteins are also oligomerized in the Golgi. Here proteins are in addition sorted for transport to different cellular compartments, or for eventual secretion.

Protein transport and retention is currently believed to take place according to the bulk flow model: There is a bulk flow of material from the ER through the Golgi to the plasma membrane, and unless a polypeptide is specifically retained in the ER or the Golgi it will flow with the bulk flow to the cell surface. It takes for instance about 10 - 20 minutes for a small peptide to travel from the ER to the cell surface (Weiland et al., 1987), while a protein takes from 30 min to several hours (Scheele and Tartakoff 1985; Yeo et al., 1985; Rose and Doms 1988). Proteins are not marked for transport after processing, but instead proper processing ensures that the protein is not retained by the processing and quality control machinery, and it is the retention and processing of proteins in the ER and Golgi that causes them to travel considerably slower than the bulk flow. Resident ER luminal proteins have for instance been shown to contain a specific retention signal (Munro and Pelham, 1987), that ensures that they are retained in the ER. It should be noted though, that the bulk flow model is not universally accepted, that arguments exists for specific transportation signals for some proteins, and that there may be several different signal systems (Rose and Doms, 1988).
2.5.2 Post-translational processing of immunoglobulins

Post translational processing for Ig’s starts already as the nascent polypeptide chain emerges from the ribosome into the rough ER. There it binds to the Binding Protein (BiP), which presumably serves to prevent the polypeptide from folding prematurely and erroneously (Hendershot et al., 1987; Kassenbrock et al., 1988; Flynn, Chappel, and Rothman, 1989; Hendershot, 1990). At this time, the first disulfide bonds are also formed on the Ig chain (Bergman and Kuehl, 1979), through the action of Protein Disulfide Isomerase (PDI) (Roth and Pierce, 1987). For immunoglobulins, also subunit assembly takes place very early. Ig light chains associate with the nascent heavy chain, and at the same time the first intra-chain disulfide bonds are formed (Bergman and Kuehl, 1979). After binding of light chains to the heavy chain, the complex is released from BiP (Hendershot, 1990). However, Ig sub-chains that are not properly folded, or that do not oligomerize to complete Ig molecules, are retained by BiP and are eventually degraded (Kassenbrock et al., 1988; Klausner and Sitia, 1990). Next, assembly of the complete Ig molecule takes place. The sequence of assembly differs depending on the heavy chain isotype. For IgG1 (the CRL 1606 antibody isotype), two heavy chains form a dimer (H₂), which then bind a light chain (H₂L), and finally the last light chain (H₂L₂) (Baumal, Potter, and Scharff, 1971; Hendershot et al., 1987). Lastly, the complete Ig molecule is transferred in transition vesicles to the Golgi for further processing of the carbohydrate side chains (Hubbard and Ivatt, 1981). After processing in the Golgi, the Ig molecules are loaded into transfer vesicles, that are transported to, and fuse with the cell plasma membrane, and the antibodies are thus secreted by the cell.
2.5.3 Rate limiting steps

There are several possible rate limiting steps in the above process, the main one probably being the quality control of proteins that take place in the ER before the secretory proteins are exported to the Golgi (Lodish, 1988; de Silva, Balch, and Helenius, 1990). In addition, the cells may also regulate the rate of Ig synthesis by the rate of heavy chain synthesis, as light chains are usually synthesized in excess (Rose and Doms, 1988). At present, it appears that the regulation of Ig secretion rate that takes place in the ER is "passive", i.e. it is limited by the presence of several rate limiting steps, without any additional controlled retention or storage of Ig molecules by the cells.

2.5.4 Components of the protein processing network

There are some indications that the components of the protein processing machinery is affected by conditions that are stressful to the culture, and that the protein processing rate can be affected by such conditions. The post translational protein processing network consists of a family of different enzymes and other factors, of which only a few have been characterized. Proteins families that are believed to be part of, or related to this network include: the glucose regulated proteins (GRP's) (Lee, 1987), the heat shock proteins (hsp's) (Lindquist, 1986), the chaperonins (GroEL's) (Ellis, van der Vies, and Hermingsen, 1989), Protein Disulfide Isomerase (PDI) and related proteins (Roth and Pierce, 1987; Mazzarella et al., 1990), and Peptidyl-Prolyl-cis-Isomerase (PPIase) (Freedman, 1987). These type of proteins can be found in all compartments of a mammalian cell, in all types of eukaryotic cells, and similar proteins are also found in bacteria.

Most of the proteineus factors involved are abundant, BiP for instance accounts for 1% of the total resident ER proteins at basal levels (Rothman, 1989). A common denominator for
these factors is that they are induced to even higher levels under conditions of stress for the cell, such as high temperature or nutrient starvation. Indeed, BiP was discovered in three different circumstances, and was for a long time described under the names of Glucose Regulated Protein 78 (GRP78), Heavy Chain Binding Protein, and immunoglobulin Binding Protein. BiP is the GRP protein that has been best characterized. BiP is closely related to the cytoplasmic heat shock protein hsp70. It has been shown to bind to nascent Ig chains, and to guide the polypeptide in its folding (Hendershot et al., 1987; Kassenbrock et al., 1988; Flynn, Chappel, and Rothman, 1989; Hendershot, 1990). BiP is believed to serve two major functions in the ER: To guide polypeptide folding and assembly; and to recognize aberrantly folded polypeptides and attempt to correct the misfolding, or else assist in their degradation (Flynn, Chappel, and Rothman, 1989; Rothman, 1989; de Silva, Balch, and Helenius, 1990). It is dependent on ATP to perform its functions, and several other GRP proteins have been shown to have ATPase activity (Dorner, Wasley, and Kaufman, 1988b). BiP is constitutively present in the ER at high concentrations, but is induced to even higher levels by glucose starvation and other conditions known to effect protein folding and glycosylation (Lee, 1987; Kozutsumi et al., 1988).

2.6 Influence of culture conditions

A couple of recent investigations have shed some light on how post translational factors can affect the rate of protein processing. Dorner, Krane, and Kaufman (1988a) showed that introduction of antisense BiP genes in CHO cells resulted in a threefold reduction in BiP levels, and in a threefold increase of tissue plasminogen activator secretion rate. Hendershot et al. (1987) demonstrated that mutant immunoglobulin heavy chains that do not associate with BiP due to sequence deletions, are secreted at a higher rate than chains that do associate with BiP in a normal fashion. The mutant Ig chains are in addition secreted in various
stages of assembly. While these results implicate that reduced interaction with BiP may result in a higher rate of protein secretion, Vogel, Misra, and Rose (1990) has demonstrated that BiP function is absolutely necessary for protein secretion in yeast. Lastly, Alberini et al. (1990), by addition of 2-mercaptoethanol to the medium at high concentrations, demonstrated that the intracellular redox state can affect the retention of IgM subunits in the ER, presumably due to the presence of critical cysteine residues on µ chains that interact with BiP. It should however be noted that addition of 2-mercaptoethanol did not affect the secretion of all Ig chain subtypes.

2.7 The use of stress response to improve process efficiency

Some of publications mentioned above have attempted to utilize the phenomena of stress related enhancement of protein production to improve process efficiency. As research on the molecular mechanisms behind this effects progresses, it should be possible to not only better explain phenomena such as those described here, but also to take advantage of the molecular biology of these cells to, in a more informed way, develop methodologies to improve the productivity of mammalian cell culture processes. In the engineering literature some approaches of this type has already appeared. Passini and Goochee (1989) investigated the distribution of heat shock proteins in hybridoma cells and the correlation with specific antibody productivity. Bibila and Flickinger (1991) has developed a model for antibody synthesis that includes a factor "F", that accounts for the levels of protein processing factors, and speculate that it could be possible to affect protein synthesis rate by manipulating these levels.
3. Experimental Approach

The experimental objective was to investigate the effects of upward step and ramp changes in the concentration of glucose, glutamine, and the combination of glucose plus glutamine, on the behavior, antibody productivity, and antibody quality of CRL 1606 hybridoma fed-batch cultures. The experimental approach was to subject fed-batch cultures directly to well controlled step and ramp changes in nutrient concentration. The experiments were designed to measure the influence of dynamic changes in glucose and glutamine concentration alone.

3.1 Basic Experimental Design

The basic experimental design is described in fig. 3.1 a. The inputs to the system are step and ramp changes of glucose, glutamine, and glucose plus glutamine concentrations. For a step change, the culture is started at a low nutrient concentration, and after a delay time the concentration is raised very quickly, and maintained at the higher level until all the feed medium is used. For a ramp change, the concentration raise is linear with time for the duration of the ramp, and the concentration is then maintained at the high level. Step change are designed to detect effects from rapid change in conditions, while ramp change are primarily designed to detect effects dependent on the time derivative of the input. The nutrient not under investigation, nutrient 2 in figure 3.1 a, is kept at a high level throughout the experiment by including it at the same concentration in both the reactor start medium and the feed medium.

The nutrient feed concentrations and feed rates were designed to maintain the desired nutrient concentration setpoints during the main growth phase of the culture, fig 3.1 a and b
Figure 3.1 a (upper) and b (lower). Desired trajectories of nutrients 1 (the controlled nutrient) or 2, total cells in reactor, and the corresponding volume trajectory.
(see also simulation below). For example, after a glutamine step change (glutamine is then nutrient 1 in fig. 3.1 a), the concentration of both glutamine and glucose (nutrient 2 in fig. 3.1 a) in the reactor are designed to be kept at a steady, high level, fig 3.1 a. This is accomplished by an exponential feed rate after the step change, fig. 3.1 b, designed to match the growth and nutrient uptake rate of the cells. The feed trajectory during the run is controlled by a computer control program, which is described below. After all the feed medium is used up, the process is allowed to run to conclusion undisturbed. For ramp changes, the procedure is the same, except in this case nutrient 1 follows the ramp change depicted in fig. 3.1 a. To maintain a linear nutrient concentration ramp with time, in a growing culture, the volume trajectory during the ramp is the sum of a linear and an exponential trajectory. The behavior of the process is followed during the whole cycle of the culture, from lag phase till well into death phase, as it is possible that a significant portion of the antibody can be produced during later phases of the culture.

3.2 Experimental Series

The experimental plan was treated a serial design rather than a factorial one. In a factorial design the whole block or sub-block of experiments has to be concluded before conclusions can be drawn about the influence of different parameters. It was not known beforehand what responses to expect or which parameters would be important. By using a serial design, it is not necessary to complete a factorial block, and the investigation can promptly focus on examining experiments that elicit the most promising response. When the response pattern is known, factorial designs can be used to further separate the influence and magnitude of responses to different inputs.

The experimental plan included a series of fed-batch cultures with step and ramp changes in
the concentration of each of the nutrients, glucose, glutamine, and the combination of glucose plus glutamine. Table 3.1 summarizes the different types of experimental designs used. For a glutamine step-change (type 2b in table 3.1) the glutamine level is kept low during the initial phase of the culture, and the glucose level is kept high. At the step-change, the glutamine concentration is increased rapidly to the higher level. By including glucose in the feed-medium at the same concentration as that in the reactor, glucose concentration is also maintained high during the course of the feed period. For a glucose step-change (type 2a in table 3.1) the nutrient concentration trajectories for glucose and glutamine are the opposite of those for a glutamine step-change. For a simultaneous step-change in glucose and glutamine concentrations (type 4 in table 3.1) both glucose and glutamine concentrations are kept low initially, and then rapidly increased at the same time. The patterns for ramp changes are the similar to those of the step-changes (type 3 a and b and type 5 in table 3.1).

3.3 Reference Processes
As references against which to judge the performance of the fed-batch cultures, two protocols were used. First, a batch culture with high initial concentrations of nutrients (type 1 in table 3.). This can be regarded as a fed-batch culture in which the concentrations of both glucose and glutamine are high, initially as well as during the main growth phase. Second, a glutamine limited fed-batch with a late step change in glutamine concentration after a shallow ramp, as described by Glacken (1987). This protocol is similar to a type 2b glutamine fed-batch in table 3.1, but with a late step change. The glutamine limited protocol was designed to minimize ammonia formation in the culture, as ammonia was found to be an inhibitor of cell growth in initial rate experiments. The protocol design further assumes that the specific antibody production rate is constant. Serum is included in the start
<table>
<thead>
<tr>
<th>Type 1. Batch culture</th>
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<tr>
<td>Type 2. Single nutrient fed-batch Step-change</td>
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<tr>
<td>Type 2a. Glucose step</td>
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<tr>
<td>Type 3. Single nutrient fed batch Ramp-change</td>
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<tr>
<td>Type 3a. Glucose ramp</td>
</tr>
<tr>
<td>Type 4. Dual nutrient fed-batch Step-change</td>
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<tr>
<td>Type 5. Dual nutrient fed-batch Ramp-change</td>
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Table 3.1. Nutrient concentration trajectories in the experimental series.
medium in the reactor at a concentration of 2.6% volume/volume, but not in the feed medium, and therefore serum levels will decline through the course of this process (see serum section below). In addition to these protocols, a low volume spinner flask culture (0.5 L), and a batch cultures with high serum content (5%) were used to compare performance.

3.4 Nutrient Concentrations

The nutrient concentrations at the start of a run were intended to limit the cells for nutrients shortly after growth commenced and thus evoke a stress response. The low initial level of the nutrient under investigation (nutrient 1 in fig. 3) was chosen to be well within a limiting regime, and the high concentration after a step change was chosen to be in a non-limiting regime. The concentration of the nutrient not under investigation (nutrient 2 in fig. 3) was set to be well inside a non-limiting regime throughout the culture. Glacken (1987) thoroughly investigated the effects of different nutrient concentrations on the steady state behavior of low cell density CRL 1606 cultures. From this data, nutrients concentrations were chosen to be limiting for growth rate, under the assumption that this would affect most aspects of cell behavior. As low levels, 3 mM was chosen for glucose, and 1.3 mM for glutamine (this is also the start glutamine concentration in the glutamine limited protocol). As high levels, 30 mM was chosen for glucose, and 10 mM for glutamine. By comparison, the initial concentrations in a typical batch culture of CRL 1606 are 25 and 6 mM for glucose and glutamine, respectively. At these concentrations there are never any stoichiometrical nutrient limitations, as determined by measured nutrient concentrations at the end of runs.
3.5 Serum Levels
Serum was included in the medium in the present investigation, as many mammalian cell processes are conducted with serum containing medium during the time period when cell growth is taking place. However, although the serum level is an important design parameter of mammalian cell cultures, it was not included as a factor in this investigation. Changing serum levels of a culture significantly affects almost every aspect of cell behavior. As this investigation focused on the effects of the major nutrients, serum was kept at one constant level throughout all experiments. This was accomplished by including serum at the same level in both the reactor start medium and the feed medium. In addition, all the serum used came from the same lot of foetal calf serum. A serum level of 2.5% volume/volume was chosen. This is an intermediary level for this cell line, and it is also the start serum concentration in the glutamine limited fed-batch protocol designed by Glacken (1987).

3.6 Step and Ramp Change Timing
The step and ramp changes were designed to be initiated in the early growth phase, after the cells experience a limitation in nutrient concentration and show a reduction in growth rate. Each experiments was intended to test the effect of only one step or ramp change, as the response time of mammalian cells limits the conditions that can be tested during one fed-batch run. The delay time after a change in culture conditions, before effects are seen on growth and metabolic rates, is usually between 6 and 24 h (see literature review). For hybridomas growing in continuous culture the time to reach 95% of the final value for most parameters is about 40 h, in response to changes in metabolites such as glucose (Miller, Wilke, and Blanch, 1988; Glacken 1987). However, longer response times should not be excluded. By initiating the step or ramp changes just after the lag phase but still well into the growth phase of the culture, it should be possible to detect responses to the change in
nutrient concentrations during the balanced growth phase of the culture.

The start cell density was chosen to be $0.1 \times 10^6$ cells/ml. This is a convenient inoculation density for the CRL 1606 cell line. It is also the start density of the glutamine limited reference protocol (Glacken, 1987). To reduce variations between different experiments, a target cell density was chosen as the criteria to induce the change in nutrient concentration. This was set at $0.3 \times 10^6$ cells/ml, which is more than one cell doubling into the growth phase. The timing of the change was varied to make up for variations in the lag times and growth rates of the cultures. Ideally, the change should occur after a clear reduction in growth rate was detected, and in most experiments this was the case although the onset of nutrient limitation varied with the state of the culture. This cell line has a lag time of about 20 h after inoculation, with a normal variation of up to 6 h. In most experiments the culture reached the target cell density after about 48 h, but variations of up to 10 h occurred.

3.7 Measurements

The goal of the present investigation is to improve the antibody production of the hybridoma process used as model system. The main performance parameter is therefore the amount, and quality, of monoclonal antibody produced by the cultures. In addition to medium and intracellular antibody concentrations (see immunoassay section), a number of other parameters were monitored in order to assess what effects the inputs were having on the system. These were the concentrations of the nutrients in the reactor, glucose and glutamine; the major waste products, lactate and ammonia; and the dissolved oxygen, pH, and cell densities of the system.
3.8 Reactor Scale

The experiments in this investigation were conducted in a 2 liter bioreactor. To facilitate comparisons with the glutamine limited protocol, a start volume of 0.93 L and a final volume of 2.0 L was used. These volumes coincides with those used in the glutamine limited protocol. Due to volume limitations in the reactor, with several probes and a silicone tube holder present, the batch cultures were run at 1.95 L volume.
4. Materials and Methods

4.1 Tissue culture media and serum
For seed and stock culture Dulbecco's Modified Eagles Media (Gibco Laboratories, Grand Island, N.Y.) was used. Glutamine was added from a 200 nm stock solution (Gibco Laboratories, Grand Island, N.Y.) just prior to use. For the bioreactor experiments a custom formulated Dulbecco's Modified Eagles Media was used, containing all ingredients except glucose and glutamine (Gibco Laboratories, Grand Island, N.Y.). Fresh media was made up prior the experiment, with added glucose and glutamine, according to the manufacturers instructions. The same batch of heat inactivated foetal calf serum was used throughout the whole investigation (Gibco Laboratories, Grand Island, N.Y.).

4.2 Seed and stock cultures
Stock and seed cultures were maintained in 175 cm² T-flasks in the same type of medium used for the bioreactor experiments. Nutrient concentrations were 25 mM glucose, 6mM glutamine, and the foetal calf serum concentration was 2.5% (v/v). The incubator gasphase contained 7% CO₂.

4.3 Bioreactor sampling and sample treatment
8 ml of bioreactor media with cells were collected in a centrifuge tube. 1 ml was transferred to a smaller tube and pH was measured for calibration of the bioreactor pH probe. 1 ml sample was transferred to a smaller test tube and was used for cell enumeration. The
remaining 6 ml where centrifuged at 200 g for 10 min to separate the cells. The supernatant was transferred to another tube and frozen for subsequent analysis. The cell pellet was transferred to an eppendorf tube washed once in cold PBS. After centrifugation for 2 min at 1000 g the supernatant was discarded and the washed cell pellet was frozen for subsequent analysis.

4.4 Bioreactor probes and calibration

The pH, redox, and polarographic dissolved oxygen (DO) bioreactor probes where from Ingold (Ingold Electrodes Inc., Wilmington, MA). All probes where calibrated before each experiment according to the manufacturers instructions. The pH probe was in addition recalibrated each time a sample was taken from the reactor. The pH of the sample was measured using a separate pH meter that had been recalibrated just prior to the sampling, and the bioreactor pH probe was adjusted accordingly. The redox probe was also calibrated in fresh medium before and after each experiments. Lastly, the DO probe was calibrated in air saturated medium after the culture as the calibration can change during autoclavage of the reactor. If there was a substantial difference in the calibration before and after the experiment, then the calibration value obtained after the experiment was used to adjust the measured data.

4.5 Cell enumeration and size determination

The media with the cells was diluted 1:2 or 1:4 in a 0.4% trypan blue solution (Zigma Co., St Louis, MO), and viable and dead cell where counted in a Neubauer hemacytometer (Reichert, Buffalo, N.Y.). In addition, cells where counted in a Coulter counter (Coulter Electronics, Hialeah, FL) as described elsewhere (Aunins, 1989). In short, the cells where
diluted 1:2 to 1:10 in isotope (Coulter Electronics, Hialeah, FL) depending on cell density, and counted using a 100 μm diameter orifice. Instrument settings where AMP=4, T=5, and APC=4. Cell size determination where done using a Coulter chanelyzer connected to the Coulter counter, as described elsewhere (Aunins, 1989).

4.6 Analysis of nutrients and waste products

Analysis of glucose and lactate was done by HPLC using a Aminex HPX-87H column (BioRad, Richmond CA) according to the manufacturers instruction, together with a refractometric detector. The carrier solvent was 0.05 mM H₂SO₄ in water. To verify the results of the HPLC measurements, glucose and lactate was also measured using enzymatic assay kits (Sigma No. 15-UV (glucose), and Sigma No. 826-UV(lactate), Zigma Co., St Louis, MO). Ammonia concentration was measured using an enzymatic assay kit (Sigma No. 170-UV, Zigma Co., St Louis, MO), according to the manufactures instructions. For all measurements standard curves were made by serial dilutions from standard solutions of the compounds.

Glutamine was assayed as detailed elsewhere (Adema, 1989) by HPLC after derivatization with ortho-phenyl-diamine (Zigma Co., St Louis, MO). Briefly, samples where deprotinated as described elsewhere (Perry, 1987) by adding trichloroacetic acid, centrifugate out the precipitated proteins, and neutralize the pH by adding a potassium bicarbonate solution. The column employed was a ODS-Hypersil C-18, operated with a gradient of two solvents; A (50 mM Na₂HPO₄, 50 mM NaOAc, 20 ml/L methanol, 20 ml/L tetrahydrofuran, pH 7.5 in water) and B (0.65 L/L methanol in water). Detection was done at 334 nm.
4.7 Immunoassay Measurements

Reliable measurements of the antibody concentration in the reactor is essential for assessing process performance. For this purpose, four different assays has been used in this investigation: an Enzyme Linked Immunosorbent Assay (ELISA), two versions of a Chemiluminescent Immuno Assay (CIA), and a Radial Immunodiffusion Assay (RID). The anti-fibronectin ELISA and the anti-IgG CIA assays are further described and compared in appendix 2.

4.7.1 Radial Immunodiffusion Assays (RID)

An old fashion but standard method was used with minor modifications (Berne, 1974; Daniels, 1979; Mancini, Carbonara, and Heremans, 1965; Springer, 1983; Vaerman, 1981). RID was done in 1% agarose (ICN, Costa Mesa, CA) with 100 μg/ml of a Rabbit-anti-Mouse IgG F(ab')2 antiserum (Pierce, Rockford, IL) in 1 mm thick gels. Wells 4 mm in diameter were cut in the gel, and were loaded with 30μl of sample, in single or multiple loadings. Diffusion was allowed to progress for four days at room temperature. The diameter of precipitate rings was read by enlarging the rings with an overhead projector, and measuring two perpendicular outer ring diameters with a micrometer on a screen. Sample immunoglobulin (Ig) concentration was obtained from a curve of standard Ig concentration of a known CRL 1606 standard preparation, versus the product of the two ring diameters.

4.7.2 The anti-fibronectin ELISA

The ELISA assay used is a direct binding anti-fibronectin heterogenous assay (α-Fn ELISA), fig 4.1. In this assay, the analyte, the CRL 1606 monoclonal antibody, binds to the immobilized antigen, fibronectin, and excess unbound antibody is washed off. After
Fig. 4.1. The anti-Fibronectin ELISA assay. Fibronectin is immobilized onto the plastic of a microtiter plate well, and excess is washed off. Analyte monoclonal antibody in the sample binds to the fibronectin. After a wash the second antibody, an anti-mouse antibody which is conjugated to an enzyme, is added to the well, and binds to the analyte. After washing, a substrate for the enzyme is applied to the well, and the amount of analyte can be quantified by the light absorption of the colored enzyme reaction product.
binding of the second enzyme conjugated antibody to the analyte, and another wash, 
substrate for the enzyme is added. The amount of enzyme retained in the ELISA plate well 
is quantified by the development of a colored reaction product. This assay has been shown 
to be somewhat less reliable and accurate than the CIA (see appendix), and therefore most 
of the analysis in this investigation were done with the RID and CIA assays.

4.7.3 The anti-IgG CIA

The CIA assay normally employed uses a different immunoassay format than the ELISA. 
This assay is an anti-mouse-IgG CIA (α-IgG CIA), which utilizes a homogenous 
competitive format, with a capturing antibody, fig. 4.2. The analyte (CRL 1606 monoclonal 
antibody) competes for binding to the capturing goat-anti-mouse antibody with a mouse 
IgG antibody that is labeled with a luminescent marker. The luminescent signal is quenched 
when the labeled antibody is retained in proximity to the bead, onto which the capturing 
antibody is immobilized. The light signal will thus be proportional to the amount of labeled 
antibody in solution, and this in turn is proportional to the amount of competing 
multoclonal antibody in the sample.

4.7.4 The anti-fibronectin CIA

To ensure that measurements could be compared directly, without interference from the 
detection systems employed by the assays, i.e. enzyme markers versus luminescent markers, 
a second CIA assay was developed to replace the ELISA assay. This is a direct binding anti 
fibronectin CIA assay (α-Fn CIA), fig. 4.3. This assay uses the same immuno assay 
format as the ELISA assay, but in a homogenous CIA assay. In this assay, fibronectin is 
immobilized to the beads. The CLR 1606 monoclonal antibody (the analyte) binds to the
Fig. 4.2. The anti-IgG chemiluminescent Immunoassay. The solid phase beads are coated with a Goat-anti-Mouse polyclonal antibody, anti-fibronectin Monoclonal antibodies (the analyte) present in the sample competes for binding to the immobilized antibody with a mouse IgG antibody labeled with the luminescent marker. Upon binding to the bead, the signal from the label is modulated. The amount of analyte in the sample will thus be proportional to the light signal.
Fig. 13. A direct binding anti-fibronectin chemiluminescent Immunoassay. The solid phase beads are coated with fibronectin. anti- fibronectin Monoclonal antibodies (the analyte) present in the sample will bind to the fibronectin. The second antibody, labeled with the luminescent marker, will bind to the analyte antibody. Upon binding, the signal form the label is modulated. If no analyte is present, or if the analyte is unable to bind to the fibronectin, no binding of the second antibody will occur, and the signal will be unmodulated.
fibronectin, and the second antibody, a goat-anti-mouse antibody labeled with the luminescent marker, binds to the analyte. The signal is again proportional to the amount of labeled antibody free in solution, and therefore in this case the amount of analyte antibody in the sample is inversely proportional to the light signal.

Due to the different immunoassay formats employed in the assays, the measurements results will differ depending on the nature of the analyte. The RID assay is dependent on a precipitation reaction for the measurement signal to develop (precipitin rings), which makes it perhaps the most reliable and accurate of the assays used. Both the anti-Fibronectin ELISA and CIA are dependent on the ability of the analyte, the CRL 1606 monoclonal antibody, to bind to its antigen, fibronectin. Analyte antibody that is not able to bind, i.e. that is not functional, will not be detected in these assays. In contrast, the anti-IgG CIA assay is dependent only on the binding function of the capturing goat-anti-mouse Ig kappa chain antibody. This antibody will bind proteins displaying epitopes present on the kappa light chain of mouse antibodies, whether these analyte antibodies themselves are functional or not. Therefore, the anti-IgG CIA assay (α-IgG CIA) measures total antibody present in the sample, while the anti-fibronectin CIA (α-Fn CIA) and ELISA (α-Fn ELISA) assays measures only functionally active antibody present in the sample. All the measurement results are related to the standard used in the assay, and are correlated accordingly. The measurements in the different assay coincided within 20% of the measured values.

4.8 SDS-PAGE
The Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was done by the standard Laemmli protocol (Laemmli, 1970; Hames and Rickwood, 1990). Gels were made 1.5 mm thick, with a gradient of cross-linked polyacrylamide ranging from
5 or 10 % up to 15 %, depending on the samples being investigated. A standard set of molecular weight markers were used (BioRad, Richmond CA). All samples were boiled for 5 minutes with SDS before being applied to the gel. For reduced samples dithiothreithol (DTT, Sigma, St. Louis, MO) at a final concentration of 25 mM was included in the sample preparation. For non-reduced samples, various blocking agents for disulfide bindings were tested, including iodoacetamide and N-ethylmaleimide. For most samples, the best result was obtained without blocking agent in the sample preparation. Except where otherwise noted, a total of 5 μl of each bioreactor sample was applied to the gel, diluted in sample buffer.

4.9 Western Blotting
For immunodetection, SDS-PAGE gels were electrophoretically blotted onto nitrocellulose membranes according to the manufacturers recommendations (BioRad, Richmond CA). Blocking of excess binding capacity of the membrane was done with a 3% gelatine solution at pH 7.8.

4.10 Immunodetection
Immunodetection of mouse immunoglobulins on nitrocellulose was done by standard methods as documented by the manufacturer (BioRad, Richmond CA). Briefly, the blocked membranes were incubated with either a alkaline phosphatase conjugated anti-mouse Ig antisera, or with a primary unconjugated anti-mouse antisera, for 1 h at 37°C. For unconjugated primary antibodies, a second incubation, after washing, was done with an alkaline phosphatase conjugated second antibody. After washing, the blot was developed using the substrates 5-Bromo-4-Chloro-3-Indolyl Phosphate and Nitro Blue Tetrazolium
(BioRad, Richmond, CA), which together react with alkaline phosphatase to form a purple insoluble salt that deposits on the nitrocellulose.

4.11 2-dimensional immunoblots

2-dimensional (2D) immunoblots were performed with the first dimension being a non-reduced gel, and the second dimension being done under reducing conditions. Fig. 4.4 illustrates how the 2D gels were produced. For the first dimension, a broad lane of the sample, and a lane with molecular weight markers, was run under non reducing conditions as described above. After the electrophoresis, the sample lane was cut into two strips. One gel strip, containing part of the sample lane, and the molecular weight marker lane, was immediately placed on a large sheet of nitrocellulose, and blotted as described above. The second gel strip, containing the rest of the sample lane, was soaked for 30 min in electrophoresis sample buffer containing 50 mM DTT, to reduce the proteins in the gel. Next, this gel strip was placed horizontally between the glass plates of a gel assembly, at the very top. The 2nd dimension gel was then immediately cast below the gel strip, and a resolving gel was cast around the gels strip. Lanes for molecular weight markers and a purified preparation of the MOPC 21 protein was also included in the 2nd dimension gel. After electrophoresis of the 2nd dimension, the whole resolving gels was placed on the same nitrocellulose sheet onto which the first dimension strip had already been transferred, in such a way that the first dimension strip lined up with the 2nd dimension gel. This assembly was then blotted and immunodetected as described above. In this manner, the 2D immunoblot contained a record of the pattern from the first dimension and molecular weight markers, in addition to the 2D electrophoresis pattern.
Fig. 4.4. The procedure for performing a 2 dimensional immunoblot, with the first dimension run under non-reducing conditions, and the second dimension run under reducing conditions. See text for details.
4.12 Immunoprecipitation

Beads coated with Goat-anti-Mouse kappa light chain antisera or fibronectin were diluted in 1:10 in Origen CIA assay diluent (see immunoassay section). To 500 μl diluent, 100 μl beads was added, and 100 μl of sample containing the CRL 1606 antibodies. The mixture was incubated on a shaker for 90 min at 37 °C, and the beads were then centrifuged and washed 3 times in cold PBS. Next, the centrifuged beads were suspended in 100 μl pH 6.8 0.05 mM tris buffer, and the preparation was boiled for 5 min. 50 μl of the bead suspension was the finally loaded onto a gel lane for electrophoresis.

4.13 N-Glycosidase F treatment

Sample were treated with N-glycosidase F to remove attached glucoside residues according to the manufacturers recommendations (Boehringer-Mannheim, Indianapolis, IN). Briefly, samples were boiled 5 min in the presence of SDS and mercaptoethanol. Next EDTA, n-octyl-glucoside, and N-glycosidase F was added, and the preparation was incubated overnight at 37 °C. The sample was finally boiled for 5 min in reducing electrophoresis sample buffer, and run on the electrophoresis gel.
5. Development of a Technology for Dynamic Operation of Bioreactors

Development of a technology for computer control of dynamically operated mammalian cell bioreactors is an integrated part of the work presented here. The guidelines for good manufacturing practices requires that a production process is reliable and highly reproducible, and that proper operation can be validated. To utilize dynamic operation for production on an industrial scale, it is necessary the process can be controlled accurately. Advanced control hardware and software cab ensure proper implementation a this operating strategy. Good design of such systems will contribute to product consistency and safety, and to enhanced process efficiency. The objective of the simple control system designed here has been limited to the core task of ensuring that the desired nutrient control trajectories are followed tightly, and that the culture environment is within the intended boundaries.

The control program evolved during the course of this investigation. The system outlined here is the final version in this development, but almost all experiments presented here were controlled by programs with the features described below.

5.1 The control problem

The major problems in controlling mammalian cell cultures are consequences of the lack of on-line sensors for important culture parameters, lack of models that can accurately describe the complex behavior of cultures, and lack of useful control strategies. A common approach to deal with these difficulties has been to design pre-programmed feeding schedules, where the cells are assumed to follow a fixed growth trajectory (Harbour, 1988; Reuveny and
Lazar, 1989; Glacken 1987). For instance, the glutamine limited process for the CRL 1606 cell line developed by Glacken (1987) follows a feeding schedule obtained from a process optimization, based on a model of culture behavior. It has to be stressed that these schedules are not controllers, as no provision is made to correct for deviations from the expected process trajectory.

To control fed-batch cultures accurately, either a model of culture behavior or a feed-back measurement of the culture state is needed. A model that describes the behavior of the culture with reasonable accuracy under the range of conditions that can be expected in the reactor can be used to construct both observers and controllers. However, mammalian cells tend to do as they please no matter what is expected of them, and most models of mammalian cells cultures break down outside a very narrow range of conditions. The other option available for control is to obtain some type of feed-back measurement of the state of the culture, and base the control strategy on this information. Considering the complexity of mammalian cell cultures under shifting conditions, as in a dynamically operated culture, such an approach seem more attractive than a model-predictive approach. The control technology developed here uses both of the approaches mentioned, feed-back measurements for long time periods, and model-predictive control for the time between the feed-back measurements. Before describing the control problem and strategy in detail, it is instructive to examine the information available to the control system.

5.2 Process Inputs and Outputs

Any control system will be limited in its performance by the information available to it. The concentration of nutrients and waste products in the reactor are some of the important control variables for a mammalian cell culture. The result of the cells metabolism can be
summarized as:

Glucose + Glutamine + O₂ → Cells + Lactate + Ammonia + CO₂ + Protein product

The main environmental parameters of the process are:

- The concentrations of the main nutrients glucose and glutamine, and the major waste products, lactate and ammonia.
- The dissolved gases in the medium, oxygen and carbon dioxide.
- The pH of the medium.
- The temperature of the reactor.
- The concentrations of viable and dead cells
- The concentration of the protein product, i.e. in this case the antibody.

In addition, it is desirable to have access to the flowrates to and from the reactor of gases (air and CO₂) and fluids (acid, base, and feed medium). If all the information listed above were available to the control system it would be possible to control the environment of the cells in detail. Unfortunately there are sensors for direct and convenient measurement for only a few of these parameters; data for dissolved oxygen, pH, redox potential, and temperature is available on-line from sterilizable probes; The flow of gases and liquids can be accessed from mass flow meters and load cells. Fig. 5.1 gives an overview of the inputs and outputs that are available to the control system used here. As can be seen by comparing it to the list of culture parameters above, most of the information needed to accurately control the bioreactor environment is not directly available. For instance, there is no technology commercially available that enables direct on-line measurement of nutrient concentration from the sterile environment of a bioreactor. Neither is there a useful technology for measuring cell densities on-line. Changes in the CO₂ content in the medium is an
Fig. 5.1. Inputs and outputs to and from the process, and to and from the control system.
indication of cell metabolism, but are difficult to measure as the media is buffered with about 5% CO₂ in the gas phase. Thus, little useful information about primary control parameters like nutrient concentrations and cell densities are available to the control system: from the sensors most conveniently used. The information that is available to the control system used here is the pH, dissolved oxygen, redox potential, and temperature of the reactor medium, the flow of gases to the reactor, the flow of medium to and the volume of medium in the reactor, and the flow of acid and base. The main problem in controlling the culture environment is lack of on-line measurement of the control variable, the concentration of glucose or glutamine. The strategy used to control these variables with the information available is outlined below, after a description of the hardware of the control system.

5.3 Hardware System Overview

Fig. 5.2 presents an overview of the hardware of the system. The bioreactor is equipped with probes for pH, dissolved oxygen, and redox potential. The gas flows are measured and controlled by three Brooks 1000E flow controllers, one each for air, CO₂, and oxygen. (The oxygen controller was used as a backup in case the dissolved oxygen levels went to low during the culture, but was never needed). The feed medium bottle is placed on a balance. The feed medium, acid, and base fluid lines are connected to the reactor via peristaltic pumps. In addition, the reactor is also equipped with a sample line for manual sample acquisition.

All probes, pumps, balances etc. are interfaced with the computer control system. This consists of two computers: An ALERT 50 Mini dedicated real time control computer (Satt Control, Malmö, Sweden). The ALERT is monitored and programmed via the Alcom
Fig. 5.2. Overview of the bioreactor and control system hardware.
control design program (Satt Control, Malmö, Sweden), residing in a Compaq 286
computer running the Venix operating system. The actual control and alarm programs
reside in the ALERT, which can operate free-standing from the Alcom program. The
ALERT automatically boots up and resumes the control tasks after a power failure, which
increases the robustness of the system. The Alcom program is used to compile and
download controller code to the ALERT, and to assemble and download the control
configuration. Supervisory control programs reside in the Alcom module. Data logs, alarm
logs, and output files from supervisory programs run under the Alcom module, and are
stored on a hard disk. Lastly, the Alcom module is used to design and build the graphic
displays of the control system parameters, which can be represented as figures, bars,
timecurves, etc.

The Bioreactor assembly is described in fig. 5.3. The reactor is a Braun Biostat M
equipped with a marine impeller and two turbine impellers, rotating at 60 rpm. To
oxygenate the culture, a spiral of silicone tubing is immersed in the medium (Goldstein,
1986; Lee, Huang, and Lee, 1990). The tubing wall acts as a semipermeable membrane,
through which oxygen and carbon dioxide will diffuse. In this way sparging the culture
with air can be avoided, as this can cause cell damage. The silicone tubing spiral used here
has a total length of 20 m, with an outer diameter of 1.96 mm and a wall thickness of 0.64
mm (SIP Medical grade Silastic Q7-4750 Silicone tubing, Baxter, McGaw Park, U.). This
surface area was enough to oxygenate these cultures at all times. The in-gases are sparged
through sterile water bottles to ensure that no liquid is lost from the culture medium. The
reactor is equipped with probes for dissolved oxygen, pH, and redox potential, as well as
fluid lines for acid, base, and feed medium. The feed-medium bottle is placed inside a
refrigerator to minimize glutamine degradation.
Fig. 5.3. Bioreactor assembly with probes, silicone tubing spiral, gas lines, acid base lines, and feed medium line.
5.4 Control Strategy

The strategy used in the design of the present control system takes advantage of the specific oxygen rate of the cells to estimate the viable cell density of the culture (described below). This information is used together with a very simple model of the culture to predict and control the concentration of the nutrient in the medium. The time constants of the oxygen transfer system, a silicone tubing coil, does not permit measurement of dynamic oxygen uptake rate (dOUR) more frequently than once every two hours (see separate section below). A model of the culture is therefore used to predict the systems behavior during the time between feed-back measurements. In addition, the model-predictive part of the control system is used as a back-up system should the measurement of dOUR fail.

An crucial feature of using the dOUR of the culture is that the tuning and performance of the control system is tied to the state of the culture. The culture system is not time invariant; as will be seen in the result section below, both growth rates and nutrient uptake rates vary with the state and runtime of the culture. This poses several difficulties for the construction and use of a controller, and any such program for a bioreactor process will have difficulties with control accuracy over time. With the use of a feed-back measurement that reflects the state of the culture most of these problems can be avoided. It also permits the use of a quite simple model predictive controller for the time periods between OUR measurements (which has been a source of great relief for the author of this thesis), as the system parameters can be considered constant for this incremental time. To deal with the variance of the culture system over longer times, a separate rule based program was designed which selects the appropriate system constants based on the culture state (described below).
5.5 Mathematical description of the cell culture

This description differs somewhat from that used to describe most bioreactor systems. Yield factors to measure the effectiveness of the cultures' metabolism and utilization of medium components is of little use for this type of cultures. It is difficult to relate the production of protein products to the utilization of medium components, and yields calculated in this manner are ridiculously low, varies little, and are thus not very informative. In addition, the cost of nutrients and other medium components (perhaps with the exception of serum) are not a major concern in the economics that govern these processes. The treatment presented here is based on simple relations that adequately describes the important aspects of these cultures. The nomenclature is defined in appendix 1. Here only those relationships of importance for the development of the control theory are described.

5.5.1 Balances

The system is governed by a set of balances and models of parameter behavior. The balances are:

Balance on cells in the reactor:

Balance on the total amount of viable, dead, and total cells in the reactor, including a cell breakdown rate k:

\[ \frac{d(N_v \cdot V)}{dt} = \mu \cdot N_v \cdot V - \alpha \cdot N_v \cdot V - k \cdot N_d \cdot V \]

In the treatment below cell breakdown is neglected, i.e. assume \( k \cdot X_d = 0 \). This holds for all phases of the culture except late death phase. If the cells are not affected by the volume changes in the reactor, the balance can be made on total cells in the reactor. The cell balance is then

80
\[
\frac{d(X_v)}{dt} = \mu \cdot X_v - \alpha \cdot X_v
\]

The corresponding balance for dead cells is

\[
\frac{d(X_d)}{dt} = \alpha \cdot X_v
\]

Assuming that the total cells are monotonously increasing and \(k \cdot X_d = 0\), write:

\[
\frac{d(X_T)}{dt} = \mu \cdot X_v
\]

Balance on nutrients and waste product in reactor:

For control purposes, only the balance on the limiting nutrient needs to be considered. In cases where glucose and glutamine are fed simultaneously, only one nutrient is used as control variable. Assume that only viable cells are metabolically active, and that dead cell do not release significant amounts of waste product or antibodies. For a general balance on a substance, write:

\[
\frac{d(C \cdot V)}{dt} = F \cdot C_{feed} - q \cdot N_v \cdot V
\]

Again, assume cells are not affected by the volume changes, and write a balance on the total amount of the substance in the reactor:

\[
\frac{d(A)}{dt} = F \cdot C_{feed} - q \cdot X_v
\]

To complete the description of the system, accurate models of the culture parameters (growth and uptake/production rates) as a functions of the bioreactor environment are needed. As stated above, the behavior of these parameters is quite complex and time variant. Such models are therefore difficult to obtain and use for control purposes. The strategy
used to control the cultures presented here avoids most of these problems by treating these parameters as piece-wise constant.

5.6 Control Program Overview

Fig. 5.4 shows an overview of the dynamic fed-batch control program features, while fig. 5.5 gives a more detailed picture. The main functions are an on-line measurement of viable cell density via the oxygen uptake rate of the culture (described in more detail below), and a model-predictive combined feed back and feed-forward nutrient feed control. The control system is divided into two layers, with different sampling and recalculation frequencies.

5.6.1 Supervisory programs

The supervisory control programs reside in the Alcom system on the Compaq 286 computer. They perform two functions, and are described in more detail in a separate section below. The dynamic oxygen uptake rate (dOUR) program measures the oxygen rate of the culture every two hours. The program turns off aeration, waits for the dissolved oxygen (DO) level to decline, calculates the slope of the DO curve, and turns aeration on again. The calculated dOUR value is passed on the cell estimation program. This is a rule based program that calculates the viable cell density based on a correlation between dOUR and viable cell density. The specific oxygen and nutrient uptake rates changes with the culture phase. The program estimates the phase of the culture based on a set of rules, and selects the appropriate specific rates correlations for calculating cell density. The program also selects a specific nutrient uptake rate correlation and calculates the growth rate. These parameters are then passed on every two hours to the nutrient feed flow control programs residing in the ALERT computer.
Fig. 5.4. Schematic overview of the control system features.
Fig. 5.5. The control system layout.
5.6.2 ALERT programs

The ALERT programs consist of two separate sets. The four nutrient feed control programs estimates and controls the nutrient concentration is the reactor, with a recalculation frequency of once a minute. The fed-batch predictor estimates the nutrient concentration in the reactor based on the known volume of the reactor (from feed bottle weight), and a balance on cells and nutrients. Every two hours the program is provided the estimated viable cell density, growth rate, and nutrient uptake rates from the cell density estimation supervisory program. Between the update times, the program calculates the cell density and nutrient concentration from the known volume of the reactor and the feed flow rate (from feed bottle weight change), based on a simple model of the culture derived from the cell and nutrient balances. This program essentially performs a real-time simulation of the culture. The predictor passes on values for cell density, volume, and nutrient concentration to the setpoint determinator and flow controller programs. The setpoint determinator contains the control objective function for the nutrient concentration trajectory of the culture. In the case of a step change run, this consists of a simple table relating the setpoint to the viable cell density. The setpoint is an input to the feed flow control program. This program calculates the feed flowrate needed to reach or maintain the nutrient concentration in the reactor during the recalculation period (one minute). The calculation is based on a model of the culture, and is essentially a combined feed-back and feed-forward controller. The flow rate is passed on as a request to the pump control program, which controls the pumping frequency of the peristaltic pump based on a signal for feed bottle weight from a balance.

The last set of programs in the ALERT are the pH and gasflow controllers. These are recalculated every second. pH is primarily controlled via the fraction carbon dioxide in the gas stream through the silicone tubing in the reactor. The fraction is controlled by a PID
controller based on the pH signal. The actual gas flows are calculated by a gas-flow controller which feeds the flow setpoints to two dedicated gas mass flow controllers. The gasflow controller also turns on and off aeration on request from the dynamic oxygen uptake rate measurement supervisory program. A backup system for acid/base control of pH sets in if the pH is outside a set range of the setpoint. A proportional on/off controller pumps in the acid or base estimated to be needed to restore pH to the setpoint range.

5.7 Control Program Descriptions

5.7.1 Fed-batch Predictor

The predictor program contains the models used to predict nutrient concentration in the reactor, as well as cell density between the dOUR measurements. In addition, the program contains features to completely estimate the culture without on-line estimation of cell density, should any of the supervisory programs fail. This would for instance happen after a power failure, as the ALERT boots up automatically and these programs thus continue to run, while the Alcom computer has to be booted manually. The program contains equations that are derived from the balances above describing the culture. Here a solution to the predictive model equations is exemplified for a glutamine controlled fed-batch. The treatment is greatly simplified by treating the culture parameters as constants for the time periods (two hours) between the dOUR measurements. Other simplifying assumptions are mentioned below.

The control system has only to consider maintaining the correct concentration of glutamine in the reactor, i.e. the control objective is the concentration of glutamine. Also, for control purposes only the amount of viable cell is interesting, and the death rate can be neglected, i.e. we can replace μ by an apparent growth rate μ_{app}. The system is governed by two
equations, where GLN is the state variable: A balance on total glutamine in the reactor:

$$\frac{d(A^{GLN})}{dt} = F \cdot C^{GLN}_{\text{feed}} - q^{GLN}_{m} \cdot X_v$$

and a balance on total cells in the reactor:

$$\frac{d(X_v)}{dt} = \mu \cdot X_v$$

where $\mu$ is the apparent growth rate (see appendix 1). Assuming that the cells are not affected by the volume changes and that the growth rate is constant for the incremental time period, the differential cell balance can be solved to give

$$X_{vt} = X_{v0} \cdot e^{\mu \cdot t}$$

or in terms of cell densities

$$N_{vt} = \frac{V_0}{V_t} \cdot N_{v0} \cdot e^{\mu \cdot t}$$

By making some further assumptions, the nutrient balance can easily be solved analytically;

- Assume that the cell number is constant for the time period considered, i.e. neglect growth for the short time-period between cell density estimations. This is a reasonable assumption based on the time-constants of the system. For cell number the time constant is on the order of hours; for nutrient concentration changes from feed flow the time constant is on the order of minutes.

- Assume that the feed flowrate is constant for the time period considered;

- Assume that the glutamine uptake rate is constant.

The expression can then be integrated and solved for glutamine concentration at time $t$:

$$C^{GLN}_t = \frac{1}{V_t} \left[ C^{GLN}_0 \cdot V_0 + F_{t=0 \to t} \cdot C^{GLN}_{\text{feed}} \cdot t - q^{GLN}_{m} \cdot N_v \cdot V_0 \cdot t \right]$$

This expression for glutamine concentration can be used as a predictor. It is valid for an
incremental time $t$. Given the start conditions, a constant flow rate, and the assumptions above, the expression predicts the glutamine concentration at time $t$. The expression lumps the maintenance and growth related nutrient uptake rates into one parameter ($q_m$). A similar expression can be derived with separate constants for these uptake rates, but this is of no consequence for control purposes. Glutamine degradation is further neglected. In early versions of the program, a first order decomposition expression for glutamine decomposition was included. This had very little impact on the glutamine control, and no consequence for glucose control; it was later excluded for simplicity. (The model equations can be solved in a number of other ways; with cell growth included in the nutrient balance; or as difference equations. The method used here has the advantage of making the analysis of the transfer functions of the system much easier. The solution method has no consequence for the control accuracy.)

The complete predictor consists of these equations together with an expression for volume change. The predictor with inputs and outputs is described in fig. 5.6. It is in essence a real-time simulation of the culture. The input to the program is the feed medium flow rate. External parameters set from supervisory programs are growth rate, feed glutamine concentration, and glutamine uptake rate. The program runs calculates the present volume based on the flowrate for the previous time period, the cell density based on the growth rate, and the present glutamine concentration based on the previous concentration, the amount fed, and the amount consumed by the cells. The calculation is repeated once a minute, which gives the simulation more than adequate accuracy for control purposes. In addition, the program monitors the weight signal from the feed medium balance to ensure that the correct reactor volume is used in the calculations. The predictor program also contains a number of safeguarding features. For instance, if any of the supervisory programs fails and
Fig. 5.6. Description of the fed-batch predictor simulation block. Inputs are the feed medium flowrate, outputs are the reactor medium volume, the viable cell density, and the glutamine concentration in the reactor. Parameters set from supervisory programs are growth rate, glutamine uptake rate, and feed medium glutamine concentration. Recalculation frequency is once a minute.
the viable cell density and other parameters are not updated, the program continues to control the culture using a set of default values. Also, if parameters calculated in other programs goes outside reasonable bounds, default values are exchanged.

5.7.2 The setpoint determinator
The setpoint determinator block contains the setpoints for the desired nutrient concentration trajectory, as a function of cell density. For a step change, the block simply raises the setpoint from the low to the high value once the cell density has passed the point selected for introducing the change, fig. 5.7. The block also contains functions to guard against premature induction of the concentration change due to temporary faults in the control system, for instance sensor faults. It in addition contains a trig safeguard, so that the setpoint is not lowered as the cells are diluted immediately following a step change in nutrient concentration. For the glutamine limited reference runs (Glacken 1987) the block is loaded with a more complicated setpoint function, reflecting the shallow glutamine concentration ramp followed by a step in concentration that is used for this type of experiments.

5.7.3 Flow controller blocks
The flow controller block is a dedicated controller for the fed-batch system. The feed flow control problem can be stated as: Control the feed flow \( F \) so that the difference between predicted nutrient concentration and nutrient concentration setpoint is within a set dead zone,
IF \( N_v < 0.3 \cdot 10^9 \) THEN \( C_{set} = 1.3 \text{ mM} \)

IF \( N_v \geq 0.3 \cdot 10^9 \) THEN \( C_{set} = 10 \text{ mM} \)

Fig. 5.7. Description of the setpoint determinator block. The input is viable cell density from the predictor block. The output is the setpoint concentration for nutrient (here glutamine). The block is designed as a trig function. Once the cell density goes above the trig point (here \( 0.3 \cdot 10^9 \text{ cell/L} \)), the setpoint does not go down again when the cells are diluted during nutrient feeding.
i.e. control $F$ so that

$$\Delta C_{\text{GLN}}^{\text{GLN}} = C_{\text{set}}^{\text{GLN}} - C_0^{\text{GLN}} < \text{dead zone}$$

Using $F = V_0 + F_t$ and $\Delta C_{\text{GLN}}^{\text{GLN}} = C_{\text{set}}^{\text{GLN}} - C_0^{\text{GLN}}$ the expression for the glutamine concentration derived for the predictor block above can be solved for the flow rate:

$$F_{t+1} = \frac{V_0}{(C_{\text{feed}}^{\text{GLN}} - C_{\text{set}}^{\text{GLN}})_t}[\Delta C_{\text{GLN}}^{\text{GLN}} + q_{\text{in}}^{\text{GLN}} N_v t]$$

This expression can be viewed as a combined proportional and feed-forward controller. The first part inside the parenthesis is proportional to the difference between the setpoint and the concentration at time 0, the second part compensates for the glutamine that the cells will consume during the feed time (without compensation for cell growth). The feed flow is calculated for the time period the feed is to last (one minute recalculation frequency was used), a shorter time available to reach the setpoint the will increase the flow. The expression was used to construct a feed flow control program, together with standard control expressions for derivative and integral control based on $\Delta C_{\text{GLN}}^{\text{GLN}}$. The derivative and integral part were intended to compensate for drift, for instance to compensate for lagging peristaltic pumps. In reality they were never needed and were tuned to zero. An offset, a gain for each control term, and a dead zone where the controller does not react was also added. The complete control algorithm is described in fig. 5.8. The calculated flow rate is fed to a separate pump control program. This program runs a peristaltic pump to feed the requested amount of medium for that time period (one minute), based on the weight signal from the feed medium balance.
FIG. 5.8. Description of the fed-batch flow controller block. Inputs are the reactor medium volume, the viable cell density, and the glutamine concentration in the reactor. Parameters set from the predictor block are, glutamine uptake rate, and feed medium glutamine concentration. The output is the feed medium flowrate for the next time period. Recalculation frequency is once a minute.
5.7.4 pH control

1. Gas flow control

pH is primarily controlled via the fraction of carbon dioxide in the gas flow through the silicone tubing in the bioreactor. Gas flow is controlled in three steps. First, a PID controller controls the fraction CO\textsubscript{2} in the gas stream based on the signal from the pH probe. The fraction signal is fed to a gasflow control block, that determines the flowrates of air and CO\textsubscript{2} based on a set total flowrate. The gasflow controller also can switch from air to air/oxygen control should the dissolved oxygen level in the medium go below the set minimum value (20% of air saturation). (This guard feature was never evoked by the system and is not further described here). The block also turns off all gasflow and vents the gas lines on a trig signal from the dynamic oxygen uptake rate measurement program (see below). The gasflow pH control is usually able to maintain measured pH within 0.02 units of the setpoint. The response time and accuracy of pH control via gas flow, is improved by mixing the two gas streams (air and CO\textsubscript{2}) just before the reactor, i.e. the connection point for joining the two lines is placed as close to the reactor as possible.

2. Acid/Base Control

The secondary pH control is via acid/base addition. The acid and base solutions where 320 mM HCl and NaOH. The acid/base pH controller is a proportional on/off controller with a delay time and a dead zone. The dead zone was set to 0.1 pH units around the pH setpoint (7.2). The acid/base controller will be idle as long as the gasflow control manages to contain the pH within ±0.1 units of the setpoint. The acid/base control was needed in a few instances where events such as empty gas bottles or leaking gas lines disrupted gas flow. In a few runs there was also a need for base addition towards the end of the runs due to high production of lactate by the culture.
5.7.5 Other features

The control system also contains a number of functions not further described here. Alarm and safety features warn the operator of system problems, and also serve to turn off pumps and other flows when significant problems are detected. All control variables are logged and stored on a hard disk. Finally, the system used a set of menus to display the parameters of the control system. These are shown in timecurves, relation curves, and as graphic system descriptions.

5.8 Control of glutamine limited cultures

For control of the glutamine limited reference process, the approach developed by Glacken (1987) was used with some adaptations. In this process, feed-back measurement of viable cell density is not used. Instead, a model of growth rate as a function of culture environment is used to calculate cell growth. The concentration of nutrients and waste products in the reactor are estimated from expressions for specific uptake and production rates.

Glacken developed an expression for growth rate from initial rate data of low density T-flask cultures. This expression relates the growth rate to the concentrations of cells, serum, lactate, ammonia, and glutamine.

\[
\mu = \frac{\mu_{\text{max}} C_{\text{serum}} C_{\text{GLN}}}{[K_{\text{serum}} (N_v)^{\beta} + C_{\text{serum}}][1 + \frac{(C_{\text{AMM}})^2}{K_{\text{AMM}}}][1 + \frac{(C_{\text{LAC}})^2}{K_{\text{LAC}}}][K_{\text{GLN}} + C_{\text{GLN}}]}
\]
\[
\begin{align*}
\mu &= \text{apparent growth rate (h}^{-1}) \\
\mu_{\text{max}} &= 0.055 \text{ h}^{-1} = \text{specific growth rate} \\
K_{\text{serum}} &= 6.5 = \text{monod constant for serum} \\
\beta &= 0.21 = \text{cell density monod exponent} \\
K_{\text{AMM}} &= 26 \text{ mM}^2 = \text{monod constant for ammonium} \\
K_{\text{LAC}} &= 12000 \text{ mM}^2 = \text{monod constant for lactate} \\
K_{\text{GLN}} &= 0.15 \text{ mM} = \text{monod constant for glutamine}
\end{align*}
\]

As can be seen, glutamine, serum, and cell density are modeled as simulators of growth rate, whereas ammonia and lactate are inhibitors. To use this model in a predictor, expressions for glutamine and ammonia concentration are needed. A glutamine balance including an expression for first order glutamine decomposition was used where glutamine is assumed to mostly form ammonia. A corresponding balance for ammonia was also used. In an optimization using these models, Glacken neglected the terms for lactate inhibition, and assumed that nutrient uptake and waste product formation rates where constant. The lactate term is therefore also excluded in this treatment. The balances for glutamine and ammonia are:

\[
\begin{align*}
\frac{d(A^{GLN})}{dt} &= F \cdot C_{\text{feed}}^{GLN} - q_m^{GLN} \cdot X_v - k^{GLN} \cdot A^{GLN} \\
\frac{d(A^{AMM})}{dt} &= q_A^{GLN} \cdot X_v + k^{GLN} \cdot A^{GLN}
\end{align*}
\]

where

\[
q_m^{GLN} = m^{GLN} + q_A
\]
\[ q_{m}^{\text{GLN}} = 7 \times 10^{-11} \text{ mmol/cell\cdot h} = \text{total specific glutamine uptake rate (same as used in the predictor for dynamically controlled runs above)} \]

\[ m^{\text{GLN}} = 5 \times 10^{-11} \text{ mmol/cell\cdot h} = \text{specific maintenance related uptake rate of glutamine} \]

\[ q_{A} = 2 \times 10^{-11} \text{ mmol/cell\cdot h} = \text{specific formation rate of ammonia from glutamine by cell metabolism} \]

\[ k^{\text{GLN}} = \text{first order degradation rate constant for glutamine} \]

With the assumptions of piece-wise constant cell density and parameters, this can be solved to give

\[
C_{t}^{\text{GLN}} = \frac{V_{0}}{V_{t}} C_{0}^{\text{GLN}} e^{k^{\text{GLN}} t} \cdot \frac{F_{0} C_{\text{feed}}^{\text{GLN}} - q_{m}^{\text{GLN}} N_{v_{0}} V_{0}}{V_{t} k^{\text{GLN}}} \left[ e^{k^{\text{GLN}} t} - 1 \right]
\]

\[
C_{t}^{\text{AMM}} = \frac{V_{0}}{V_{t}} \left[ C_{0}^{\text{AMM}} + (q_{A} - q_{m}^{\text{GLN}}) N_{v_{0}} + \frac{F_{\cdot} C_{\text{feed}}^{\text{GLN}}}{V_{0}} \right] t + \frac{1}{V_{t}} \left[ \frac{F_{\cdot} C_{\text{feed}}^{\text{GLN}} - q_{m}^{\text{GLN}} N_{v_{0}} V_{0}}{k^{\text{GLN}}} - C_{0}^{\text{GLN}} V_{0} \right] e^{k^{\text{GLN}} t}
\]

The predictor program for the glutamine limited runs consists of these two expressions, together with the expressions for cell balance and volume increase shown for the predictor program above. It should be pointed out that the control for this type of runs is entirely predictive, i.e. it only relies on models for cell behavior, with no feed-back estimation of viable cell density.

### 5.9 Process simulations

To be able to maintain the desired nutrient concentrations during the growth phase, the nutrient feed trajectory were designed to reach the maximum medium volume, just after the viable cell number reaches its maximum. In order to obtain this feed trajectory, the
appropriate concentration of nutrients in the feed medium has to be estimated. To predict the likely trajectory for the cultures under various conditions, a simulation program for fed-batch cultures was written. This program uses a set of models of cell growth and nutrients uptake rates similar to those presented above, as well as balances over the reactor and an emulation of a simple proportional controller for feed flow rate, to predict the time profiles of cell numbers, nutrient concentrations, and the medium volume. In the growth rate model, growth rate is a function of cells density: The cell growth rate is essentially held constant up to a cell density of $2 \cdot 10^6$ cells/ml, and thereafter it rapidly declines. These model do not consider cell death, as the time of interest is the growth period, after which all the feed medium should have been fed, and the culture will run to completion undisturbed. For glutamine fed-batches, the program uses the Glacken model for nutrient uptake rate (Glacken, 1987), and for glucose fed-batches a constant specific glucose uptake rate is assumed.

5.10 Control Results, Accuracy, and Robustness

The accuracy of the nutrient concentration control for dynamically operated fed-batch cultures was quite good. Usually the actual value was within 10% of the setpoint, as measured by off-line samples analyzed for nutrient concentration. A large part of the accuracy can be attributed to the use of on-line estimation of viable cell density via dOUR. Fig. 5.9 shows control results from the first run made with the control program, where the dOUR program failed early on. The experiment was completed using only model-predictive control. As can be seen there is a large overshoot in nutrient concentration both at the time of the step change, and later at the time the culture reaches stationary phase. The main reasons for this poor control result is inaccurate estimation of cell density by the predictor. Contrast this with the nutrient concentration profile shown in fig. 5.10. This data
is from a subsequent glucose plus glutamine controlled run, with glutamine concentration used as the control variable. Here the nutrient concentration stays within 15% of the setpoint until the all feed medium has been pumped into the reactor, at 125 h runtime. The accuracy was even further improved in subsequent runs. The robustness of the control system is also quite good, in part due to the safeguarding features built into the system. Things did at time go wrong, pumps failed, gaslines broke, etc. With the control system described here very few failures of this type lead to the termination of an experiment.
Fig. 5.9. Setpoints and measured values for a dynamically controlled culture without feedback estimation of viable cell density via measured oxygen uptake rate (run 90/1).
Fig. 5.10. Setpoints and measured values for a dynamically controlled culture with feedback estimation of viable cell density via measured oxygen uptake rate (run 90/7).
6. On-Line Oxygen Uptake Rate and Viable Cell Density Estimation

A program was written to obtain a reliable and accurate estimate of the volumetric oxygen uptake rate (OUR) of the culture; to use this measurement to estimate the viable cell density ($N_v$) of the culture; and to utilize this estimate to improve the control of the nutrient concentration trajectory. The program is divided into two parts: a program that automatically calculates the oxygen uptake rate of the culture by the dynamic method, and an algorithm that uses the dynamic oxygen uptake rate (dOUR) measurement together with a set of rules to estimate the phase of the culture, and to choose an appropriate correlation for estimation of viable cell density.

6.1 Theoretical considerations for oxygen uptake rate measurements

The oxygen uptake rate program is to designed to obtain an accurate measurement of the volumetric oxygen uptake rate of the culture. There are two common methodologies used for this purpose, out-gas analysis and mass transfer rate balance. Neither of these work satisfactorily with mammalian cell fed-batch cultures, but for different reasons.

The out-gas analysis approach relies on an analysis of the gases exiting the reactor and a mass balance, to measure the uptake of oxygen (Bailey and Ollis, 1986). The flow of oxygen in the gas phase can be related to the flow of nitrogen, which is constant. A mass balance on oxygen written in this manner can be expressed as

$$\frac{F_{N_2} p_{in}^{O_2}}{V_L} - \frac{F_{N_2} p_{out}^{O_2}}{V_L} = q_{O_2:N_v} - q_{\text{OUR}}$$
where the term $q^{O_2} \cdot N_v$ is the oxygen uptake rate (OUR) and

$F_N = $ molar flowrate of nitrogen

$V_L = $ the liquid volume in the reactor (L)

$p^{O_2}$ and $p^{N_2} = $ partial pressure of oxygen and nitrogen in gas stream, going in and out of the reactor

$q^{O_2} = $ specific oxygen uptake rate of the cells (mmol O2/10^9 cell·h)

$N_v = $ viable cell density (10^9 cells/L)

It here assumed that only viable cells consume oxygen. For the culture system used here the oxygen uptake rate is very low. The difference in partial pressure of oxygen between the in-going and outgoing gas streams is therefore small, and the measurement error hence becomes large. In addition, because CO₂ is used to control pH in the reactor, the pO₂ and pN₂ will change, and measurement of CO₂ flow may also be needed to accurately estimate the nitrogen flow.

The transfer rate balance (or steady state) approach assumes that the dissolved oxygen level in the medium at any time can be regarded to be in a semi-steady state and thus constant (Bailey and Ollis, 1986). If the mass transfer coefficient for oxygen is known, the oxygen uptake rate of the culture can be calculated. A transfer rate balance on the oxygen in the medium can be written as:

$$\frac{dC^{O_2}}{dt} = k_L a \left( C^{*O_2} - C^{O_2} \right) - q^{O_2} \cdot N_v \quad \text{OUR}$$
\[ \frac{dCO_2}{dt} \]

where the term \( \frac{dCO_2}{dt} \) is the rate of change in the dissolved oxygen tension, which as indicated is assumed to be in steady state for the time frame considered. The term \( q_{O_2} \cdot N \), is again the oxygen uptake rate and

\[ k_L a \]

is the mass transfer rate constant for oxygen (mmol O_2/L·h)

\[ C^{*O_2} \]

the saturation concentration of dissolved oxygen in the medium, i.e. in equilibrium with the gas phase (mmol O_2)

\[ C^{O_2} \]

the actual concentration of dissolved oxygen in the medium (mmol O_2)

The dissolved oxygen (DO) concentration can be measured with an oxygen electrode. This type of OUR measurement is reasonably reliable for a batch culture of mammalian cells. However, in the fed-batch system used in this investigation there are several difficulties in obtaining the steady state measurement. The medium fed to the reactor contains oxygen, and in the slow changing system of a mammalian cell culture this will significantly disturb the dissolved oxygen concentration. The change in liquid volume in the reactor as medium is fed also means that the area term \( a \) in the mass transfer rate constant \( k_L a \) of the reactor changes, as more and more of the silicon tubing coil used for aeration is immersed in the medium. Lastly, the use of CO_2 in the gas phase to control pH also disturbs the DO measurement, as changes in pCO_2 will affect the pO_2, which in turn will disrupt the steady state oxygen concentration.

To avoid the problems presented above, a dynamic method is used to measure the oxygen uptake rate in the culture system described here (Bailey and Ollis, 1986). In this approach the oxygen uptake rate of the culture is measured directly, by following the decline in
oxygen concentration over time after the gas flow to the reactor is stopped. With no oxygen flow to the culture the mass transfer rate constant in the oxygen balance will be zero;

\[
\frac{dC^{O_2}}{dt} = k_{L,a}(C^{*}O_2 - C^{O_2}) - q^{O_2}N_v
\]

and the volumetric oxygen uptake rate of the culture is directly proportional to the first derivative of the oxygen concentration slope

\[
\frac{dC^{O_2}}{dt} = -Q^{O_2}
\]

where \( Q^{O_2} = q^{O_2}N_v \) is the volumetric oxygen uptake rate in mmol/L·h. Solving this expression for \( Q^{O_2} \), assuming a constant oxygen uptake rate during the measurement, gives

\[
Q^{O_2} = \frac{C_0^{O_2} - C_t^{O_2}}{t}
\]

\( Q^{O_2} \) is the slope of the declining straight \( C^{O_2} \) line during the measurement. In fig. 6.1 the DO track of a dynamic oxygen uptake rate measurement is depicted. With typical oxygen uptake rates of a mammalian cell culture it takes between 10 and 60 minutes, depending on the viable cell density, to get a large enough difference in dissolved oxygen concentration to obtain a good value of the oxygen uptake rate.

6.2 The dynamic Oxygen Uptake Rate Measurement Program

A supervisory program that automates dynamic oxygen uptake rate measurements has been written. The principle of the program is simple: turn off the flow of gases to the reactor, wait until DO has declined enough to get a good slope value, calculate the dOUR value, and
Fig. 6.1. Track of the dissolved oxygen concentration curve during a dynamic oxygen uptake rate measurement.
restart the gas flow. In reality, there are a number of complications which the program have been designed to handle. Fig. 6.2 describes the basic features of the program.

One source of measurement error is exchange of oxygen from the gases left in the silicon tubing coil when the gas-flow is stopped. The program handles this by waiting until the track of the DO curve is a straight line before starting to take measurement points. A straight line is a sign that the residual transfer of oxygen is low. Another source of measurement error is aeration from the head space of the reactor. There is too much oxygen in the head space to wait until it is in equilibrium with the liquid. In this application the problem has been alleviated not venting the head space of the reactor. As the gases there are stagnant they will be in equilibrium with the liquid. There is therefore no driving force for gas exchange between the headspace and the liquid at the start of a measurement cycle. However, as the DO level in the medium declines during the measurement, a driving force will be established, both for aeration from the tubing and from the headspace. There is only a small amount of gases in the tubing, and although the amount of gases in the headspace is significant the mass transfer coefficient for surface aeration is quite low, and neither of these factors do therefore contribute significantly a residual gas exchange (Goldstein, 1986). Nevertheless, gas exchange between both the tubing and headspace and the liquid does introduce a small measurement error, estimated to be about 1% at maximum cell densities and about 5% at low cell densities. While this affects the absolute measurement value of OUR, it has no consequence for control purposes. To acquire a good estimate of viable cell density in the reactor, only a reliable and reproducible correlation between cell density and dOUR is needed, while the absolute value of the correlation is not important.
Fig. 6.2. The features of the dOUR program. The line is a trace of dissolved oxygen over time, with start and stop of aeration of the culture. Values for dissolved oxygen (DO) are shown as percent of air saturation.
Other errors in the dOUR measurement can be caused by premature data collection and disturbances in the DO track. Just after the gas-flow to the reactor has stopped oxygen is still being transferred to the medium, and the DO track is not yet a straight line. The program therefore waits until DO has declined 2% of its initial value before taking the first measurements, as residual mass transfer from the silicone tubing coil has stopped at that time. If the DO track is a straight line thereafter, the program starts collecting measurement values. To further guard against measurement errors due to disturbances in the DO track, the program continuously checks the curvature of the DO line by calculating the second derivative of the track. If the curvature is too large, the program re-starts the measurement from this point, and re-takes the first measurement value. If the curvature continues to be too large, the program will stop, and issue an alarm.

The program also contains features to ensure that the dOUR measurements does not disturb the performance of the culture. The DO must not reach such low levels during a measurement cycle that it affects cells metabolism. Dissolved oxygen concentrations that affects metabolism and growth rate vary by cell line, and a range of values have been reported in the literature (Ozturk and Palsson, 1990; Miller, Wilke, and Blanch, 1987; Siano, and Mutharasan, 1991; Yamada, K. et al., 1990; Meilhoc, Wittrup, and Baily, 1990; Butler and Jenkins, 1989). CRL 1606 is influenced by DO levels below 5% of air saturation, while a level of 10% appears to have no significant effect (Glacken 1989, own observations). In this work 20% was used as the lowest permissible level for DO. To obtain a reasonably accurate dOUR value the program waits for a 10% decline in absolute DO value, or 20% of the start DO value. The measurement is not started if the DO value is below 25% of air saturation, and the program stops if DO goes below 20% during the measurement. There is also a possibility that the cycles in DO levels introduced by the dOUR measurements may affect the culture. Only limited tests has been done to ensure
that no such effects exists, but no differences has been discovered when the same type of experiments was done with and without dOUR measurements. There is no fundamental reason to expect such an influence as the DO cycles are less than 20% of the absolute DO value, and in a region where the cells metabolism is saturated for oxygen.

The measurement frequency of the dOUR program is limited by the time constants for oxygen transfer of the silicone tubing devise used. In the set-up used in this investigation the time constant for reaching 95% of the semi-steady-state DO value is about 30 min at low cell densities (and high steady state DO) and 10 min at maximum cell densities. To track the semi steady-state DO value throughout the culture the DO value has to be stable before the next dOUR measurement can be taken. Including the time for the dOUR measurement itself, a measurement cycle takes about 20 minutes at maximum cell densities and 90 min just after inoculation. The frequency of measurement has therefore been set to once every two hours. This frequency gives a more than adequate response time for the control system.

The dOUR measurement program also contains other safeguarding features and alarm functions. For instance, if a dOUR measurement takes more than 90 minutes, the program delays the next measurement by one cycle, i.e. the next measurement will start four hours after the previous one. If a measurement takes more than 2 h, for instance due to very low cell densities, the program will time out, stop, and delay the next measurement. Table. 6.1 shows a listing of the main steps and functions of the program.
1. Check start DO value. If below 25%, do not start measurement. If below 30% issue alarm and start measurement.

2. Turn off air, CO2, and nutrient feed flow. Monitor gas-flows to ensure that they are off.

3. Check initial DO drop and wait until it is below 2% of the start DO value.

4. Start data acquisition. Collect one DO data point every minute.

5. Start DO line curvature check. Continuously calculate second derivative of DO trace. If the value is too large, re-start data acquisition at that time, and continue measurement. If the curvature is out of bounds several times in a row, stop measurement and issue alarm.

6. If DO measurement takes more than 90 min. total, time-out, issue alarm, re-start aeration, and stop program.

7. If DO goes below 20% air saturation during measurement, issue alarm, restart aeration and stop program.

8. After a decline in DO of 10% air saturation, or 20% of start DO value, take final data point and calculate dOUR. If curvature has been out of bounds, take final data point at 5% decline in DO from re-start of data acquisition, or at 10% of DO value re-start of data acquisition.

9. If dOUR measurement took more than 60 min or timed-out, delay next measurement by one cycle.

10. Turn on air, CO2, and feed medium flow again.

11. Wait until next measurement (cycle at present 2h).

Table 6.1. Main functions and features of the dOUR measurement program. Values for dissolved oxygen concentrations (DO) are given in percent of air saturation.
6.3 OUR measurements and consequences for automatic control

To obtain a reliable estimate of viable cell density ($N_v$), a correlation between dOUR and $N_v$ is needed. As shown below, this correlation may differ from the specific OUR, which is the OUR divided by the cell density. There is sometimes a significant offset from a straight line through the origin in the dOUR vs $N_v$ correlation, and the slope of this track will therefore differ from the specific oxygen uptake rate value. The offset is a reflection of errors and disturbances in the dOUR measurement. Tracks cf volumetric OUR (measured in mmol $O_2$/L·h) versus viable cell density are used here to highlight the behavior of the dOUR versus $N_v$ correlation. The behavior is more visible in such plots than in plots of total oxygen uptake rate (measured in mmol $O_2$/h), although they are qualitatively the same.

Fig. 6.3 shows a time-series of dOUR measurements, together with viable cell densities and the volume track, for a glucose plus glutamine controlled dynamic fed-batch run (run 91/2). The nutrient concentration change was introduced late in the growth phase in this run, and it therefore has a long period of nutrient limited growth which highlights some interesting features. The dilution of the viable cells at the time of the step change in nutrient concentration is clearly visible. From this graph it appears that dOUR is a good indicator of viable cell densities. There are however shifts in the correlation between dOUR and $N_v$. After the step change the dOUR measurement shifts upward compared to the viable cell density, and just before the cell reach their peak density the dOUR values shifts downwards. These effects are shown more clearly in fig 6.4, where dOUR has been plotted against a curve fitted to the viable cell density. The dOUR measurements are more frequent (every 2 h) than the manually measured viable cell density (every 8 h), and more information is revealed by using a fitted curve. The fit between the curve and the measured cell data is
Fig. 6.3. Viable cell density and oxygen uptake rate vs runtime, for a glucose plus glutamine fed-batch (run 91/2). Dark balls (*) are measured cell density, thick line is fitted cell density curve.
Fig. 6.4. dOUR vs viable cell density for a glucose plus glutamine fed-batch (run 91/2). The data is divided into three phases: nutrient limitation phase, non-limited growth phase, and death phase.
quite good as can be seen in fig 6.3. The correlation between dOUR and Ny shown in fig. 6.4 can be divided into three phases. During the nutrient limited phase of the culture the dOUR follows a somewhat declining curve against Ny until the step change in nutrient concentration. Here the cells go from a limiting to a non-limiting stage and there is a large shift in the dOUR correlation. The dOUR values move upward versus Ny, and the track goes "backwards" momentarily due to the dilution of the cells during the step change. Thereafter the dOUR vs Ny correlation follows a reasonably straight line during the main non-limited growth phase of the culture. Just before the viable cells reach their maximum density there is another shift in the correlation. The dOUR track shifts sharply downwards, and then follows a different correlation during the death phase of the culture. It is interesting to note that the dOUR correlations for the death phase and the nutrient limited phase to a large extent coincide. Fig. 6.5 shows the same data as in fig. 6.4 plotted as total oxygen uptake rate versus total cells in the reactor. The data is qualitatively the same but the offset in the correlations from a line through the origin is less evident than in fig. 6.4.

Fig. 6.6 zooms in on the beginning part of the dOUR track shown in fig. 6.4, and shows the additional information hidden there. At the beginning of the culture, the cell density is low and there is a significant scatter in the dOUR data. Once the culture starts growing the scatter is reduced, perhaps reflecting a more stable specific oxygen uptake rate of the cells. The increasing nutrient limitation of the culture is evidenced by the declining slope of the dOUR track, as the aerobic metabolism of the cells slows down. At a density of about \(0.75 \cdot 10^9\) cells/L (runtime 58 h) the cells become more severely nutrient limited, and the dOUR track here has a negative slope. At \(0.84 \cdot 10^9\) cells/L (runtime 62 h) the limitation is discovered as a decline in the growth rate, and a slow feed of nutrient is initiated manually to maintain a minimum nutrient concentration. The culture responds rapidly to the very slow
Fig. 6.5. Total OUR vs total viable cell number for a glucose plus glutamine fed-batch (run 91/2). The data is divided into three phases: nutrient limitation phase, non-limited growth phase, and death phase.
Fig. 6.6. Oxygen uptake rate trajectory vs viable cell density for a late step glucose plus glutamine fed-batch (run 91/2). Zoom in on the start phase with nutrient limitation and slow nutrient feed.
Fig. 6.7. Comparison of the dOUR vs viable cell density track for two runs: a glucose plus glutamine late step-change dynamic fed-batch (GLU+GLN, run 91/2), and a glucose step-change dynamic fed-batch (GLU, run 90/6).
feed as evidenced by the increased slope of the dOUR track. At a cell density of 1.1 \times 10^9 
cells/L (runtime 73 h) the culture is subjected to a sharp increase in nutrient concentration,
from less than 0.5 mM glutamine and 1 mM glucose to 10 and 25 mM respectively, and the
sharp shift in the dOUR track is clearly seen in fig. 6.6.

The graphs shown above demonstrate how the dOUR vs measured Nv correlation can
change during a single run. However, the correlation also changes between different type of
runs. Fig. 6.7 shows the same data as in fig. 6.4, together with the dOUR track of a glucose
controlled dynamic fed-batch. The correlation for dOUR versus viable cell density differs
significantly but the same trends are evident in both runs, notably the difference between the
tracks for growth and the death phases of the culture. These changes in the correlation
between dOUR and Nv measured from samples pose some difficulties for automatic
estimation of viable cell density. Fortunately, for a given type of run and a given phase of
this run, the correlation appears to be reliable and reproducible (data not shown). For
instance, glucose change cultures will display the same type of correlation, while glutamine
change cultures will display a different but equally consistent correlation. A separate rule
based program was written for automatic estimation of viable cell density from dOUR data
(described below).

6.4 Specific oxygen uptake rate measurements
Oxygen uptake rate measurements provide valuable information about the performance of
the fed-batch process. Fig. 6.8 shows an example of specific oxygen uptake rates (qO_2) for
a dynamic fed-batch with a normal step-change. The pattern exhibited of qO_2 here is typical
for most types of dynamically operated fed-batches, as well as for batch runs. Shortly after
Fig. 6.8. Specific oxygen uptake rate, viable cell density, and volume vs runtime, for a glucose step-change dynamic fed-batch (run 9/6). Dark balls (•) are measured cell number, thick line is fitted viable cell number curve.
the lag phase the $q_{O_2}$ value increases to about 0.1 pmol $O_2$/cell·h, and thereafter declines throughout the growth phase. At the end of the growth phase, as the growth rate rapidly decreases, the $q_{O_2}$ value drops sharply to about 0.06 pmol $O_2$/cell·h and remains at this value until the end of the run. Similar findings have been reported by Ramirez and Mutharasan (1990) and Yamada et al., (1990). In some other runs the value for $q_{O_2}$ is somewhat higher and rising during the death phase. The $q_{O_2}$ data together with the growth rate of the cells also can be used to obtain an estimate of the maintenance requirement for oxygen of the cells, and the yield of cells on oxygen consumed. The specific oxygen uptake rate is defined as

$$q_{O_2}^{O_2} = \frac{1}{X_v} \frac{dC_{O_2}}{dt} = \frac{OUR}{X_v}$$

where the parameters are defined as above. $q_{O_2}$ can be divided into a maintenance term, and a growth related term with a yield factor for number of cells obtained per amount of oxygen consumed:

$$\frac{OUR}{X_v} = q_{O_2}^{O_2} = \frac{\mu}{Y_{O_2}} + m_{O_2}$$

By plotting $q_{O_2}$ against the real growth rate $\mu_{\text{real}}$ (see appendix 1), $Y_{O_2}$ can be obtained from the slope of a fitted line, and of $m_{O_2}$ from the intercept at $\mu_{\text{real}} = 0$. In reality, a linear relationship is not always obtained as for instance the growth rate may vary during a run. The data can therefore be difficult to interpret and the meaning of values obtained is not always clear. Fig. 6.9 shows the result of such an exercise. Here $q_{O_2}$ from a glutamine controlled dynamic fed-batch has been plotted against the real growth rate (compensated for cell death) for the main part of the growth phase and separately for the beginning of the
Fig. 6.9. Plot of specific oxygen uptake rate versus growth rate for a glutamine step-change dynamic run (90/5). Data is from main growth phase and early death phase.
death phase. For the growth phase, a yield coefficient of 0.8 cells/pmOL O₂ consumed is obtained, and a maintenance coefficient of 0.035 pmOL O₂/cell·h. For the death phase the corresponding values are 5.7 cells/pmOL O₂ and 0.07 pmOL O₂/cell·h. These values have to be interpreted with care. For instance, the value for cells yield on oxygen during the death phase is probable an artifact of the analysis method. In this phase, culture growth has not stopped entirely, and the cells are dividing very slowly as most of them are dying. Most of the oxygen is probably used for maintenance by the cells, and there may be no real growth related oxygen uptake at this time. The values obtained for the growth phase are probably more meaningful, and can be used as a first estimate of the oxygen needs of cultures growing at different growth rates.

6.5 The cell density estimation program

As was shown above, the correlation between the viable cell density and the dynamically measured oxygen uptake rate varies during the course of a run. Also the specific nutrient uptake rates change during a run, as shown below. This poses several problems for the use of dOUR measurements and its use in predictive models for feed-back control. Using only one set of correlations may introduce a significant error in both the cell estimation and nutrient control calculations. The best possible control accuracy can only be achieved by using the appropriate correlations at different stages of a culture.

The main determinant for the behavior of specific uptake rates appears to be the phase of the culture. The rates changes between different phases, but during a given phase they appears to be reasonably constant. The phase of the culture can therefore be used as an indicator for choosing correlations for cell and nutrient concentration estimation. A batch or fed-batch
mammalian cell culture can be divided into four general phases: lag, growth, peak, and death phase. Fig. 6.10 shows an example of a correlation between a growth curve and an oxygen uptake rate track. In the lag phase, the cells are not growing, the oxygen uptake rate is low and the values are unstable. As the cells start to grow and enter the growth phase the OUR increases and the OUR signal stabilizes. The OUR values follows the cell number quite well during the main part of the growth phase. The transition to peak phase is marked by a decline in growth rate, and a leveling off of the cell number curve. Just before the growth rate declines there is also a sharp shift downward in the OUR curve, which can therefore be used as an indicator of this transition. Lastly, death phase is here defined by a marked decrease in viable cell number, negative apparent growth rate, and a downward sloping OUR curve.

To enable the use of Nv vs dOUR correlations for cell density estimation under a wide range of culture conditions and in different types of experiments, a program was written that determines the phase of the culture. The program chooses the appropriate correlations for viable cell density estimation and specific nutrient uptake rate depending on the phase. The program is a rule based, consisting of a set of IF-AND/OR-THEN statements that are tested and executed serially. For reasons of simplicity and speed of development, a simple rule base design was used in this first version rather than a fuzzy logic or full expert system approach. The main input used by the program is the present value and history of the total OUR (in mmol O₂/h) of the culture, calculated from the volumetric dOUR measurements (in mmol O₂/L·h) and the known volume of the medium. The program divides the culture into a series of phases and sub-phases, and each phase is associated with a number called the phase estimator, fig. 6.11. The criteria for estimating the phase the culture are based on the total viable cell number (from dOUR and the known media volume), the history of the
Fig. 6.10. Example of the relation between viable cell number and oxygen uptake rate, vs runtime. Lines are from real data, but have been edited to highlight important features.
Fig. 6.11. The culture phases included in the phase estimation part of the cell density estimation program. Each phase is assigned a number. The program can only progress forward between phases. Within one phase the estimator can go back and forth between sub-phases.
dOUR track, and the direction of the dOUR track. The program is designed to bring the phase estimator from one phase to the next in one direction only. The estimator can for instance go from lag phase to growth phase, but not the other way around. Within a phase the estimator can go back and forth between sub-phases. The estimator can also jump from any phase to phase 10 (infection). Based on the selected culture phase, the program then uses a set of correlations for dOUR vs \( N_v \) and nutrient uptake rate correlations pre-programmed for that phase.

The first phase, inoculum, is at present used only as a start stage, and as soon as the first dOUR value is obtained a rule brings the state to lag phase. When a rise is detected in the total OUR curve the estimator is brought to growth phase. The sharp shift in total OUR at the end of the growth phase is the signal that brings the estimator to peak phase, where it will stay for at least twelve hours. When the total OUR level has declined more than 20% from its peak level and has a negative slope, the estimator is brought to the death phase where it will stay to the bitter end. The sub-phases shown in fig. 6.1 are more than the program uses at present. They are intended for a more advanced program version as indicators for further shifts in control strategies. There are a number of rules in the program designed to ensure that the estimator does not shift phase too early or too late. These rules evaluate parameters such as the absolute level of the OUR, the first and second derivative of the total OUR curve, etc. There is a substantial amount of scatter in the real OUR data, which can confuse the program. Earlier versions had a problems with this, and in simulations the estimator often went into death phase halfway into the growth phase. Filtering techniques helped, but the initial result was that the estimator did not go into peak phase until mid death phase. To detect phase changes early and reliably, several rules and calculations must be used and conflicts between rules must be resolved. The present
program is not well equipped to handle such difficulties. An expert system approach could be used with advantage to address these problems, as such programs are equipped with an inference engine and with mechanisms for rule-conflict resolution. Using such techniques most of the disadvantages with the present approach can be avoided, and a more reliable system can be built. However, this is also a more time consuming task and has therefore not been part of this work.

Fig. 6.12 shows an example of the performance of the phase estimator program. The path of the phase estimator is tracked as a thick line through the different stages of the culture. The program correctly identifies the right phases and shifts phase at the appropriate times. The program jumps between different sub-phases at a few times, but this has no consequence for the control system. The estimation of viable cell density is quite accurate as can be seen, although it varies a bit during the peak phases due to scatter in the dOUR signal. The on-line estimation of viable cell density by this program has been superior to any other method available, including the use of models for growth rate or using a fixed growth rate. The feed-back estimation of cell density from dOUR measurements and rule based choice of correlation is a major contributor to the accuracy of the control system presented here. Examples of the performance of the control system with and without on-line cell estimation has already been shown above in fig’s. 5.9 and 5.10.
Fig. 6.12. Phase and cell density estimation for a glucose plus glutamine step-change run. Thick line is a trace of the assigned phase number. Dark balls (*) are measured viable cell density. Un-solid line (--) is viable cell density calculated by the cell estimation program.
7. Results and Discussion

7.1 Introduction and summary

The main goal of the research presented here has been to improve the production of antibodies in a fed-batch process. The primary parameter by which performance is judged is therefore the final antibody concentration in the reactor, i.e. the antibody production. In addition, performance parameters such as volumetric productivity (amount of antibody produced per day) and product quality also are evaluated. The nutrient concentration changes introduced in the different cultures (also called runs) examined in the experimental series have a profound impact on the metabolism, size, and product formation rates of the hybridoma cells. The main finding is that antibody production is improved by about 40% in a dynamically operated culture, as compared to a batch process. The improved production is the result of different mechanisms for different types of dynamic conditions. For glucose change runs, i.e. runs with a step- or ramp-change (also called just “changes”) in the glucose concentration, the total viable cell number in the reactor is increased compared to a reference batch run, as well as the specific antibody productivity of the cells. For glutamine change runs, the viable cell number is decreased, but this is offset by a large increase in the specific antibody productivity, leading to an overall increase in the final antibody concentration. Glucose plus glutamine change runs, i.e. runs with simultaneous step- or ramp-changes in both the glucose and glutamine concentrations, behave either as a glucose or as a glutamine change runs. No difference was detected in the performance of cultures subjected to a step-change or a ramp-change in the concentration of a nutrient. For instance, a glutamine ramp-change culture behaves the same as a glutamine step-change culture. However, the timing of the concentration change appears to be import. No effect on antibody production is seen if the change is introduced too early or too late. The
different cultures in the experimental series therefore can be divided into three basic behavioral patterns: 1. Batch runs and runs that behave as batch runs; 2. Glucose change runs; 3. Glutamine change runs. The behavior and performance of these three basic types of runs will be exemplified below using three typical cultures, one from each group.

To explain the mechanisms underlying the overall results, several different techniques will be used. Overall and specific rates are calculated by standard definitions and methods, as explained in appendix 1. To compare different batch and fed-batch cultures, total amounts in the reactor are compared, rather than densities and concentrations. In this way the volume changes during fed-batch cultures can be neglected, as all cultures have the same final volume. As mentioned in the experimental approach chapter, this implicitly assumes that the cells are not significantly affected by the changes in volume occurring during a fed-batch culture. Process parameters can be analyzed and compared against the runtime of the cultures, which is initiated at the time of inoculation.

In addition to these traditional methods of comparing bioreactor runs, the data will be compared in what is called $\tau$-space. $\tau$ is defined as the integral of the viable cell density over time;

$$\tau = \int_{t=0}^{t_{\text{fin}}} X_v \, dt$$

where $X_v$ is the total amount of viable cells in the reactor. Thus, $\tau$ is a measure of the "integral viability time" of the culture, which is called "viability time" for short. Similar approaches have to some extent been used by others (Renard, et al., 1988; Ozturk, and Palsson, 1991 a and b). Fig. 7.1 shows an example of the viable cell number and the
viability time of a culture plotted against runtime. The viability time can be used as a numerical transfer function to transform the runtime to \( \tau \). This transfer function is numerically intrinsic to the viable cell number in the culture, and the function therefore will differ from one run to another. Fig. 7.2a shows an example of total cell numbers and total antibody in the reactor plotted against runtime, and fig. 7.2b shows the same data transformed to \( \tau \)-space. The space in fig. 7.2a has been transformed from the normal runtime to \( \tau \)-space in fig. 7.2b by the transformation \( d\tau = X_v \, dt \). In a \( \tau \)-space plot the x-axis is related to the viable cell density and it highlights differences between runs during the times when the number of viable cells are highest, and consequently when volumetric production and uptake rates are the highest. Moreover, the specific rates for cell growth, antibody production, and nutrient and waste product formation can be identified as the slopes of total amount curves in this space. For instance, the slope of the total cell number in \( \tau \)-space is \( \frac{d(X_v)}{d\tau} \). But as mentioned above, \( d\tau = X_v \, dt \), and therefore

\[
\frac{d(X_v)}{d\tau} = \frac{d(X_v)}{X_v \, dt} = \mu
\]

which is the normal definition of growth rate. In the same way the slope of the antibody amount curve can be identified as

\[
\frac{d(A^{Ab})}{d\tau} = \frac{d(A^{Ab})}{X_v \, dt} = q^{Ab}
\]

where \( q^{Ab} \) is the specific antibody productivity, and \( A^{Ab} \) is the total amount of antibody in the reactor. Thus, in \( \tau \)-space graphs both amounts and rates can be compared directly. Such plots may to some extent mask what happens at the beginning of a culture, as the \( \tau \)-axis compresses the beginning and end of the curves. It is therefore informative to analyze the data in runtime space as well as in \( \tau \)-space.
Fig. 7.1. Example of a numerical transfer function from runtime $t$ to viability time $\tau$ for a fed-batch culture (run 90/1).
Fig. 7.2. Comparison of data plotted in runtime space and τ-space for a fed-batch culture (run 90/1).
7.2 Overview: dynamic operation increases antibody production

Table 7.1 gives an overview of the final antibody concentrations and total cell amounts reached in the different cultures in the experimental series. The experiments have been divided into two main groups. The first (upper) group contains reference runs and dynamically operated runs with very early or late nutrient concentration changes. The second (middle) group contains experiments operated under dynamic conditions. In addition, two other reference cultures have been listed separately (lower group). Within each group, runs are listed by increasing final antibody concentration. Other performance parameters included are the maximum number of cells obtained in the cultures and the final viability times, i.e. $\int X_v \, dt$ up to the point where the antibody amount does not increase anymore. Two numbers are listed for the maximum cell numbers: the maximum viable cell number in the reactor, and the maximum total cells number i.e. viable plus dead cells. The maximum number of total cells is higher than the maximum number of viable cells as the cells continue to divide for some time after the peak in viable cell number has been reached.

7.2.1 Final antibody levels

The main finding in this investigation is that antibody production can be increased by about 40% over that of a batch culture, using dynamic operation of the process. Fig. 7.3 shows the final antibody concentrations in a bar graph; the order is the same as in table 7.1. The values are composites from several immunoassay, weighted by the inverse of the measurement variances (for data treatment see appendix 1). The average final antibody concentrations are $119\pm3$ mg/L for dynamically operated runs, and $86\pm1$ mg/L for the reference runs. (Two runs have not been included in this average, 91/3 and 90/5, as they are more than 3 standard deviations below the minimum for each group.) This corresponds to an increase in antibody production of between 25 to 50% over the average of the reference
<table>
<thead>
<tr>
<th>Run Type</th>
<th>Run Number</th>
<th>Final antibody concentration (mg/L)</th>
<th>Maximum Viable Cell Number (10^9 cells)</th>
<th>Maximum Total Cell Number (viable+dead) (10^9 cells)</th>
<th>Final Integrated Cell Viability Time (dXv/dt) (10^9 cells*h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference runs and early/late step changes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early glucose plus glutamine batch</td>
<td>91/3</td>
<td>68</td>
<td>4.64</td>
<td>5.09</td>
<td>484</td>
</tr>
<tr>
<td>Early glutamine step change</td>
<td>89/1</td>
<td>82</td>
<td>3.34</td>
<td>4.25</td>
<td>420</td>
</tr>
<tr>
<td>Glacken fed-batch</td>
<td>89/7</td>
<td>83</td>
<td>2.20</td>
<td>2.96</td>
<td>235</td>
</tr>
<tr>
<td>Glacken fed-batch</td>
<td>89/6</td>
<td>87</td>
<td>2.00</td>
<td>3.50</td>
<td>181</td>
</tr>
<tr>
<td>Batch, 2.5% FCS</td>
<td>89/5</td>
<td>87</td>
<td>4.24</td>
<td>4.80</td>
<td>471</td>
</tr>
<tr>
<td>Late glucose + glutamine step change</td>
<td>91/2</td>
<td>89</td>
<td>4.89</td>
<td>6.39</td>
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<td>Dynamically operated runs</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Glutamine step change</td>
<td>91/5</td>
<td>88</td>
<td>2.92</td>
<td>3.33</td>
<td>298</td>
</tr>
<tr>
<td>Glucose + Glutamine step change</td>
<td>90/7</td>
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<td>4.07</td>
<td>5.03</td>
<td>502</td>
</tr>
<tr>
<td>Low glucose step change</td>
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<td>111</td>
<td>4.51</td>
<td>6.07</td>
<td>563</td>
</tr>
<tr>
<td>Glucose + glutamine step change</td>
<td>91/1</td>
<td>115</td>
<td>4.24</td>
<td>5.30</td>
<td>337</td>
</tr>
<tr>
<td>Glutamine ramp change</td>
<td>90/2</td>
<td>118</td>
<td>2.68</td>
<td>4.26</td>
<td>392</td>
</tr>
<tr>
<td>Glucose step change</td>
<td>90/6</td>
<td>120</td>
<td>4.58</td>
<td>5.40</td>
<td>517</td>
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<tr>
<td>Glutamine step change</td>
<td>90/1</td>
<td>128</td>
<td>2.27</td>
<td>3.88</td>
<td>373</td>
</tr>
<tr>
<td>Glucose + glutamine ramp change</td>
<td>90/9</td>
<td>132</td>
<td>3.23</td>
<td>4.96</td>
<td>475</td>
</tr>
<tr>
<td>Other reference runs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Batch, 5% FCS</td>
<td>89/2</td>
<td>112</td>
<td>4.98</td>
<td>5.51</td>
<td>730</td>
</tr>
<tr>
<td>Batch 0.6 L spinner flask culture</td>
<td>91/4</td>
<td>133</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

Table 7.1. Comparison of performance parameters between batch runs, glutamine limited fed-batches, and dynamically operated fed-batch runs. Some data is in total amounts in reactor (final volume is 2 L, to get concentrations, divide amounts by 2).
Fig. 7.3. Final antibody concentrations in the different bioreactor runs.

Key: GLU = glucose; GLN = glutamine.
Example: GLU+GLN step = glucose plus glutamine dynamically controlled fed-batch run with nutrient concentration step-change.
cultures. In those dynamically operated runs where the nutrient concentration change is introduced very early or late, the culture behaves mostly as a batch run and does not display an increased antibody production. The variation in final antibody concentration is larger among the dynamically operated runs than among the reference runs. This may in part reflect to what extent the nutrient concentration change was introduced at the appropriate time for the dynamically operated runs. The difference in final antibody concentration between the reference runs and the dynamically operated runs is well within three standard errors of the measurements used and is therefore statistically significant (Bevington, and Robinson, 1992). Fig. 7.4 shows the same data as fig. 7.3, together with the standard errors calculated from the coefficient of variation of the RID assays. The common way to calculate standard errors for this type of data (see appendix) yields much lower errors than those shown here, and probably underestimates the error in the measurements. As the RID measurements contribute the most to the mean data, the error of this measurement is therefore used.

The data presented here shows that dynamic reactor operation reactor is responsible for improved process performance, compared to batch runs. For instance, cultures perform essentially as a batch cultures if the nutrient concentration change is introduced too early to influence the cells (see runs 89/1 and 91/3). There is no indication in the data in fig. 7.3 of a difference in performance between a step-change or a ramp change in nutrient concentration (see also below). The glutamine limited fed-batch runs, designed by Glacken (1987), did not result in an improved antibody production. While an increase in specific antibody productivity was seen, there was a large decrease in the maximum viable cell number. The overall result was a reduced antibody production.
Fig. 7.4. Standard errors for the composite immunoassay measurements shown in fig. 7.3, estimated from the standard errors of the RID immunoassay (~4%), which is larger than the calculated intrasample measurement standard error.

Key: GLU = glucose; GLN = glutamine.
Example: GLU+GLN step = glucose plus glutamine dynamically controlled fed-batch run with nutrient concentration step-change.
7.2.2 Cells densities

The viable cell numbers and the final integrated viability times of the runs in the experimental series provide a first clue to the factors behind the antibody result. A ranking of the different runs based on final integrated viability times differs markedly from a ranking based on final amount of antibody produced. In fig. 7.5 the runs have been ranked based on final integrated viability times, within the high and low antibody producing groups. The emerging pattern is that a glucose change run produces a somewhat higher final viability time than the reference batch run, whereas a glutamine change run yield a significantly lower value. Glucose plus glutamine change runs (i.e. cultures where the concentration of glucose and glutamine was changed simultaneously) behave either as glucose or as glutamine change runs, although some intermediary values are seen. Runs 90/7 and 91/1 are example of such runs behaving as glucose change cultures, while run 90/9 behave as a glutamine change culture. This pattern is perhaps best shown by viable cell data. Fig. 7.6a shows a comparison of two glucose change cultures (90/6 and 90/8) and a glucose plus glutamine change culture (91/1) that behaves as a glucose change culture. Fig 7.6b shows a similar comparison of glutamine change cultures (90/1 and 90/2) and a glucose plus glutamine change culture (90/9) that behaves as a glutamine change culture. (The residual growth that is seen in culture 91/1 and 90/9 have been observed in other change cultures, but the reason for it is not known.) This pattern holds also for process parameters other than cell density or antibody concentrations, as will be shown below. The glutamine limited reference runs yield very low final viability times. The results are essentially the same if the runs are ranked based on maximum viable or total cell numbers. Based on the values of final antibody amounts, maximum cell numbers, and final viability times, it is clear that there must be a significant difference in the specific antibody production of the different types of runs. For instance, run 90/2 (a glutamine ramp change) and 90/6 (a glucose step change) have almost the same final antibody amounts in the
Fig. 7.5. Viability time for the different cultures, calculated until the culture viability has declined to 50% (See text for definitions).

Key: GLU = glucose; GLN = glutamine.
Example: GLU+GLN step = glucose plus glutamine dynamically controlled fed-batch run with nutrient concentration step-change.
Fig. 7.6 a (upper) and b (lower). Comparison of viable cell numbers for a; glucose change cultures (90/6 and 90/8) and glucose plus glutamine change cultures behaving as glucose change cultures (91/1). b; glutamine change cultures (90/1 and 90/2) and glucose plus glutamine change cultures behaving as glutamine change cultures (90/9).
reactor, but the final viability time and maximum viable cell number of run 90/2 is only 76% and 59% of the values for run 90/6, respectively.

### 7.3 Different mechanisms behind increased antibody production

#### 7.3.1 Runtime analysis

The mechanisms underlying the increased antibody production in dynamically operated runs will be analyzed using three experiments representative of the patterns observed in the experimental series: a batch reference run (89/5), a glucose step-change run (90/6), and a glutamine step-change run (90/1). The final total antibody amount in a reactor \( A_{\text{Ab}} \) is the integral of the volumetric antibody productivity \( Q_{\text{Ab}} \) over runtime, which in turn can be divided into the specific antibody productivity \( q_{\text{Ab}} \) times the viable cell number \( X_v \), i.e.

\[
A_{\text{t,Ab}} = \int_{0}^{t} Q_{\text{Ab}} \, dt = \int_{0}^{t} q_{\text{Ab}}X_v \, dt.
\]

The mechanisms behind the final antibody production levels can be elucidated by analyzing these parameters as a functions of runtime. Fig. 7.7 shows the viable cell number in the reactor for the three representative runs. The growth curves for the batch run and the glucose change run are quite similar, the glucose change producing somewhat higher viable cell numbers. By contrast, the glutamine change run displays not only a much lower maximum number of viable cells, but also a prolonged growth period. These differences are reflected in the apparent growth rates of the cultures, shown in fig. 7.8. The apparent growth rate (called just growth rate below) is based on the viable cell number, and is therefore the sum of the real growth rate and the death rate (see appendix 1). A positive apparent growth rate indicates an overall growth of the cells, while a negative number
Fig. 7.7. Viable cell densities for three representative cultures.
Fig. 7.8. Apparent growth rates for three representative cultures.
indicates an overall death of the culture. In fig. 7.8 it can be seen that the average growth rate of the glucose change run is comparable to that of the batch run, while the growth rate of the glutamine step-change runs is clearly lower at the beginning of the run, although the decline is slower.

The other factor governing the antibody production is the specific antibody production rate, which is shown in fig. 7.9. Here another difference between glutamine change runs and glucose change runs is visible; the specific antibody productivity of a glutamine change run is significantly higher than that of a glucose change run, which in turn is somewhat higher than the reference batch run. However, as the volumetric antibody productivity is the product of the specific antibody productivity and the viable cell number, it is the specific productivity during the period of highest viable cell number that is most important. Fig. 7.10 shows the volumetric antibody productivities for the three runs, and summarizes the influence of the specific antibody productivity and the viable cell number. The explanation behind the antibody production is evident from this graph. The glucose step-change run has the same pattern of antibody productivity as the batch runs, but at a higher level. By contrast, the glutamine step-change run has a lower but prolonged volumetric productivity. The antibody production curves are the integral over time of the volumetric production curves, fig. 7.11. Both the dynamically operated runs reach the same final antibody concentrations in the reactor, but the glutamine change run achieves that level about two days later than in the glucose change run. The overall productivity, measured in mg antibody produced per day, is therefore higher for a glucose change run than for a glutamine change run (more on this below). Fig 7.12 summarizes the differences between the runs that have been outlined above, and shows how the different mechanisms for glucose and glutamine change cultures ends up producing the same final amount of antibody.

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Fig. 7.9. Comparison of specific antibody productivities for three representative cultures.
Fig. 7.10. Comparison of volumetric antibody productivities for three representative cultures.
Fig. 7.11. Comparison of antibody production for three representative cultures.
Fig. 7.12. Explanations of the antibody production data. Comparison in runtime of the behavioral patterns seen in the cultures. The data is from fitted curves of the data seen in fig 7.6 to 7.11.
7.3.2 Integral viability time analysis

The differences between batch reference runs, glucose change runs, and glutamine change runs shown above can perhaps be more comprehensively analyzed in $\tau$-space. By transforming the x-axis from runtime to $\int Xv \, dt$, the parts of the cultures where the cell numbers and productivities are highest are emphasized and compared. Also, as explained in the introduction to this chapter, in $\tau$-space the specific growth and production rates can be identified as the slopes of the curves. Fig. 7.13 shows the same viable cell number data as fig. 7.6, plotted against $\tau$. In this space the changes in nutrient concentrations come very early, within the first 5 units ($10^9$ cells/h) of the x-axis. The graph clearly shows the difference in cell numbers between the cultures, and from the slopes of the curves the difference in growth rates also is evident. The glucose change run achieves a higher viable cell number and final viability time from a prolonged growth period compared to the batch run, while the growth rate is the same. In contrast, the glutamine change run has both a lower growth rate and a shorter growth period in this space, resulting in lower total cell numbers and a lower final viability time, compared to the reference batch run. The growth rates are shown separately in fig. 7.14. The batch and the glucose change culture have essentially the same growth rate, apart from the variations in the beginning of the batch culture. The reason for prolonged growth period of the glucose change culture can be seen in the extended period of positive apparent growth rate, compared to the batch culture. It is interesting to note that the influence of the step change in the beginning of this culture extends up to this point. This may be an example of a dependence of these cells on the history of the culture. In fig. 7.14 the reduced growth rate of the glutamine culture also can be seen, which is the cause of the lower numbers for this culture.
Fig. 7.13. Comparison of viable cell numbers in τ-space for three representative cultures (see text for definitions).
Fig. 7.14. Comparison of apparent growth rate in $\tau$-space for three representative cultures (see text for definitions).
The differences in both antibody levels and productivities are evident in fig. 7.15, which shows the total antibody amounts in the reactor versus $\tau$. Both the dynamically operated runs reach higher antibody levels and display a higher specific productivity (the slope in fig. 7.15) than the reference batch run. From the slopes, especially at the beginning of the cultures, it is evident that the glutamine change run has the highest specific productivity. These differences are further emphasized in a plot of the specific productivities against the viability time, fig. 7.16. Here the central events determining the specific antibody productivity in the three runs is evident. Both the dynamically operated runs show a stimulation of the specific productivity shortly after the change in nutrient concentration, from limiting to non-limiting conditions. This stimulation is larger for a glutamine change culture than for a glucose change culture. After the nutrient concentration change, the stimulation decays with viability time, and eventually the specific antibody productivity of the dynamically operated runs approaches that of the batch run. The specific productivities are essentially the same shortly after the cultures have reached the peak in their viable cell numbers. Fig. 7.17 summarizes the data presented above, and shows how the specific antibody productivity contributes to the final antibody concentrations reached in the three processes.

7.4 Timing of nutrient concentration changes affects process performance

The data shown above suggest that there is a dependence of the antibody production levels on the timing of the nutrient concentration changes. This conclusion is further re-enforced by the result presented in table 7.2. Here the time of the introduction of the step- or ramp-changes in nutrient concentration is listed together with the viable cell densities in the reactor at that time. The timing of the concentration changes vary substantially. The objective of the experimental design was to introduce the changes at a cell density of
Fig. 7.15. Comparison of antibody production in $\tau$-space for three representative cultures (see text for definitions).
Fig. 7.16 Comparison of specific antibody production rates in $\tau$-space for three representative cultures (see text for definitions).
Fig. 7.17. Explanation of the antibody production data in $\tau$-space. The three behavioral patterns seen in the data. Data is from curves shown in fig. 7.14 to 7.16 (see text for definitions).
<table>
<thead>
<tr>
<th>Run Type</th>
<th>Run Number</th>
<th>Final antibody concentration (mg/L)</th>
<th>Final Integrated Cell Viability (10^9 cells*h)</th>
<th>Time of Nutrient Concentration Change (h)</th>
<th>Cell density at time of change (10^9 cells/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference runs and early/late step changes</td>
<td>91/3</td>
<td>68</td>
<td>484</td>
<td>22</td>
<td>0.2</td>
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<tr>
<td>Early glucose plus glutamine batch</td>
<td>89/1</td>
<td>82</td>
<td>420</td>
<td>22</td>
<td>0.18</td>
</tr>
<tr>
<td>Early glutamine step change</td>
<td>89/7</td>
<td>83</td>
<td>235</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Glacken fed-batch</td>
<td>89/6</td>
<td>87</td>
<td>181</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Glacken fed-batch</td>
<td>89/5</td>
<td>87</td>
<td>471</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
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<td>91/2</td>
<td>89</td>
<td>449</td>
<td>74</td>
<td>0.8</td>
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<tr>
<td>(Run 91/2: Very slow feed starting at 61 h, 0.7*10^9 cells/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Dynamically operated runs</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamine step change</td>
<td>90/5</td>
<td>88</td>
<td>298</td>
<td>56</td>
<td>0.3</td>
</tr>
<tr>
<td>Glucose + Glutamine step change</td>
<td>90/7</td>
<td>107</td>
<td>502</td>
<td>50</td>
<td>0.25</td>
</tr>
<tr>
<td>Low glucose step change</td>
<td>90/8</td>
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<td>563</td>
<td>33</td>
<td>0.33</td>
</tr>
<tr>
<td>Glucose + glutamine step change</td>
<td>91/1</td>
<td>115</td>
<td>337</td>
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<tr>
<td>Glutamine ramp change</td>
<td>90/2</td>
<td>118</td>
<td>392</td>
<td>48</td>
<td>0.25</td>
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<tr>
<td>Glucose step change</td>
<td>90/6</td>
<td>120</td>
<td>517</td>
<td>32</td>
<td>0.5</td>
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<tr>
<td>Glutamine step change</td>
<td>90/1</td>
<td>128</td>
<td>373</td>
<td>38</td>
<td>0.3</td>
</tr>
<tr>
<td>Glucose + glutamine ramp change</td>
<td>90/9</td>
<td>132</td>
<td>475</td>
<td>35</td>
<td>0.32</td>
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<tr>
<td>Other reference runs</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Batch, 5% FCS</td>
<td>89/2</td>
<td>112</td>
<td>730</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Batch 0.6 L spinner flask culture</td>
<td>91/4</td>
<td>133</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

Table 7.2. The performance parameters of the cultures, listed together with the timing and cell densities for the nutrient concentration changes.
about $0.3 \times 10^9$ cells/L. A secondary objective was to observe a reduction in growth rate before the introduction of the concentration change, although this could not always be achieved. The investigation of timing effects was limited to establish that there is a dependence on the change timing. Again three patterns are emerging. Although there is some variation in the results, in general a glutamine change culture displays a longer lag period and slower growth during the nutrient limitation period before the change has occurred, compared to the glucose change cultures. Cells that are limited for glutamine appears to decrease their growth rate more than cells limited for glucose. It is possible that cells are able to compensate for a for a glucose limitation by utilizing glutamine, more readily than in the reverse case. The results in table 7.2 also suggest that the height of the nutrient concentration change is not important. The size of the nutrient concentration change is defined as the difference in nutrient concentration just before and just after the change. This is explained in a separate section.

**7.4.1 Nutrient concentration change timing**

The effect nutrient concentration change timing on process performance is evident from the results shown in table 7.2. In cultures where the concentration change was introduced very early or too late in the culture, there is no improvement in antibody productivity. This was examined in three cultures, runs 91/3, 89/1, and 91/2. The results from the early step changes in glutamine concentration (run 89/1) and in glucose plus glutamine concentration (run 91/3) indicate that if the change is introduced too early in the culture, there is no increase seen in antibody productivity. However, as shown below, the glucose plus glutamine step-change run (91/3) has a metabolic pattern consistent with a normal glucose change culture, with high final concentrations of lactate and ammonia. Thus, it is appears possible to affect the metabolic rates of the cells, without affecting antibody productivity.
This also may be an explanation for the low antibody production of run 90/5. This glutamine step-change culture has cell growth and metabolism patterns similar to those of other glutamine change cultures. The low antibody production may therefore be the result of suboptimal timing of the nutrient concentration step.

There is no improvement in antibody production if the nutrient concentration change is introduced too late; this is seen from the result of run 91/2. In this glucose plus glutamine step-change culture the concentration change was introduced very late, after more than one third of the growth phase. To maintain the cell viability in this culture, a very slow feed was maintained before the step-change. During this period the cells were still limited for nutrients as evidenced by a reduced growth rate. This culture did not display either elevated antibody production or elevated metabolic rates. In addition, the glutamine limited cultures used as a reference process here may be viewed as dynamic glutamine change cultures with an extremely late step-change in glutamine concentration. In these cultures, a low feed of glutamine is maintained until the cells are close to their maximum cell density, then the glutamine concentration is stepped up to 10 mM, the same set-point as the high concentration set-point used in glutamine change cultures. The fact that these cultures do not show increased antibody production supports the other findings presented here.

Taken together, this data indicate that there is a window in time when a change in nutrient concentration may affect the antibody production of the culture. If a change is introduced too early, it has no effect on antibody production. If it is introduced too late, it also has no effect on antibody production. The reasons underlying this correlation are not clear. In the case of an early nutrient concentration change, it may be speculated that the cells have not experienced a nutrient limitation long enough to influence the production of secreted proteins. In the case of a prolonged nutrient limitation period, it is possible that the state
and metabolism of the culture is negatively affected. The variation in the final antibody concentrations between different glucose change cultures, and different glutamine cultures, may be an indication that there is an optimal time period to limit a culture for nutrients, before the concentration is increased. In the experiments presented there was a substantial difference in the growth rates at the time of the concentration change, as well as in the time the cells have been subjected to a nutrient limitation. It is therefore possible that in the cultures showing the highest final antibody concentration the cells were subjected to the nutrient concentration change at a more appropriate time than in other cultures. The possibility to optimize change timing and culture performance will be discussed further in the chapter on suggested future work.

7.4.2 Nutrient concentration change levels

In the results presented here there is no apparent difference in the behavior in cultures with step or ramp changes in nutrient concentration. In table 7.3 the results of the high producing dynamically operated step- or ramp-change cultures have been listed separately. For instance, the glutamine ramp-change culture 90/2 behaves the same as the glutamine step change culture 90/1. Further, the height of a nutrient concentration step-change does not appear to be of importance, with the ranges investigated here. In run 90/8 a lower step-change of glucose concentration was tried. Here the glucose concentration after the change was controlled at 10 mM, instead of 30 mM as used in other cultures. This had no apparent influence on the culture performance.

The reasons that neither ramp changes or lower step-changes display any different behavior, is probably that the nutrient concentration differences used in this investigation are much larger than the difference between the real limiting and non-limiting concentrations for these
<table>
<thead>
<tr>
<th>Run Type</th>
<th>Run Number</th>
<th>Final antibody concentration (mg/L)</th>
<th>Maximum Viable Cell Number (10^9 cells)</th>
<th>Maximum Total Cell Number (viable+dead) (10^9 cells)</th>
<th>Final Integrated Cell Viability Time (Xv dt) (10^9 cells*h)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dynamically operated step-change runs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose + Glutamine step change</td>
<td>90/7</td>
<td>107</td>
<td>4.07</td>
<td>5.03</td>
<td>502</td>
</tr>
<tr>
<td>Low glucose step change</td>
<td>90/8</td>
<td>111</td>
<td>4.51</td>
<td>6.07</td>
<td>563</td>
</tr>
<tr>
<td>Glucose + glutamine step change</td>
<td>91/1</td>
<td>115</td>
<td>4.24</td>
<td>5.30</td>
<td>337</td>
</tr>
<tr>
<td>Glucose step change</td>
<td>90/6</td>
<td>120</td>
<td>4.58</td>
<td>5.40</td>
<td>517</td>
</tr>
<tr>
<td>Glutamine step change</td>
<td>90/1</td>
<td>128</td>
<td>2.27</td>
<td>3.88</td>
<td>373</td>
</tr>
<tr>
<td><strong>Dynamically operated ramp-change runs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamine ramp change</td>
<td>90/2</td>
<td>118</td>
<td>2.68</td>
<td>4.26</td>
<td>392</td>
</tr>
<tr>
<td>Glucose + glutamine ramp change</td>
<td>90/9</td>
<td>132</td>
<td>3.23</td>
<td>4.96</td>
<td>475</td>
</tr>
</tbody>
</table>

Table 7.3. Comparison of performance parameters between step-change runs and ramp-change runs. Some data is in total amounts in reactor (final volume is 2 L, to get concentrations, divide amounts by 2).
cells. Thus, even during the slower nutrient concentration changes occurring during a ramp change the cells probably will go from limiting to non-limiting conditions within much shorter time frames than their response times. The typical time constant for response of mammalian cells to changes in culture conditions is on the order of 4 to 24 h (Glacken 1987, Miller et. al. 1987, 1988, 1989 a and b; own observation and personal communications). The Monod constant for growth limitation of CRL 1606 is about 0.15 mM for glutamine and about 0.2 mM for glucose, which means that at concentrations above 1 mM there will be almost no change in growth rates with increased nutrient concentration. The step changes used in this investigation are completed in less than two hours, and even for the ramp changes used the change in nutrient concentration exceeds 1 mM within 24 h. Therefore, the cells will not see much difference between a ramp change and a step change, nor between step changes of different heights. The cell metabolism is probably saturated for the limiting nutrient in a few hours after the increase in its concentration. From this perspective, it has to be concluded that the design for ramp change cultures used here did not work. To investigate possible effects from stepping or ramping up nutrient concentrations within a range where there is a notable influence on growth rate and other parameters, in the normal response time of the cells, a different type of experiment has to be designed. However, this may be quite difficult, as the nutrient concentrations has to be kept within tight limits for at least 12 h, the apparent minimal response time for the culture, in order to be able to distinguish the effect of different concentration levels.

7.5 Antibodies are not stored intracellularly
The cellular mechanism responsible for the increased specific antibody productivity seen in dynamically operated runs is not known. Antibodies are believed to be constitutively secreted by plasma cells, and not stored in any intra-cellular vesicles. Nevertheless, one
possible explanation for the increased specific antibody productivity in response to nutrient concentration changes could be a release of antibody or antibody sub-chains from intracellular stores, as opposed to increased production of antibody. To investigate this possibility, the cells from samples taken throughout the course of runs where centrifuged out of the media and stored separate from the supernatant. The cells in the cell pellet where subsequently lysed in a mild lysing buffer, known not to denature proteins (G. Grampp, personal communication), and the lysed cell suspension were analyzed for antibodies using the same α-IgG CIA assay as was used for the analysis of the supernatant. Fig. 7.18 shows the results of such an analysis for a representative culture. Only a minute fraction of the antibodies are found in the cell pellet, at no time does it exceed 25 parts per million. From these results it can be concluded that the increased antibody productivity seen in the dynamically operated fed-batches is due to an increased production and secretion of antibody, and not due to a release of stored antibody from live or dying cells. Similar results have been published elsewhere (Coco-Martín et al., 1991).

7.6 CRL 1606 hybridomas secretes chimeric antibodies

The CRL 1606 hybridoma secretes chimeric antibodies, consisting of two different antibody heavy chains, and two different light chains. Lanes D to F and G to I in fig. 7.19c shows an immunoblot of reduced samples from two different runs, from different runtimes during these runs. The upper bands are the two heavy chains (marked with α-FN Heavy and MOPC21 Heavy respectively), and the two lower bands are the light chains. Lane A in fig 7.19c shows a sample of purified MOPC 21 protein (Sigma, St. Louis, MO), secreted from the X-63 myeloma cell line (Harlow and Lane, 1988; Horibata and Harris, 1970; Köhler and Milstein, 1975), the myeloma parent of the CRL 1606 hybridoma (Schoen, Bentley, and Klebe 1982). As can be seen, the migration rate of these bands matches that of the lower of
Fig. 7.18. Analysis of antibody present in cells. Cells from reactor samples were separated from the medium by centrifugation, and subsequently lysed with a mild buffer. The lysis suspension was analyzed for antibody using the α-IgG CIA immunoassay. The left y-axis is the total antibody present in the reactor medium. On the right y-axis the total antibody present in the cell pellet is plotted, as well as the total antibody in the cell pellet as a fraction of the total antibody in the reactor (in percent). The total antibody in the supernatant, and the total antibody in the cell pellet, are shown on separate scales.
Figure 7.19 a to d.

Immunoblots and Coomassie stain of 5-15% SDS-PAGE gels of samples from two different runs, taken at three times during these runs. Immunoblots probed with Rabbit-anti-Mouse IgG Heavy and Light Chain alkaline phosphatase conjugate.

Fig. 7.19a. Immunoblot of non-reduced samples.

Fig. 7.19b. Coomassie stain of non-reduced samples.

Fig. 7.19c. Immunoblot of reduced samples.

Fig. 7.19d. Coomassie stain of reduced samples.

Lanes are:

MW: Molecular weight markers
A: Purified preparation of MOPC 21 protein from the X-63 myeloma.
B: Whole mouse serum control
C: Control; Culture medium with 2.5% foetal calf serum, not inoculated with cells.
D: Sample from middle of a batch run with 2.5% FCS (run 89/5), runtime 81 h.
E: Sample from late in a batch run with 2.5% FCS (run 89/5), runtime 105 h.
F: Sample from end of a batch run with 2.5% FCS (run 89/5), runtime 163 h.
G: Sample from middle of a glucose plus glutamine step change run (run 90/7), runtime 103 h.
H: Sample from late in a glucose plus glutamine step change run (run 90/7), runtime 136 h.
I: Sample from end of a glucose plus glutamine step change run (run 90/7), runtime 240 h.
Fig. 7.19a

Fig. 7.19b

Fig. 7.19c

Fig. 7.19d
both the heavy and the light chains bands. The upper of the heavy and light chain bands are thus derived from the spleen cell parent of the hybridoma. The molecular weights of the MOPC 21 chains are 52 and 27 kdalton, and those of the spleen cell chains are 57 and 28 kdalton, as measured from reduced (glycosylated) samples, in 5-15 % SDS-PAGE gradient gels.

The antibodies secreted by CRL 1606 are scrambled chimeric antibodies, i.e. they are assembled from all possible combinations of the different heavy and light chains. In addition, this hybridoma secretes both incompletely assembled antibodies, consisting of two heavy chains and one light chains, as well as heavy chain dimers and free heavy and light chains. Fig. 7.20 shows the composition of a mouse IgG antibody, and fig. 7.21 shows the possible combinations of antibody chains. In table 7.4 the different combinations of antibodies and their molecular weights are listed, predicted from the measured molecular weights of the heavy and light chains. Proof of the nature of the secreted antibodies comes from several lines of evidence; streaks of antibody band in immunoblots of non reduced samples, estimation of migration rates of different antibody chain combinations, immunoprecipitation of antibodies, and 2-dimensional gels.

7.6.1 Non-reducing immunoblots

Lanes D to I in fig. 7.19a show unreduced preparations of the same samples used in fig. 7.19c. The free secreted heavy and light chains are clearly visible at the lower part of the gel (marked H and L), while the complete and incomplete antibodies, and the heavy chain dimers, form a continuous streak in the upper part of the gel. The streak is further made up of three major groups of bands, which are composed of complete antibodies.
Composition of an Mouse IgG antibody.
Molecular weight about 150 kdaltons

Reducing Agent;
Mercapto-Ethanol C2H5-SH

Free heavy chain.
Molecular weight about 50 kdaltons

Free light chain
Molecular weight about 25 kdaltons

Fig. 7.20. Composition of, and reduction products from a mouse IgG antibody.
Fig. 7.21. Possible combinations of antibody chains in chimeric antibodies
### Molecular weights

<table>
<thead>
<tr>
<th></th>
<th>Heavy chain</th>
<th>Light chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen cell cha</td>
<td>57 = H</td>
<td>28 = L</td>
</tr>
<tr>
<td>MOPC 21 cha</td>
<td>52 = h</td>
<td>27 = l</td>
</tr>
</tbody>
</table>

Table 7.4a. Molecular weights in kdaltons for the different antibody chains secreted by the CRL 1606 hybridoma, and their designation in table 7.4b.

### Antibody chain combination

<table>
<thead>
<tr>
<th>Antibody chain combination</th>
<th>Predicted Molecular Weights (kilodaltons)</th>
<th>Active fibronectin binding antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2L2</td>
<td>170</td>
<td>Active</td>
</tr>
<tr>
<td>H2L1</td>
<td>169</td>
<td>Active</td>
</tr>
<tr>
<td>H2L2</td>
<td>168</td>
<td></td>
</tr>
<tr>
<td>hHL2</td>
<td>165</td>
<td>Active</td>
</tr>
<tr>
<td>hHL1</td>
<td>164</td>
<td>Active</td>
</tr>
<tr>
<td>hHl2</td>
<td>163</td>
<td></td>
</tr>
<tr>
<td>hhL2</td>
<td>160</td>
<td></td>
</tr>
<tr>
<td>hhL1</td>
<td>159</td>
<td></td>
</tr>
<tr>
<td>hhl2</td>
<td>158</td>
<td></td>
</tr>
<tr>
<td>H2L</td>
<td>142</td>
<td>Active</td>
</tr>
<tr>
<td>H2I</td>
<td>141</td>
<td></td>
</tr>
<tr>
<td>hHL</td>
<td>137</td>
<td>Active</td>
</tr>
<tr>
<td>hHl</td>
<td>136</td>
<td></td>
</tr>
<tr>
<td>hhL</td>
<td>132</td>
<td></td>
</tr>
<tr>
<td>hhl</td>
<td>131</td>
<td></td>
</tr>
<tr>
<td>H2</td>
<td>114</td>
<td></td>
</tr>
<tr>
<td>Hh</td>
<td>109</td>
<td></td>
</tr>
<tr>
<td>h2</td>
<td>104</td>
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</tr>
<tr>
<td>H</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>h</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>l</td>
<td>27</td>
<td></td>
</tr>
</tbody>
</table>

Table 7.4b. Molecular weights for different antibody chain combinations, sorted by molecular weight. The chains are assumed to combine in all possible ways. A spleen cell heavy and light chain bound to each other can form an active fibronectin binding site. Multiple chain combinations with same molecular weights are not shown.
(marked H₂L₂ in fig. 7.19), incomplete antibodies (marked H₂L), and heavy chain dimers (marked H₂), from top to bottom. In addition, fig. 7.19b and 7.19d show the same samples as in fig. 7.19a and 7.19c, in Coomassie stained reduced and non-reduced gels. The dominant band in these samples is bovine serum albumin. Weaker bands of immunoglobulin (Ig) chains, both mouse and bovine, are visible in fig. 7.19d, and weaker bands of complete antibodies (marked IgG) are visible in fig. 7.19b.

7.6.2 Predicted migration distances

Further clues to the composition of the antibodies in the streak on the immunoblots can be derived from predictions of migration distances of different antibody chain combinations. Lanes MW to C in fig. 7.22a shows immunoblots of reduced molecular weight markers, a non-reduced antibody sample, and two immunoprecipitated antibody preparations, respectively. Unfortunately, the migration rates of the reduced molecular weight markers correlate poorly with the migration rates of non-reduced proteins on such a blot. However, the free heavy and light chain bands are clearly identifiable in lane A, and it known that the top of the antibody streak corresponds to the heaviest antibody, which consists of the two heaviest heavy chains, and the two heaviest light chains, and has a molecular weight of about 170 kdalton. From the migration distances of these known bands on this gel, the migration distances for all other possible antibody chain combinations can be estimated. Lane D shows the predicted position of these molecules on this gel, while lanes F identifies the predicted position of each separate antibody molecule. As can be seen, the predicted migration distance of the main clusters in lane D of the blot corresponds very well with the predicted position of the complete antibodies, incomplete antibodies, and the heavy chain dimers.
Figure 7.22 a and b.

Composite figure of immunoblots and calculated position of immunoglobulin molecules, as predicted by the migration distances of known bands on the immunoblots, as well as active chimeric antibodies, i.e. antibodies that are able to bind to fibronectin. Immunoblots probed with Rabbit-anti-Mouse IgG heavy and light chain alkaline phosphatase conjugate.

Fig 7.22a. Immunoblot and predicted migration distances of a 5-15 % SDS-PAGE gradient gel with non-reduced samples. Lanes are:

MW: Molecular weight markers.
A: Sample from end of a glucose plus glutamine step change run (run 90/7).
B: Same sample as in A, immunoprecipitated with latex beads coated with a Goat-anti-Mouse kappa light chain antisera. Antibodies released from beads by boiling.
C: Same sample as in A, immunoprecipitated with latex beads coated with fibronectin. Antibodies released from beads by boiling.
D: Predicted migration distances of all chimeric antibodies, including incompletely assembled antibodies.
E: Predicted migration distances of active chimeric antibodies, i.e. antibodies that are able to bind to fibronectin, including incompletely assembled antibodies.
F: Individual lanes of predicted migration distance for each chimeric antibody.
G: Individual lanes of predicted migration distance for each active chimeric antibody.

Fig. 7.22b. Same samples as in lane MW to C in fig. 7.22a, run under reducing conditions in a 10-15% SDS-PAGE gel.
7.6.3 Immunoprecipitation

Lane B and C in fig. 7.22a shows immunoblots of antibody samples that have been immunoprecipitated with latex beads with bound anti-mouse IgG, and with bound fibronectin, respectively (see materials and methods). Samples immunoprecipitated with anti-Mouse IgG beads, lane B, show only one major band, from complete antibodies, perhaps indicating that the antisera immobilized on the beads react predominately with complete assembled antibodies. Samples immunoprecipitated with beads coated with fibronectin, lane C, show three major bands, and a much weaker lower band. The chimeric antibodies can form a total of six different active antibodies, i.e. antibodies that retain an active fibronectin binding site. These antibodies are also shown in table 7.4, and lane E in fig. 7.22a show the predicted migration distances of these active antibodies, while lanes G show the predicted distance of each active antibody molecule. These migration distances are to a large extent consistent with the bands shown in lane C, while the fourth, lower band could be explained as co-precipitated heavy chain dimers. Fig. 7.22b shows the same samples as in fig. 7.22a, run under reducing conditions. As can be seen in lane C in fig. 7.22b, a sample immunoprecipitated with fibronectin coated beads contain mostly the fibronectin binding antibody chains stemming from the spleen cell parent of the hybridoma.

7.6.4 2-dimensional immunoblots

Lastly, evidence of the composition of the chimeric antibodies comes from 2-dimensional gels, with the first dimension being a non-reduced gel, and the second dimension run under reducing conditions. Fig. 7.23 shows an example of an immunoblot of such a gel, and fig. 4.4 shows how this gel was produced (see materials and methods). In the upper part of the figure is a blot of a part of the first dimension gel, run under non-reducing conditions, along with molecular weight markers. The streak of chimeric antibodies are clearly
identifiable, as are the bands for free heavy chains, while the free light chains are barely visible. A strip of the sample lane from the first dimension gel was soaked in DTT for 30 min to reduce the proteins in the gel, and thus separate the antibody chains from each other. This strip was electrophoresed perpendicular to the first dimension direction under reducing conditions, along with lanes for molecular weight markers and a reduced sample of a MOPC 21 antibody preparation. In the 2-D blot, the series of spots of heavy and light chains stemming from the compete antibodies are marked H, h, L and l respectively. The two lower spots below the H and h markers are artifacts, resulting from proteins that has migrated with the chlorine ion front in the stacking gel used (see materials and methods), and that were not focused properly at the top of the resolving gel. The spots stemming from incompletely assembled antibodies, consisting of one light chain and two heavy chains, are visible to the right of the leftmost heavy and light chain spots. The heavy chain spots further to the right of these are stems from the heavy chain dimers, as is evidenced by the fact that there are no corresponding spots for light chains in this position. The spots resulting from free heavy and light chains are barely visible on the blot. This 2-D analysis shows that the upper portion of the first dimension antibody streak consists of both heavy and light chains, as does the middle portion, while the lower portion only consists of heavy chains. Also, a diagonal displacement of spots between the heavier and lighter molecules of both the heavy and light antibody chains is clearly visible. This displacement is the result of the faster migration in the first dimension gel of molecules made of increasingly lighter chains.

7.6.5 Low molecular weight immunoblots

To investigate if there are any low molecular weight antibody fragments present in the reactor samples, an immunoblot was done from a gel run according to the method of
Fig. 7.23. 2-Dimensional immunoblot of sample from end of a glucose plus glutamine step change run (run 90/7), prepared as described under methods, see also fig. 4.4. 1st dimension run under non-reducing conditions in a 5-10% SDS-PAGE gel, together with molecular weight markers (MW). A strip of this sample was blotted on the top of the blot (marked Sample) as a record of the first dimension. A second strip from the first dimension gel was cut out, soaked in reducing agent (DTT) for 30 min, and run in the 2nd dimension in a 10-15% SDS-PAGE gel under reducing conditions, together with molecular weight markers (MW) and a preparation of the MOPC 21 antibody. The blot was immunodetected with a Rabbit-anti-Mouse IgG Heavy plus Light Chain alkaline phosphatase conjugate.
Fig. 7.23
Schräger and von Jagow (1987). No low molecular weight components able to bind the antisera used for probing were detected (data not shown).

7.7 Dynamic operation does not affect antibody quality

While there is a substantial difference in final antibody concentrations between dynamically operated cultures and batch cultures, there is no detectable difference in antibody quality as determined by probing immunoblots of reducing and non-reducing SDS-PAGE gels with different antibodies. Nor is there any significant difference in glycosylation levels of antibodies, judging from immunoblots of samples treated with N-glycosidase F. This investigation of antibody quality is however limited as the methods used are intended to examine broad differences. There are some indications that culture method affect antibody quality. There was for instance an unspecific binding seen in some immunoassays, predominately for samples from glucose plus glutamine change cultures. Further investigation of the relationship between product quality and the bioreactor operating protocol is therefore warranted (see suggested future research).

7.7.1 Immunoprobing with different antibodies

Fig 7.19, and 7.22 above are examples of immunoblots of samples from different bioreactor runs probed with a restrictive antisera, highly specific for mouse antibody chains (Biorad Rabbit-anti-Mouse IgG Heavy and Light chains). Further probing of final samples from all bioreactor runs with this antisera did not reveal any significant difference in band number and composition between the different runs, although high producing runs did display somewhat heavier immunoglobulin (Ig) bands, consistent with the observations from the immunoassays (data not shown). Probing of immunoblots of a number of samples from
different bioreactor runs with less restrictive antisera also failed to detect any significant
difference in antibody composition and quality. Fig. 7.24 a and b show non-reduced
samples from a series of different runs probed with two less restrictive antisera. The
immunoblot in fig. 7.24a has been probed with a Rabbit-anti-Mouse IgG F(ab')\(_2\) antisera,
the same antisera used in the RID assays (see above). The blot shown in fig. 7.24b has
been probed with a Goat-anti-Mouse kappa light-chain antisera, the same antisera that is
bound to the latex beads used in the Origen anti-IgG CIA immunoassay. As can be seen,
neither of these antisera is very restrictive for IgG's, and they bind to a number of non-Ig
bands. However, while some bands in some samples bind more heavily than others, there
are no unique bands in any samples, and there appears to be no correlation between band
intensities and run-types. The only significant difference between samples that has been
detected in this analysis is seen in fig. 7.24c, which shows the same samples used in fig.
7.24 a and b, but this time reduced, and probed with the Rabbit-anti-Mouse IgG F(ab')\(_2\)
antisera. Reducing the proteins in the samples eliminates a lot of the non-Ig cross reactions,
and only one major non-Ig band around 130 kdalton is seen, apart from some minor bands.
In lanes L to O this band is much weaker than in the other samples, and these lanes all
contains samples from glucose plus glutamine fed-batches. However, lanes K and P also
contain samples from glucose plus glutamine fed-batches, and here the 130 kdalton band is
as strong as in the other lanes.

7.7.2 Antibody glycosylation

Treatment of samples from bioreactor runs with N-glycosidase F, which digest glycoside
side chains on proteins, reveal that the heavy chains of the antibodies are glycosylated, while
the light chains are not, as expected (Harlow and Lane, 1988). Fig. 7.25 show
Fig. 7.24. Immunoblots of samples from several different bioreactor runs probed with less restrictive antisera. Fig. 7.24 a. Immunoblot of a non-reducing 5-15% SDS-PAGE gel probed with a Rabbit-anti-Mouse IgG F(ab')\textsubscript{2} antisera. Fig. 7.24 b. Immunoblot of a non-reducing 5-15% SDS-PAGE gel probed with a Goat-anti-Mouse Kappa Light Chain antisera. Fig. 7.24 c. Immunoblot of a reducing 5-15% SDS-PAGE gel probed with a Rabbit-anti-Mouse IgG F(ab')\textsubscript{2} antisera. Lanes are:

MW: Molecular weight markers.
A: Control; Medium with 2.5% FCS, not inoculated with cells.
B: Sample from end of a batch run with 5 % FCS (run 89/2).
C: Sample from end of a batch run (run 89/5).
D: Sample from end of a 600 ml spinner batch culture (run 91/4).
E: Sample from end of a Glacken fed-batch run (run 89/6).
F: Sample from end of a early glutamine concentration step change run (run 89/1).
G: Sample from end of a glutamine concentration step change run (run 90/5).
H: Sample from end of a glutamine concentration ramp change run (run 90/2).
I: Sample from end of a glucose concentration step change run (run 90/6).
J: Sample from end of a low glucose concentration step change run (run 90/8).
K: Sample from end of a glucose plus glutamine concentration step change run (run 90/7).
L: Sample from end of a glucose plus glutamine concentration step change run (run 91/1).
M: Sample from end of a late glucose plus glutamine concentration step change run (run 91/2).
N: Sample from end of an early glucose plus glutamine concentration step change run (run 91/3).
O: Sample from end of a low glucose plus glutamine concentration step change run (run 90/12).
P: Sample from end of a glucose plus glutamine concentration ramp change run (run 90/9).
Fig. 7.25. Immunoblot of untreated reduced samples, and the same samples treated with N-Glycosidase F to remove attached glucoside residues. Blot of a 10-15% SDS-PAGE gel immunodetected with Rabbit-anti-Mouse IgG Heavy and Light Chain alkaline phosphatase conjugate.

Lanes are:

MW: Molecular weight markers.

A: Untreated sample from end of batch run (run 89/5).

A: N-Glycosidase F treated sample from end of batch run (run 89/5).

C: Untreated sample from end of glutamine concentration step change run (run 90/5).

D: N-Glycosidase F treated sample from end of glutamine concentration step change run (run 90/5).

E: Untreated sample from end of glucose plus glutamine concentration step change run (run 90/7).

F: N-Glycosidase F treated sample from end of glucose plus glutamine concentration step change run (run 90/7).

G: Untreated sample from end of late glucose plus glutamine concentration step change run (run 91/2).

H: N-Glycosidase F treated sample from end of late glucose plus glutamine concentration step change run (run 91/2).
both untreated samples, and samples digested with N-glycosidase F, run under reducing conditions. As can be seen, treating the sample with glycosidase result in faster migration of the heavy chains, due to the removal of glucoside residues bound to this chain. The level of glycosylation of different samples appears to be quite homogenous, as judged by both absolute migration distances, and the increase in migration distance resulting from glycosidase digestion. Further, glycosidase treated samples run under non-reducing conditions do not reveal any significant difference in composition (data not shown).

7.8 Dynamic operation affects cell metabolism

The nutrient limitations and changes in nutrient concentrations that cells go through in the dynamic operation of a bioreactor significantly affect their metabolism. Three main patterns can be distinguished: batch reference cultures, glucose change cultures, and glutamine change cultures. Glucose plus glutamine change cultures behave mostly as a glucose or as a glutamine change culture as seen for the viable cell results above, although there is some variation in the data. A thorough investigation and explanation of the metabolic effects in these cultures could fill a thesis on its own. Here two main points will be made. First, dynamic operation of the culture affects the formation of metabolic waste products, although for different reasons in different types of operation. This has implications for the waste product concentrations in the reactor, which will be explained in more detail in a separate section. Second, there are large increases in the uptake rates of the limiting nutrient at the time of a step or ramp change, and these effects have an impact on the cultures metabolism for a significant part of the culture. In essence, it appears that the state of the cell during the nutrient limitation period before the concentration change sets a pattern for the metabolism that persist for most of the culture time after the nutrient concentration change has occurred, slowly decaying towards the rates seen for a normal batch culture.
The overall effects on cell metabolism from dynamic operation can be conveniently analyzed in $\tau$-space. As explained in the introduction to this chapter, in this space the slope of curves of total amounts in the reactor are equal to the specific production or uptake rate of the compound. Both absolute levels and specific rates can be visualized in one graph. The same three cultures used to exemplify the antibody production patterns above will be used here to exemplify the metabolic changes (an additional glutamine step-change culture will be used to show oxygen uptake result). Although there is some deviations in the data for other runs, they all fall within the basic categories described here. Next, the events at the time of a change in nutrient concentrations are exemplified using a glucose change culture. The effects seen in a glutamine change culture are quite similar, and a glucose plus glutamine change cultures will exhibit one main limiting nutrient. For this example the focus is on the early stages of the culture, at a time when viable cell numbers are quite low. It is therefore more illustrative to use runtime as the basis for this analysis.

### 7.8.1 Dynamic operation alters the overall metabolism of cultures

The batch culture is used as the reference in the analysis of metabolism resulting from glucose changes and glutamine changes. In summary, a glutamine change culture exhibits lower metabolic activity than a batch or a glucose change culture, consistent with the lower cell growth of this culture. The glutamine change culture also uses more glutamine after the nutrient concentration change, leading to an increased level of ammonia formation. Although a glucose change culture has a similar level of metabolic activity as a batch culture, the metabolism is shifted toward a more anaerobic state, with a corresponding higher production of lactate. A glucose change culture also utilizes more glutamine than the batch culture, resulting in higher concentrations of ammonia.
Fig. 7.26 shows the accumulated oxygen uptake of three representative cultures. (The glutamine step-change culture (90/1) used to explain the antibody production data had a failure of the oxygen uptake rate measurement program. Here data from another glutamine change culture (90/5) is used instead. The metabolic data for the cultures is very similar, but the glutamine change culture shown in fig. 7.26 had low antibody production for reasons not understood. The oxygen uptake rate data for this culture is much more frequent than for the other glutamine step-change culture (90/1) and it therefore visualizes the important effects better). The accumulated oxygen uptake is the integral of oxygen uptake at \( \tau \), i.e.

\[A_{\text{O}_2}(\tau) = \int \text{OUR} \, dt.\]

The batch culture uses significantly more oxygen than the dynamically operated cultures, at a higher rate throughout the course of the culture. Also the glucose uptake rate of the batch culture is higher than for glutamine change or glucose change cultures, fig. 7.27. In contrast, the lactate production rate of a glucose change culture, fig. 7.28, is higher than that for a batch or a glutamine change culture. Thus, from these results it can be concluded that the batch culture has a more aerobic metabolism than a glucose change culture. Further indications as to the nature of the metabolism of the cultures can be seen in graphs of glutamine uptake and ammonia production, fig. 7.29 and 7.30. Both the glucose and the glutamine change culture have elevated glutamine uptake rates compared to the batch culture. Interestingly, the uptake rate for the glutamine change culture is especially high toward the middle of the culture, long after the step-change in nutrient concentration has occurred (the change is at \(5 \times 10^3 \text{ cell\cdot h}^{-1}\), or 38 h runtime). The higher glutamine uptake rate towards the middle of this culture is also reflected in the ammonia production data, fig. 7.30. The higher glutamine uptake rate of the glucose change culture is reflected in an elevated ammonia formation rate.
Fig. 7.26. Comparison of accumulated oxygen uptake in the reactor versus viability time $\tau$ for three representative runs.
Fig. 7.27. Comparison of total glucose uptake in the reactor versus viability time $\tau$ for three representative runs.
Fig. 7.28. Comparison of total lactate production in the reactor versus viability time $\tau$ for three representative runs.
Fig. 7.29. Comparison of total glutamine uptake in the reactor versus viability time $\tau$ for three representative runs.
Fig. 7.30. Comparison of total ammonia production in the reactor versus viability time $\tau$ for three representative runs
The level of oxygen, nutrient and waste product metabolism of the cultures can be used to estimate an ATP formation rate, given some basic assumptions (Fleischaker, 1982; Glacken 1985 and 1987). ATP can be generated from both lactate via the glycolysis pathway, and from the oxidation of NADH or FADH$_2$ by oxygen. The amount of ATP formed from the reduction of one mole of oxygen in these reactions can vary somewhat (Miller, Wilke, and Blanch, 1987). Under most circumstances one mole of NADH oxidized by 1/2 moles of oxygen will result in the formation of three mole of ATP. The amount of ATP generated from the oxidation of FADH$_2$ can vary more, but generally one mole of FADH$_2$ is oxidized with 1/2 mole of oxygen to form only two moles of ATP. However, in the citric acid cycle the formation of one mole of FADH$_2$ occurs simultaneously with the formation of one mole of GTP. The generation of GTP is equivalent to the generation of ATP, as the high energy phosphate group of the GTP can be transferred to ADP without significant energy loss via nucleoside diphosphate kinase. Thus, the overall result of oxidative phosphorylation of NADH and FADH$_2$ is the generation of 6 moles of ATP for each mole of oxygen consumed. As one mole glucose yields two mole of ATP for two mole of lactate formed in glycolysis, the ATP production rate could be calculated as

$$\text{ATP PR} = 6 \text{OUR} + 1 \text{LPR}$$

where OUR is the oxygen uptake rate, and LPR is the lactate production rate. However, in the metabolism of mammalian cells in culture, a significant part of the energy is also derived from glutamine. It is harder to assess the amount of ATP generated from glutamine anabolism (Glacken 1987). The NADH generated in glutamine metabolism will result in the formation of ATP via oxidative phosphorylation, yielding 6 moles of ATP per mole of oxygen consumed, no different from glucose metabolism. The problem is to estimate the amount of lactate derived from glutamine (Glacken, 1985, 1987). Lactate generated from glutamine does not contribute to ATP formation. The formation rate of ATP from
glutamine metabolism will be dependent on the relative flux of carbon through the pentose phosphate pathway and the glycolysis. It is not possible to correctly estimate these fluxes here as the true CO₂ evolution rate of the culture is not known. To account for the amount of lactate generated from glutamine, and thus not contributing to ATP formation, the expression for ATP generation can be written on a cellular basis as

\[ \dot{q}^{\text{ATP}} = 6 \dot{q}^{\text{O}_2} + k_{\text{GLN}} \dot{q}^{\text{LAC}} \]

where the \( q \)'s stand for specific production or uptake rates. \( k_{\text{GLN}} \) is a factor compensating for the amount of lactate generated from glutamine. In the literature, \( k_{\text{GLN}} \) has been reported to vary between 0 for cells grown on fructose (all lactate formed from glutamine) to close to 1 for cell with a significant glucose metabolism (Glacken, 1986, 1987 and 1991; Miller, Wilke, and Blanch, 1987). To estimate the ATP formation rates in the cultures here, a value of 0.5 has been assumed for \( k_{\text{GLN}} \). Several authors have used a value of 1 for this factor, assuming that glutamine does not contribute to lactate production (Glacken, 1986, and 1991; Miller, Wilke, and Blanch, 1987). However, CRL 1606 cells derives a significant part of their energy from glucose metabolism as evidenced by the glucose uptake rates. It is clear therefore that glutamine does contribute to lactate formation, although the exact extent is not known. A factor of 0.5 probably does not underestimate the influence of glutamine metabolism. To visualize the ATP formation rate during the course of a culture, it is informative to show the accumulated ATP formed over time, rather than to plot actual formation rates. This is simply the total amount of ATP formed at each point in time from the start of the culture, plotted against \( \tau \). Thus the accumulated ATP can be calculated as

\[ A^{\text{ATP}} = 6 A^{\text{O}_2} + k_{\text{GLN}} A^{\text{LAC}} \]

where \( A \) denotes the accumulated amount at each point in time. For lactate this is the same as the amount of lactate in the reactor at that time. \( k_{\text{GLN}} \) is assumed here to be 0.5 as
mentioned. Fig. 7.31 shows an example of such a graph (again data from the glutamine step change culture 90/5 has been used, as for the oxygen uptake graph). The slope of the curves are the specific ATP production rates. From this graph is can be seen that the metabolic activity (measured as ATP formation) is higher for the batch culture. The glucose change culture has a somewhat lower activity, while the metabolic activity from oxidative and glycolytic metabolism of the glutamine culture is clearly lower. The contribution of glycolysis to ATP formation may be underestimated in this graph. In the graph, $k_{GLN}$ was set to 0.5 for all cultures. It is likely that this factor is higher for a glucose change culture than for a glutamine change culture, which would further emphasize the lower metabolic activity of the glutamine change culture. This would also be consistent with the lower number of cells formed in the glutamine change culture. An idea of the yield of cells on ATP in the cultures can be obtained from the total amounts of cells and the total amount of ATP generated in the cultures, at the time the maximum number of total cells has been reached. Table 7.5 shows such a comparison for three representative cultures. The data seems to indicate that a glutamine change culture and especially a glucose change culture uses the available energy a bit more effectively for cell formation than a batch culture. However, it has to be remembered that most of the energy used by these cells goes to maintenance and futile cycles (Darnell, Lodish, and Baltimore, 1986), and this comparison may therefore not reflect the true yield factors involved.
Fig. 7.31. Comparison of calculated ATP production for three representative cultures (see text for calculation methods).
<table>
<thead>
<tr>
<th>Run Type</th>
<th>Run Number</th>
<th>Final antibody concentration (mg/L)</th>
<th>Integrated Cell Viability Time at max Total Cells (lxv dt) (10^9 cells*h)</th>
<th>Maximum Total Cell Number (viable+dead) (10^9 cells)</th>
<th>Accumulated ATP Formed at max Total cells (mmol)</th>
<th>Yield of cells on ATP 10^9 cells/mmol ATP formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch, 2.5% FCS</td>
<td>89/5</td>
<td>87</td>
<td>183</td>
<td>4.80</td>
<td>143.00</td>
<td>3.36E-02</td>
</tr>
<tr>
<td>Glutamine step change</td>
<td>90/5</td>
<td>88</td>
<td>130</td>
<td>3.33</td>
<td>88.00</td>
<td>3.78E-02</td>
</tr>
<tr>
<td>Glucose step change</td>
<td>90/6</td>
<td>120</td>
<td>200</td>
<td>5.40</td>
<td>130.00</td>
<td>4.15E-02</td>
</tr>
</tbody>
</table>

Table 7.5. Comparison of accumulated ATP production and cell yield on ATP for three representative cultures.
7.8.2 Nutrient concentration changes affect metabolic rates

A number of interesting events occur before and after the nutrient concentration changes in dynamically operated cultures. As cell metabolism has not been a focus of this investigation, these events are exemplified with a description of one culture, a glucose step change run. The effects seen in a glutamine change culture are similar, but not as dramatic. This type of metabolic effects are best visualized by analyzing specific metabolic rate data in runtime. Fig. 7.32a and b shows the nutrient uptake and waste product formation rates of the glucose change culture, plotted versus runtime. Before the step change in glucose concentration, the increasing limitation of the cells for glucose is visible by the decrease in the specific glucose uptake rate (at about 18 h runtime). The cells compensate for this limitation in part by utilizing more glutamine, as seen in the specific glutamine uptake rate. These effects also are evident in the waste product formation. The lactate production rate decreases, while the ammonia formation rate increases. Just before the glucose step-change an increase in the glucose consumption rate is seen. The reason for this is not clear. It may be an artifact of the measurements, as this increase is determined from only two data-points. It is also possible that the cells switch back to higher glucose utilization as they start to utilize more oxygen at this point. This latter explanation is consistent with the ammonia and oxygen uptake data. The specific oxygen uptake rate data at this time, fig. 7.33b, indicates a change to a more aerobic metabolism with time. Hybridoma cells are usually found to utilize less glucose and more oxygen at low glucose concentrations (Wohlpert, Kirwan, and Gainer, 1990; Fleischaker, 1982; Frame and Hu 1985), and the data shown in fig. 7.32 are consistent with this pattern. The specific ATP production rate, calculated as

\[ q^{ATP} = 6q^{O_2} + 0.6q^{LAC} \]

is shown in fig. 7.33a. A factor of 0.6 has been used here to estimate the contribution of lactate formation to ATP production as this culture utilize significantly more glucose than glutamine (although this factor probably changes over the course of the culture).
Fig. 7.32 a (upper) and b (lower). Specific nutrient uptake rates and waste product formation rates for a culture with a step-change in glucose concentration (run 90/6). Data derived from the accumulated uptake and production data shown in fig. 7.26 to 7.31 above.
Fig. 7.33 a (upper) and b (lower). Specific ATP Production rate and oxygen uptake formation rate for a culture with a step-change in glucose concentration (run 90/6). Data derived from the accumulated uptake and production data shown in fig. 7.26 to 7.31 above.
The ATP production rate appears to be quite constant before the step change in glucose concentration, and is in agreement with the findings of Sonderhoff, Kilburn, and Piret (1992). Shortly after the step-change in glucose concentration, at 32 h runtime, there is a dramatic shift in the metabolism of the cells. The discontinuity in the curves in fig. 7.32 is due to the sampling frequency for manual samples. A sample was taken 2 h before the step change, and another 4 h after. The exact rates just after the concentration change are not known, as no samples was taken right at the time of the step change. The control system initiates the nutrient step-change automatically based on the estimated cell density, as explained in the chapter on control of dynamic cell cultures. However, the specific oxygen uptake rate curve in fig. 7.33b is continuous, as this measurement is done automatically by the control system.

Within two hours after the step-change there is a dramatic but transient increase in the specific oxygen uptake rate, fig. 7.33b. The increase is almost 50% of the level before the step-change, but within a few hours the oxygen uptake rate decreases to a level that is about 10% higher than before the step-change. The increase in specific glucose uptake rate is as dramatic, but lasts longer, fig. 7.32a. There is a doubling in the uptake rate after the step change. The corresponding effect is seen in the lactate production rate, fig. 7.32b. There is a delay between the peak in the glucose uptake rate and the peak in lactate production rate. The sampling frequency used in this experiment does not permit an exact measurement of this delay. However, from the results of this and other cultures (data not shown), it can be estimated to be between 4 and 12 h, depending on the exact culture conditions and whether a step or ramp change was used. This is in agreement with results presented by Miller et al (1989 a and b) for another hybridoma cell line. There is no corresponding change in the specific rates for glutamine uptake or ammonia production, indicating that the culture was limited only by glucose.
The effects seen in the specific oxygen, nutrient, and waste product metabolism rates are summarized in the specific ATP production rate curve. Shortly after the glucose concentration step-change there is a marked increase in the ATP production rate, which decays with time as the culture progresses. A slow change back to a more balanced metabolism between glucose and glutamine is evident in the increased glutamine uptake rate towards the later stages of the culture, at about 70 h runtime. Finally, Fig. 7.34 shows the specific ATP production rate in viability time. The decay in ATP production with viability time is apparent here. The rate of decay is similar to the rate of decay in the specific antibody production rate shown above for this culture. It is tempting to speculate that there may be a connection between the metabolic activity of the cells and the specific antibody production rate. It should be pointed out though that any such connection also would depend on the type of dynamic operation of the culture, as a glutamine change culture will show a lower overall metabolic activity. However, this type of culture has an increase in uptake rate just after a nutrient concentration change, with a similar decay in glutamine consumption rates, although the effects on the oxygen uptake rate and ATP formation rate are not quite as dramatic. In addition, the glucose plus glutamine change culture 91/3 has metabolic rates that are similar to the glucose change culture shown here (data not shown). However, this culture does not display increased antibody production. Therefore, a connection between the metabolic activity of the cells and the specific antibody production rate remains a speculation at this stage.
Fig. 7.34. Specific ATP Production rate versus viability time $\tau$ for a culture with a step-change in glucose concentration (run 90/6). Data derived from the accumulated uptake and production data shown in fig. 7.26 to 7.31 above.
7.9 Dynamic operation cause higher waste product formation

It has already been implied in the section on metabolism above: dynamic operation of a
culture causes a higher rate of metabolic waste product formation, and higher final waste
product concentrations. However, there are again differences between different types of
dynamic operation. In comparison to a batch culture: A glucose change culture has a
higher level of both lactate and ammonia formation, and higher final concentrations. A
glutamine change culture has a rate of lactate formation comparable to that of a batch
culture, while the ammonia formation rate is higher, leading to a higher final ammonia
concentration. The result of the differing metabolic rates can be seen in table 7.6. Here the
different cultures are listed in the same order as in table 7.1, together with the final lactate
and ammonia concentrations in the reactor. The reference batch culture has a final
concentrations of 16 mM for lactate and 4 mM for ammonia. In comparison, the glucose
change cultures all have higher final concentrations of both lactate and ammonia, about 32
and 11 mM respectively. The values are the same for glucose plus glutamine cultures that
behave as a glucose culture, runs 90/7 and 91/1 in table 7.6. By contrast, the glutamine
change cultures have a lower final lactate concentration, about 18 mM which is close to that
of a batch culture. The final ammonia concentrations of these cultures are similar to those
of glucose change cultures, about 10 mM. The glucose plus glutamine ramp-change culture
90/9 behaves like a glutamine change culture, and displays final lactate and ammonia
concentrations accordingly. For cultures not showing increasing antibody formation rates
the pattern is somewhat more disperse. In particular, the glucose plus glutamine culture
90/3 with an early step-change shows elevated final concentrations of both lactate and
ammonia, consistent with the behavior of a glucose change culture. However, this culture
did not display increased antibody production. This may indicate that the metabolic activity
of the cells and the production rate of secreted proteins do not correlate.
<table>
<thead>
<tr>
<th>Run Type</th>
<th>Run Number</th>
<th>Final antibody concentration (mg/L)</th>
<th>Maximum Total Cell Number (10^9 cells)</th>
<th>Final Integrated Cell Viability Time (h * (Xv + Xd))</th>
<th>Final Lactate Conc. (mM)</th>
<th>Final Ammonia Conc. (mM)</th>
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<tbody>
<tr>
<td>Reference runs and early/late step changes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early glucose plus glutamine batch</td>
<td>91/3</td>
<td>68</td>
<td>5.09</td>
<td>484</td>
<td>35</td>
<td>9</td>
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<tr>
<td>Early glutamine step change</td>
<td>89/1</td>
<td>82</td>
<td>4.25</td>
<td>420</td>
<td>19</td>
<td>3</td>
</tr>
<tr>
<td>Glacken fed-batch</td>
<td>89/7</td>
<td>83</td>
<td>2.96</td>
<td>235</td>
<td>31</td>
<td>5</td>
</tr>
<tr>
<td>Glacken fed-batch</td>
<td>89/6</td>
<td>87</td>
<td>3.50</td>
<td>181</td>
<td>21</td>
<td>6</td>
</tr>
<tr>
<td>Batch, 2.5% FCS</td>
<td>89/5</td>
<td>87</td>
<td>4.80</td>
<td>471</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>Late glucose + glutamine step change</td>
<td>91/2</td>
<td>89</td>
<td>6.39</td>
<td>449</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Dynamically operated runs</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamine step change</td>
<td>90/5</td>
<td>88</td>
<td>3.33</td>
<td>298</td>
<td>17</td>
<td>9</td>
</tr>
<tr>
<td>Glucose + Glutamine step change</td>
<td>90/7</td>
<td>107</td>
<td>5.03</td>
<td>502</td>
<td>35</td>
<td>12</td>
</tr>
<tr>
<td>Low glucose step change</td>
<td>90/8</td>
<td>111</td>
<td>6.07</td>
<td>563</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>Glucose + glutamine step change</td>
<td>91/1</td>
<td>115</td>
<td>5.30</td>
<td>337</td>
<td>37</td>
<td>11</td>
</tr>
<tr>
<td>Glutamine ramp change</td>
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<td>118</td>
<td>4.26</td>
<td>392</td>
<td>22</td>
<td>12</td>
</tr>
<tr>
<td>Glucose step change</td>
<td>90/6</td>
<td>120</td>
<td>5.40</td>
<td>517</td>
<td>30</td>
<td>11</td>
</tr>
<tr>
<td>Glutamine step change</td>
<td>90/1</td>
<td>128</td>
<td>3.88</td>
<td>373</td>
<td>19</td>
<td>10</td>
</tr>
<tr>
<td>Glucose + glutamine ramp change</td>
<td>90/9</td>
<td>132</td>
<td>4.96</td>
<td>475</td>
<td>18</td>
<td>11</td>
</tr>
<tr>
<td>Other reference runs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Batch, 5% FCS</td>
<td>89/2</td>
<td>112</td>
<td>5.51</td>
<td>730</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Batch 0.6 L, spinner flask culture</td>
<td>91/4</td>
<td>133</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 7.6. Comparison of waste product formation between cultures. Some data is in total amounts in reactor (final volume is 2 L, to get concentrations, divide amounts \( \times 2 \)).
Finally, it should be noted that the glutamine limited operating strategy designed by Glacken does achieve its primary design objective: a low ammonia concentration.

The final waste product concentrations shown in table 7.6 are at a level that has been reported as inhibitory for most cell lines (see reviews by Reuveny and Lazar, 1989; Ozturk, Riley, and Palsson, 1992). In particular, Glacken (1987) found ammonia to inhibit growth of CRL 1606 hybridomas at low cell densities, and in addition found lactate to be a weak inhibitor of antibody production. For dynamically operated cultures it would instead appear that ammonia and lactate stimulate cell growth and antibody formation, judging only from the results presented in table 7.6,. This is of course not the case. The real determinant of waste product inhibition are the actual concentrations of lactate and ammonia in the reactor during the main growth and antibody production period of the culture. Fig. 7.35 and 7.36 shows the actual concentration of lactate and ammonia for the three cultures previously used to exemplify process behavior. The cell densities in the reactor during the cultures are shown for reference. Compared to a batch culture, the glucose culture has a higher level of both lactate and ammonia present during the main growth period of the cells. The glutamine change culture by contrast has lactate concentrations similar to that of a batch culture for the major part of the culture (which is delayed compared to the batch culture). However, the glutamine concentrations of this culture are clearly higher than for a batch culture, especially towards the end of the run. The pattern seen in fig. 7.35 and 7.36 holds for all the cultures investigated in this work (data not shown). A higher concentration of waste products at the end of a culture is indicative of a higher concentration during the main growth and antibody formation period of the culture. The differences between cultures are less than what the figures in table 1 might indicate. Nevertheless, all the high antibody producing cultures have higher concentrations of ammonia during the main antibody production phase than a reference batch culture, or a glutamine limited culture.
Fig. 7.35 a (upper) and b (lower). Comparison of lactate concentrations over runtime in three representative cultures. Viable cell data shown for reference.
Fig. 7.36 a (upper) and b (lower). Comparison of ammonia concentrations over runtime in three representative cultures. Viable cell data shown for reference.
For glucose change cultures, also the lactate levels are higher than seen in a batch culture. In terms of cell densities, the cultures that produces the most cells, glucose change cultures, have the highest concentrations of both lactate and ammonia during the main growth and antibody production period.

These findings are in contrast to the waste product inhibition reported in a number of investigations (see literature review). Several of these investigations were specifically designed to investigate such inhibition, and they are well documented. This investigation on the other hand was designed to investigate stimulatory effects on antibody production resulting from dynamic operation of cultures. The work presented here is therefore not intended to elucidate waste product inhibitions, and the levels of such inhibitions can not be directly determined from the results at hand. Thus, care has to be taken in interpreting the influence on lactate and ammonia on cell growth and antibody formation in dynamically operated cultures, as cause and effect can not be separated. Nevertheless, it can be concluded that waste product inhibition does not play a major role in dynamically operated cultures. The effects on antibody formation and cell growth resulting from this operating strategy obviously outweigh possible inhibitions resulting from higher lactate and ammonia concentrations. However, the data does not exclude inhibitory influences of lactate and ammonia.

There may be further reasons for the discrepancies seen between the results presented here and the conclusions reached in the investigations mentioned above. In most experimental designs intended to investigate the influence of lactate and ammonia, these compounds are added to the medium before the inoculation with cells. Thus, the cells will go through a lag phase with ammonia and lactate present in the medium at high concentrations. However, in this investigation it has been shown that influences of culture conditions may last for a
significant time. Therefore, the results from such cultures may be more indicative of the influence of lactate and ammonia on cells in lag phase than on an actively growing culture. But for process design it is the influence of lactate and ammonia during the main growth and production phase that is important. In a production culture, the start concentrations of these compounds are always close to zero. A ramp change of lactate or ammonia concentration induced during the main production phase of a culture may therefore be a more realistic approach to investigate real inhibitory effects of lactate and ammonia. This will be further elaborated upon in the chapter on future work below. Interestingly, Glacken investigated the effect of ammonia step-changes on the growth and antibody formation of CRL 1606 cells. The influence of a given concentration of ammonia was found to be much less when added to an actively growing culture, than when added to the start medium of a culture. This appears to support the speculation presented above regarding the influence of ammonia on cells in lag phase.

7.10 Osmolarity

The results at hand appear to exclude other causes than the nutrient step-and ramp changes for the effects seen on cell growth and specific antibody productivity. It should be mentioned, however, that changes in osmolarity have not been completely ruled out as a possible, albeit unlikely, cause. The start and feed medium used in this investigation where not adjusted to the same osmolarity. This was judged unnecessary, as the osmolarity changes during the culture from the medium changes are within the normal tolerance range for hybridomas. The medium used for a batch culture has an osmolarity of 320 mOsmol. The start medium with the lowest concentration of glucose and glutamine medium used here has a calculated osmolarity about 300 mOsmol, while the final media mixture in the reactor with the highest nutrient concentrations, has an osmolarity of 345 mOsmol. The normal
osmolarity range for optimal growth of hybridomas is 290 - 350 mOsmol, and is quite flat (Waymouth, 1973; Schlaege and Schupmp, 1988). Increasing osmolarity to 480 mOsmol completely inhibits cell growth, while specific antibody production increases (Øyaas et al., 1988). The largest change in osmolarity during a nutrient concentration change in this investigation occurs during a simultaneous glucose plus glutamine concentration change. Such a fed-batch culture has a calculated increase in osmolarity from 300 to 345 mOsmol during the course of the run from the addition of nutrients. In comparison, the osmolarity during a normal batch changes by 10 to 20 mOsmol during the run due to breakdown of glucose and glutamine to lactate and ammonia.

7.11 Dynamic conditions affects cell size distribution

The nutrient concentration changes introduced in dynamically operated fed-batch cultures have profound effects on the size distribution of the cells in the culture. The analysis of cell sizes from Coulter counter chanelizer data presented here emphasizes the qualitative aspects of the size distributions. The focus of this investigation was on antibody production, and cell size data for every experiment performed are not available.

Fig. 7.37a shows the viable and dead cell curves for a small scale batch culture, together with the average volume of the cells. Fig. 7.37b displays the total cell number and total volume of the cells for the same culture, while fig. 7.38 shows the relative distribution of cell sizes for some selected samples. In fig. 7.37 and 7.38 it can be seen that the cells shift to a larger size shortly after inoculation, and then start growing. They reach their largest peak in the size distribution quite early in the culture, at 26 h. As the cells continue to grow, the size distribution shifts to towards smaller sizes, and the viable cell size reaches a minimum just before the peak in viable cell density (81 h). At this time dead cells start to appear, which
Fig. 7.37 a (upper) and b (lower). Cell and cell volume data for a 0.6 L spinner batch culture (run 91/4).
Fig. 7.38. Cell size distribution over time for a batch run in a 0.6 L spinner flask (run 91/4). Each cell size curve has been normalized by the most frequent size, and therefore shows relative size distribution. Numbers represent runtime at which the sample was taken.
is increasingly visible in the size distribution curves as a separate peak in left end of the curve (106 h). As the culture dies, the average size of the cells continue to decrease, and the dead cell and debris peak at the left end of the size distribution curves continue to grow. Similar results have been obtained elsewhere (de la Broise et al., 1991). Throughout the culture the total integrated volume of the cell mass is a good indicator of the total number of cells (viable plus dead) in the reactor, As seen in fig. 7.37b.

Fig. 7.39 a and b together with fig. 7.40 shows the same results as presented in fig. 7.37 and 7.38 but for a dynamically operated culture, a glucose plus glutamine change run with an early step change (run 91/3). The influence of the nutrient change can be seen in the average cell volume curve. At the time of the nutrient concentration step-change, the average cell volume does not immediately increase (21 h). However, after about 8 h a significant increase is seen in the average cell volume, which reaches a peak at about 46 h at a value of 2,000 $\mu$m$^3$; which is 200 $\mu$m$^3$ larger than the maximum average cell volume for the batch culture. The largest peak size in the size distribution curve is reached a few hours before this point, at 35 h. Also after the peak in average cell volume, the cell volume of the dynamic culture remains larger than the corresponding volume of the batch culture, and in addition decreases slower. Evidence of this can be seen in the size distribution curve at 115 h, which has a distinct peak of dead cells.

The difference in cell size distribution between a batch and a dynamically operated culture is perhaps not immediately visible by comparing the figures presented above. Fig. 7.41 shows the relative size distribution of the two cultures at the time of their largest peak in the size distribution curve. Here the larger cell size in the dynamic culture as compared to the batch culture is clearly visible. The difference in average cell volume between these two culture over time is compared in fig. 7.42. To compensate for the longer lag time of the dynamic
Fig. 7.39 a (upper) and b (lower). Cell and cell volume data for a dynamically operated fed-batch culture with an early glucose plus glutamine step-change (run 91/3).
Fig. 7.40. Cell size distribution over time for a glucose plus glutamine change run with a early step-change at 24 h (run 91/3). Each cell size curve has been normalized by the most frequent size, and therefore shows relative size distribution. Numbers represent runtime at which the sample was taken.
Fig. 7.41. Comparison of the maximum peak cell diameter for a batch culture (run 91/4) and a dynamically operated culture (91/3) with an early nutrient change. Cell sizes have been normalized to the most frequent size.
Fig. 7.42. Comparison of average cell volumes versus runtime for a batch culture (90/4) and a dynamically operated culture (91/3) with an early nutrient change. The curve relating the cell sizes has been offset so that the peak in the average cell volume curves of the culture coincide.
culture, the cell volume curve of this run has been shifted to the left in the graph, so that the peak in the cell volume of the two runs coincide. The true difference in average cell size is thus represented in the curve shown of the cell volume of the dynamic culture as a fraction of that of the batch culture. The difference is largest during the main growth period of the cultures. The average cell volume of the dynamic culture is about 30% larger than that of the batch culture during this period. However, the large difference in cell size during this period is also reflective to some extent of the prolonged growth period of the dynamic culture. A more correct value for the true difference in average cells volume between a dynamic culture and a batch culture is therefore probably closer to 20%, as seen at the time of the peaks in the average cell sizes of the cultures. When the dynamic culture has reaches its maximum viable cell number the cells of this culture shrink rapidly, and the average size of the cells approaches that of the batch culture over a period of about 12h, at 120 h runtime in fig. 7.42.

A more comprehensive way to display the changes in cell size and growth during the course of a culture is to plot the data in a 3-dimensional graph. The cell sizes measured in the Coulter chanalyzer determines the frequency of particles for a given particle size, over a range of sizes. With knowledge of the total number of particles counted, and the cell density for that sample, the number of cells of each size can be calculated for every sample taken from the culture. The total number of particles (cells) of each size in the reactor can then be plotted against the runtime of the culture in a 3-D plot. This will graphically describe the complex events during the culture in a manner similar to a landscape photo. Fig. 7.43 shows such a 3-D graph for the batch culture described above. The “upward” z-axis in this graph is the total number of cells in the reactor for each size, which is plotted on the “transverse” y-axis. These size distribution curves are plotted against the runtime of each sample on the “inward” going x-axis. The two “mountains” seen in this figure then
describes the growth and death of the cells. In fig. 7.43 the cells are inoculated with a peak cell size shown by the arrow at point 1. Fig. 7.44 zooms in on the beginning part of this culture, and here the events just after inoculation are more clearly visible, point 1 in fig. 7.44. Just after inoculation the cells go through a lag phase where they do not divide. Instead, they shift in size to larger volumes, and start dividing first after about 15 h. The growth of the culture is next seen as a steeper slope of the “mountain” in fig. 7.44. A continued shift in cell sizes to larger sizes up to about 35 h is also visible in fig. 7.44. The subsequent fate of the culture can be followed in fig. 7.43. The “growth mountain” continue to rise up to the point of maximum cell density (point 2), with a reduction in growth rate before the peak visible as a leveling off of the slope. The events during the early death of the culture is better depicted in fig. 7.45, which zooms in on this phase and shows the data from a different angle than the previous graphs. The beginning of the death phase can be seen as a reduction in the maximum size of the cells at the time the culture approaches the peak in viable cell number, point 1 in fig. 7.45. At the same time the dead cell number starts to increase, seen as the increase of the “death mountain” in the inner corner of fig. 7.45. As the culture passes the maximum viable cells number, the “peak” of the cell “growth mountain” is passed, and the viable cell slope descends downward rapidly. At the same time the “wall” of dead cells and debris towards the inner corner of fig. 7.45 and 7.43 rises rapidly, and the culture progresses towards its sad end.

The same data for the dynamic culture described above reveals a somewhat different pattern, fig. 7.46. The “growth mountain” is delayed compared to the previously described batch culture, and the cell size distribution is shifted towards larger sizes, as was seen in the analysis of the average cell sizes in fig. 7.42 above. The difference compared to the batch culture is further highlighted in fig. 7.47 which zooms in on the beginning of the dynamic culture. The response to the nutrient concentration step change that was seen as an
Fig. 7.43. 3-dimensional graph of the total cell number and size distribution for a 0.6 L spinner batch culture (run 91/4). Overview of the culture (see text for description).
Fig. 7.44. 3-dimensional graph of the total cell number and size distribution for a 0.6 L spinner batch culture (run 91/4). Zoom in on the inoculum and lag phase of the culture (see text for description).
Fig. 7.45. 3-dimensional graph of the total cell number and size distribution for a 0.6 L spinner batch culture (run 91/4). Zoom in on the peak phase and beginning of death phase of the culture, from a different angle (see text for description).
Fig. 7.46. Total cell size distribution for a glucose plus glutamine concentration step-change fed-batch (run 91/3). Total cells per channel versus runtime. Overview of the culture (see text for description).
Fig. 7.47. Total cells per size for a glucose plus glutamine concentration step-change fed-batch (run 91/3), plotted versus runtime. Zoom in on the start of the run, and the nutrient step change (see text for description).
increased average cell size in fig. 7.39 above, is seen as a period of slow growth with a less steep slope of the “growth mountain”, and a shift in cell size towards larger sizes, point 3 in fig. 7.46. First about 24 h after the nutrient step change does the cells start dividing at their full rate (point 4).

The results presented above on the cell size distribution of the CRL 1606 hybridoma during dynamic operation of a fed-batch culture show that the main impact on cells size distribution is a shift towards larger sizes as compared to a batch culture. After a nutrient concentration change there is a delay of the cell growth, during which the cells shift toward larger sizes. First about 24 h after the nutrient concentration change do the cells start dividing, at a higher rate than for a batch culture and (for a glucose change culture) reaching a higher peak viable cell number and a longer viability times, as was shown in the cell growth section above.

The most dramatic influence on cell size distribution from dynamic operation of a culture seen in this investigation was found for a different cell line than CRL\textsuperscript{*}1606, the FN-A2C2 hybridoma. This cell line also produces an antibody against fibronectin, but at such low levels that it is not useful as a model cell line. The result for cell growth and size distribution obtained for this cell line does, however, illustrate the effects from dynamic operation of hybridoma cultures better than the results obtained for CRL 1606. Fig. 7.48 shows similar data as that presented for CRL 1606 in fig.’s 7.37, 7.39, and 7.42 above. This figure compares a 2 L batch culture of FN-A2C2 with a 2 L final volume dynamic fed-batch culture with a normal glucose plus glutamine step-change. The total cell volume reached for the dynamic culture is considerable larger than that of the batch culture, almost twice as much at the time of the peak in the viable cell number (fig. 7.48b), in accordance with the results obtained for CRL 1606. The average cell volumes for the two cultures are

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Fig. 7.48 a (upper) and b (lower). Comparison of average and total cell volumes of a batch culture (91/5) and a glucose plus glutamine change culture (91/11) for the FN-A2C2 hybridoma.
shown in fig. 7.48a together with a curve of the average cell volume of the dynamic culture as a fraction of that of the batch culture. During the nutrient starvation period of the dynamic culture, the cells shrink in volume. After the nutrient concentration step change a dramatic increase is seen in the average cell volume, while cell growth does not yet resume. The cell volume curve levels out about 24 h after the step-change, and at that time the cells start to divide. The average cell volume of the dynamic culture remains larger than that of the batch culture throughout the growth phase. First after the peak in the viable cell number of the dynamic culture does the cell volume start to approach that of the batch culture.

The 3-D data for the FN-A2C2 batch culture is presented in fig. 7.49. The FN-A2C2 cell line grows less well than CRL 1606 and has a large number of non-viable cell present in the culture, which is visible as the “slope” against the inner wall of the graph. Otherwise the events of this culture are essentially the same as those describe above for the CRL 1606 batch culture. The death of the cells can be seen as the dramatic shift in the particle size distribution towards the back corner of the graph. Fig. 7.50 zooms in on the beginning phase of the batch culture, and shows the shift in cell size during the lag phase.

The 3-D picture of the dynamic culture of the FN-A2C2 cells is quite different from that of the batch culture. In fig. 7.51 the stimulatory effect on cell growth from the dynamic operation can be seen by comparing the height and with of the viable cell peak at the left inner part of the graph, to that of the batch culture shown in fig. 7.49. However, the difference in behavior of the cultures is most remarkable at the time of the step change in nutrient concentration. Fig. 7.52 zooms in on this part of fig. 7.51. After the inoculum has gone through the lag phase, where the cells increase in size before they start growing (point 2 in fig. 7.52), growth starts just as for a batch culture (point 3). However, at about 34 h
Fig. 7.49. Total cell size distribution for a batch run (hybridoma FN-A2C2, run 91/5). Total cells per size versus runtime. Overview of the culture (see text for description)
runtime the nutrient limitation of the culture is visible as a leveling off of the growth slope of the cells (point 4). At 36 h (point 5) the culture is fed a very small amount of nutrients in order to prolong the nutrient limitation phase of the culture, with approximately a 0.15 and 0.07 mM increase in glucose and glutamine concentrations respectively. The effect of even this small amount of nutrient addition is seen as an increase in the slope of the “cell growth surface”, before nutrient limitation sets in and growth slows again (point 6). At 48 h after inoculation the nutrient step change is fed to the culture; in 30 min the nutrient concentration is increased to 30 mM and 10 mM for glucose and glutamine respectively. The significant effect of this change in culture conditions is clearly visible in the 3-D graph. The cells do not start growing immediately after the step change. Instead, they increase their volume (point 8) and first at about 74 h runtime, after about one generation time (26 h), do they start dividing again (point 9 in fig. 7.51). The increase in growth rate at this runtime compared to that of a batch culture can be visualized by comparing the slope of the “growth mountain” in fig. 7.52 to that in fig. 7.50.

In summary, dynamic operation of a hybridoma fed-batch culture has dramatic effects on the cell size distribution of the culture, as well as on the average size of the cells. During the nutrient limitation period before the nutrient concentration change the cell size decreases, and after a while the growth rate of the cells also decrease. For the cell lines and the conditions used here, this occurs about 36 h after the inoculation of the culture, when the cells have grown from $0.1 \cdot 10^9$ cells/L to about $0.2 \cdot 10^9$ cell/L. When the nutrient concentration change is introduced, at about 48 h runtime and $0.3 \cdot 10^9$ cells/L, there is a dramatic response of the culture. The cells do not immediately start dividing, instead, they shift towards larger sizes, and first about 24 h after the nutrient concentration change do
Fig. 7.50. Cell size distribution over time for a batch run (hybridoma FN-A2C2, run 91/5), for the start of the culture. Total cells per size channel, versus runtime. Zoom in on the beginning of the culture (see text for description).
Fig. 7.51. Total cell size distribution for a glucose plus glutamine concentration step-change fed-batch (hybridoma FN-A2C2, run 91/11). Total cells per size versus runtime. Overview of the culture (see text for description).
Fig. 7.52. Total cells per size for a glucose plus glutamine concentration step-change fed-batch (hybridoma FN-A2C2, run 91/11), plotted versus runtime. Zoom in on the start of the run, and the nutrient step change (see text for description).
they begin to divide. The stimulation from the dynamic operation is here evident as an increased growth rate, except for a glutamine change culture which has a lower growth rate but instead has a prolonged growth period. For a glucose change run (and for a glucose plus glutamine change run that behaves as a glucose change run), the stimulation of the culture is also evident as an increased peak viable cell number. The average cell volume for a dynamic run remains larger than that for a batch run throughout the whole growth phase of the culture, and first after the peak in viable cell number does the stimulation of the dynamic culture decay enough that the cell size approaches that of a batch culture. The increase in the cell volume for a dynamic culture compared to that of a batch culture is between 10 to 30%, with an average of about 20%. This corresponds to an increase in the cell membrane surface area of about 10% to 20%. The data presented here is consistent with the findings of others (Dalili and Ollis 1990; Ramirez and Mutharasan, 1990; Frame and Hu, 1990) including those of Needham et al. (1991), who found an increase in cell volume and cytoplasmic content of individual cells as the cell divisional cycle progresses.

7.12 The final result: glucose change cultures have highest productivity

With the results presented above it is possible to summarize the performance and productivity of the dynamically operated cultures. In the preceding the antibody production, i.e. final antibody concentrations, have been used as the primary performance to evaluate the different processes. Judging from this parameter all dynamically operated cultures presented here perform essentially equally well, with some variation. Another performance parameter of interest in evaluating processes for industrial use is volumetric productivity. This is the amount of antibody produced per reactor volume and day. This parameter is determining for the process economy of the mammalian cell culture process as an isolated unit operation, not considering the influence of product concentration on downstream
operations. In table 7.7 the volumetric productivity of the cultures is listed together with some additional performance parameters. The variations in the final antibody concentrations to some extent obscures the pattern that can be seen in this parameter. From the runtimes at which the antibody concentration has reached its final value, it can be seen that glucose change cultures (or glucose plus glutamine cultures behaving like glucose change cultures) reach their final antibody concentration one to two days faster than glutamine change cultures. The volumetric productivity of a glucose change culture is therefore higher than that of a glutamine change culture, given similar final antibody concentrations. For instance, run 90/6, a glucose step-change culture, has a final antibody concentration of 120 mg/L which it reached after 127 h, giving a volumetric productivity of 0.95 mg/L-h. The glutamine change culture 90/1 has a similar antibody concentration, 128 mg/L which was reached after 180 h, resulting in a volumetric productivity of 0.71 mg/L-h.

By comparison, the batch culture 89/5 has a volumetric productivity of 0.62 mg/L-h. Glucose change cultures are thus the best performing processes, considering both final antibody concentrations and volumetric antibody productivity.

An overview of the performance of the cultures can also be obtained by plotting the final antibody concentrations versus the final viability times, fig. 7.53. Here the processes can be divided into three distinct groups, disregarding the outliers 90/5 and 91/3. The batch culture and cultures behaving as batch cultures fall into one cluster. Another larger cluster consist of the dynamically operated cultures. The glutamine limited cultures form their own cluster. This figure summarizes the conclusion that can reached from examining the data presented above: by utilizing the dynamics of mammalian cell fed-batch cultures it is possible to significantly improve process performance.
<table>
<thead>
<tr>
<th>Run Type</th>
<th>Run Number</th>
<th>Final antibody concentration (mg/L)</th>
<th>Final Integrated Cell Viability (10^9 cells*h)</th>
<th>Runtime when final antibody concentration was reached (h)</th>
<th>Volumetric Antibody Productivity (Final Ab conc./ Ab amount) (mg/L.*h)</th>
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<tr>
<td>Reference runs and early/late step changes</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>91/3</td>
<td>68</td>
<td>484</td>
<td>177</td>
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<td>82</td>
<td>420</td>
<td>153</td>
<td>0.53</td>
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<tr>
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<td>235</td>
<td>168</td>
<td>0.49</td>
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<tr>
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<td>181</td>
<td>150</td>
<td>0.58</td>
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<tr>
<td>Batch, 2.5% FCS</td>
<td>89/5</td>
<td>87</td>
<td>471</td>
<td>140</td>
<td>0.62</td>
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<tr>
<td>Late glucose + glutamine step change</td>
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<td>89</td>
<td>449</td>
<td>143</td>
<td>0.62</td>
</tr>
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<td>Dynamically operated runs</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Glutamine step change</td>
<td>90/5</td>
<td>88</td>
<td>298</td>
<td>171</td>
<td>0.52</td>
</tr>
<tr>
<td>Glucose + Glutamine step change</td>
<td>90/7</td>
<td>107</td>
<td>502</td>
<td>180</td>
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<tr>
<td>Low glucose step change</td>
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<td>111</td>
<td>563</td>
<td>135</td>
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</tr>
<tr>
<td>Glucose + glutamine step change</td>
<td>91/1</td>
<td>115</td>
<td>337</td>
<td>140</td>
<td>0.82</td>
</tr>
<tr>
<td>Glutamine ramp change</td>
<td>90/2</td>
<td>118</td>
<td>392</td>
<td>179</td>
<td>0.66</td>
</tr>
<tr>
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<td>517</td>
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<td>0.95</td>
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<td>Glutamine step change</td>
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<td>373</td>
<td>180</td>
<td>0.71</td>
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<td>475</td>
<td>160</td>
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<td>91/4</td>
<td>133</td>
<td>--</td>
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</tr>
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Table 7.7. Comparison of runtime and volumetric productivities for the cultures.
Fig. 7.53. Plot of culture performance in a space of final antibody concentration versus final viability time.
A mathematical model of a mammalian cell culture process should serve a number of purposes. First, it must provide an accurate and adequate description of process behavior. It should further provide insight into the mechanisms behind process behavior. A good model can serve as a conceptual framework for investigation and process design. The model should also make it possible to predict process behavior within the valid parameter space, and it should ideally be possible to extrapolate from this parameter space to other process conditions of interest. A common example in using models for extrapolation is process scale-up. Lastly, a model can serve as a guide for future investigations and an aide for further experimentation.

8.1 Basic model

The model presented below has been designed to meet several requirements. The main parameters that determine antibody productivity of a hybridoma culture are the viable cell density and the specific and volumetric antibody productivity of the process. A model should therefore adequately describe the behavior over the course of the culture of the cells' growth rate, the viable cell density, the specific and volumetric antibody productivity, as well as the amounts of antibody produced in the reactor. As a significant part of antibody production in hybridoma cultures takes part during the peak and early death phases of the culture (see literature review), the model should describe these phases in addition to the growth phase of the culture. To aid in the interpretation of experimental data, the model parameters should have a physical meaning.
The approach followed here is similar to that used by Volterra in the 1920's to describe predator-pray systems. The behavior of a mammalian cell culture over runtime is quite complex, as has been shown in the results presented above. However, the behavior is somewhat simpler to describe in the $\tau$-space that was used to compare different cultures (see introduction to the results chapter). The model presented here is based on viability time rather than runtime. This has the advantage of automatically relating the model parameters to the course of the culture. It does, however, have the drawback of making the mathematics of the model in runtime-space complex, and most of the expressions cannot be solved analytically in this space. Fig. 8.1a shows a $\tau$-space graph of viable cell number and the apparent specific growth rate for a typical dynamic fed-batch culture. Fig. 8.1b shows the amounts of antibody in the reactor as well as the specific antibody productivity of the same culture. The model built below is designed to describe this data.

The apparent growth rate in fig. 8.1 can be described by a combination of a maximum growth rate $\mu_{\text{max}}$ and a decay factor that is a function of $\tau$, i.e. $\mu = \mu (\mu_{\text{max}}, \tau)$. As a first approximation, the decay in the growth rate can be modeled as a linear function of $\tau$:

$$\mu_{\text{app}}(\tau) = \mu_{\text{max}} - k \cdot \tau$$

where $k$ is the decay constant for the growth rate and $\mu_{\text{max}}$ is a constant. The apparent growth rate used here to model cell growth is the sum of the real growth rate and the death rate of the cells (see appendix 1), i.e.

$$\mu_{\text{app}} = \mu_{\text{real}} - \alpha$$

This follows the definitions for these variables that is presented in appendix 1 below. The apparent growth rate can accordingly be both positive and negative. A positive $\mu_{\text{app}}$ describes an overall growth of viable cells, a negative $\mu_{\text{app}}$ describes an overall death of the
Fig 8.1 a (upper) and b (lower). a) Cell growth and growth rate versus runtime for a batch culture (89/5). b) Cell growth and growth rate versus viability time $\tau$ for a batch culture
cells. From the definition of cell growth in $\tau$-space:

$$\mu_{\text{app}} = \frac{d(X_v)}{d\tau}$$

the expression for cell growth and death can be derived as

$$\frac{d(X_v)}{d\tau} = \mu_{\text{app}} - k \cdot \tau$$

This equation can easily be solved in $\tau$-space by separating the variables, and integrating; from $X_{v0}$ to $X_v(\tau)$ and from $\tau = 0$ to $\tau$:

$$\int_{X_{v0}}^{X_v} d(X_v) = \int_0^{\tau} (\mu_{\text{app}} - k \cdot \tau) \, d\tau$$

and

$$X_v(\tau) = X_{v0} + \mu_{\text{app}} \cdot \tau - \frac{k}{2} \cdot \tau^2$$

This expression is a parabola in $\tau$-space, which essentially describes the viable cell number curve in this space throughout most of the culture, as will be shown below. An expression for the maximum cell density of the culture is implicit in this model. Differentiating with respect to $\tau$ and setting the derivative to zero, solving the expression for $\tau$, and plugging this value into the model gives

$$X_{v,\text{max}} = X_{v,0} + \frac{\mu_{\text{max}}^2}{2k}$$

The antibody production can be described in a similar manner as for the viable cells. As the viable cell density is implicitly built into the independent parameter $\tau$, it is not necessary here
to include the viable cell number $X_v$ explicitly in the expression for specific antibody production. In fig. 8.1b it can be seen that the specific antibody productivity can be modeled as a maximum productivity that decays with $\tau$.

$$q^{Ab}(\tau) = q_{max}^{Ab} - k^{Ab} \cdot \tau$$

and thus

$$\frac{d(A^{Ab})}{d\tau} = q_{max}^{Ab} - k^{Ab} \cdot \tau$$

where $q_{max}^{Ab}$ is a constant, and $k^{Ab}$ is a decay constant. To see how this expression implicitly depends on $X_v$, remember that $d\tau = X_v \, dt$, and thus

$$\frac{1}{X_v} \frac{d(A^{Ab})}{dt} = q_{max}^{Ab} - k^{Ab} \cdot \tau$$

The equation for $A^{Ab}$ can be solved in the same way as for the viable cell number above, to give

$$A^{Ab}(\tau) = A_0^{Ab} + q_{max}^{Ab} \cdot \tau - \frac{k^{Ab}}{2} \cdot \tau^2$$

where the initial antibody concentration $A^{Ab}_0$ can be assumed to be zero. This simple model describes the system adequately in $\tau$-space, as exemplified in fig. 8.2.
Fig. 8.2 a (upper) and b (lower). Model fit to data from a batch culture (89/5) in runtime (a) and τ-space (b).
8.2 Transformation to runtime

The inverse transformation $d\tau = Xv \, dt$ from $t$-space to runtime for the basic model described above results in the expressions;

$$\frac{d(X_v)}{dt} = \mu_{app}X_v - k \cdot X_v \cdot \int_0^t X_v \, dt$$

$$\frac{d(A^{Ab})}{dt} = q_{max}^{Ab} \cdot X_v - k^{Ab} \cdot X_v \cdot \int_0^t X_v \, dt$$

This is a set of Volterra integral equations. Some equations of this type can be solved analytically, for instance by differentiating repeatedly and solving the resulting differential equations. In many cases the equations have only numerical solutions though. For the data presented here numerical transformations $\tau = \int X_v \, dt$ have been used initially, where $X_v$ is the viable cell number for each individual culture. Therefore, the inverse transformation has also been done with the numerical values of $X_v$ for each run. A more general transformation can be obtained by using an expression of $X_v$ as a function of $t$, or simply an expression relating $\tau$ to $t$. For a discussion of the relevant mathematic the interested reader is referred to Bronshtein and Semandyayev (1985) an Mikhlin (1957).

8.3 Model interpretation

The parameters in the above model can be interpreted in several ways. The integral $\int X_v \, dt$ can be viewed as a memory factor intrinsic to the culture. This factor incorporates the influence of culture history. It is interesting to note that this integral also can describe the
influence of an accumulating substance that causes the cells to die. If such an substance is produced at a constant rate by the viable cells it could be described by the expression

$$\frac{dC}{dt} = K \cdot X_v$$

where $C$ is the amount or concentration of the substance, $K$ is a constant, and $X_v$ is the viable cell number. Without knowledge of the functionality of $X_v$, this equation can be integrated to give

$$C(t) = K \int_0^t X_v dt$$

for $C(0) = 0$. The influence of the substance on the growth rate can be assumed to be linear, giving an expression for the apparent growth rate as

$$\frac{1}{X_v} \frac{dX_v}{dt} = \mu - C$$

inserting the expression for the concentration gives

$$\frac{1}{X_v} \frac{dX_v}{dt} = \mu - K \int_0^t X_v dt$$

which is equivalent to the expressions developed above. This model is consistent with the presence of inhibitory autocrine factors in cell cultures, which have been suggested as a cause for the maximum cell density most mammalian cell cultures can reach, irrespective of nutrient and waste product concentrations (Dodge, Ji, and Hu, 1987; for review of early literature see Bettger and McKeehan, 1986). In should be noted that such an accumulation also could take place intracellularly.
While the model can be said to be consistent with the presence of inhibitory factors for cell growth, it does not necessarily predict the presence of such factors or explain the behavior of the culture. The model can be interpreted in other ways. The integral term can for instance be considered simply to be a description of a death constant. It also can be interpreted as a description of the limited lifespan of mammalian cell cultures. Regardless of the interpretation integral equations offers a satisfactorily and general paradigm for modeling the generic behavior of the full growth cycle of mammalian cell cultures, and a simple way to synthesize the description of both the growth and decay phases of the cultures.

8.4 Model expansion

The model outlined above can be expanded to include the influence of dynamic operation of the culture. Fig. 8.3 a and b show a comparison of the growth rates and the specific productivities of the three culture patterns outlined in the result chapter, for period of the cultures with highest cell numbers and antibody productivities. These profiles are obviously better fitted by a decay function than by a linear expression. Here, an extension of the linear expressions presented for the basic model above have been used. The dependence of \( \mu \) and \( q^{Ab} \) have been modeled as power functions. Such functions can adequately describe a decay of the parameters, and the solution of the integrals of these functions behave well in the limits of \( \tau \to 0 \) and \( \tau \to \infty \). The extended models are:

\[
\mu_{app} = \mu_{max} - k \cdot \tau^\beta
\]

\[
q^{Ab} = q^{Ab}_{max} - k^{Ab} \cdot \tau^{\beta^{Ab}}
\]
Fig. 8.3 a (upper) and b (lower). Profiles of growth rate (a) and specific antibody production rates (b) in τ-space for three representative cultures.
These expressions solve to give

\[ X_v = X_{v0} + \mu_{\text{max}} \tau - \frac{k}{1+\beta} \tau^{(1+\beta)} \]

\[ A^{Ab} = q_{\text{max}}^{Ab} \tau - \frac{k^{Ab}}{1+\beta^{Ab}} \tau^{(1+\beta^{Ab})} \]

### 8.5 Process description by model

The enhancement of antibody production seen in the dynamically operated cultures is consistent with a stimulation of the specific antibody productivity. Such stimulation is in this model described by the parameters for the maximum rates, \( \mu_{\text{max}} \) and \( c_{\text{max}}^{Ab} \), and by the decay functions for these rates which is described by the \( k \) and \( \beta \) parameters. Fig's 8.4 to 8.6 shows the fit of the model in \( \tau \)-space to results from three representative cultures. Fig's 8.7 to 8.9 shows the translation of the models to runtime space.

The model parameters for the three runs are summarized in table 8.1. The glucose change culture has about the same maximum growth rate as the reference batch culture, and the extended growth period of glucose change culture can be seen in the lower decay constant. By contrast, the glutamine change culture has a much lower maximum growth rate, while the extended growth phase of this culture also can be seen in the lower decay constant. The effect of dynamic operation on antibody production is seen in the maximum specific antibody productivities. The stimulatory effect of a glutamine nutrient concentration change result in a maximum specific antibody productivity that is about 2 times higher than that for a batch culture, while a glucose nutrient change result in a boosting of this parameter by about 75% over the batch culture. However, the decay in the specific antibody productivity
Fig. 8.4 a (upper) and b (lower). Fit of power decay function model in \( \tau \)-space to data from a batch culture (89/5).
Fig. 8.5 a (upper) and b (lower). Fit of power decay function model in $\tau$-space to data from a glutamine change culture (90/1).
Fig. 8.6 a (upper) and b (lower). Fit of power decay function model in $\tau$-space to data from a glucose change culture (90/6).
Fig. 8.7 a (upper) and b (lower). Fit of power decay function model in runtime space to data from a batch culture (89/5).
Fig. 8.8 a (upper) and b (lower). Fit of power decay function model in runtime space to data from a glutamine change culture (90/1).

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Fig. 8.9 a (upper) and b (lower). Fit of power decay function model in runtime space to data from a glucose change culture (90/6).
<table>
<thead>
<tr>
<th>Model parameter</th>
<th>Batch culture (89/5)</th>
<th>Glucose change Culture (90/6)</th>
<th>Glutamine change culture (90/1)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell growth model</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Start cell density</td>
<td>X&lt;sub&gt;vo&lt;/sub&gt;</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Maximum growth rate</td>
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<td>0.036</td>
</tr>
<tr>
<td>Decay constant</td>
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<td>1.45E-06</td>
<td>1.05E-06</td>
</tr>
<tr>
<td>Decay exponent</td>
<td>β</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><strong>Antibody production model</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum specific production rate</td>
<td>q&lt;sub&gt;Abmax&lt;/sub&gt;</td>
<td>0.95</td>
<td>1.7</td>
</tr>
<tr>
<td>Decay constant</td>
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<td>0.003</td>
<td>0.075</td>
</tr>
<tr>
<td>Decay exponent</td>
<td>b&lt;sub&gt;Ab&lt;/sub&gt;</td>
<td>0.95</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Table 8.1. Comparison of model parameters for three representative culture types
of the dynamically operated cultures can be seen in the decay constants and exponents. The
decay constants are much higher for the dynamic cultures, and the increased curvature of the
specific antibody productivity with viability time is seen in the lower decay exponents.

8.6 Model advantages and usefulness

The model presented here provides a conceptual framework and an accurate description of
the cultures using a small set of parameters. This description is valid for all the important
phases of the culture, including the death phase up to the point where antibody production
has ceased. The model provides some insight into the mechanisms of culture performance
and can serve as a conceptual framework for performance analysis. In addition, a physical
meaning can be assigned to the parameters of the model.

There are some requirements that are not met by this model though. The model in its
present form can not be used to completely predict process behavior. The data generated in
this investigation does not permit the incorporation of the functionality of the model
parameters on culture conditions. However, a model of the type presented here can provide
a vehicle for such process descriptions, and can thus serve as a guide for future
experimentation. To accurately describe and predict process behavior, the influence of
several parameters has to be elucidated. While the height of the nutrient concentration
change docs not appear to have a major influence on process performance, the timing if the
nutrient concentration change clearly has an influence. It is also possible that the initial
concentration of the limiting nutrient is of importance. To investigate the effects of initial
nutrient concentrations and of the nutrient starvation period, a set of factorially designed
experiments can be performed for each of the main types of cultures presented above. The
data then can be incorporated in one or more of the model parameters. For instance, the
stimulation of the specific antibody productivity can as a first estimation be modeled as a function of the concentration of the limiting nutrient and the time period of the limitation
\[ q^{Ab} = q^{Ab}_o + q^{Ab}_{dyn}(C_{lim}, t_{lim}) \]

where \( q^{Ab}_o \) is a basal antibody productivity seen in batch cultures, \( q^{Ab}_{dyn} \) is the enhancement of antibody productivity resulting from the dynamic operation, \( C_{lim} \) is the concentration of the limiting nutrient during the limitation period, and \( t_{lim} \) is the time period of the limitation, in runtime \( t \) or viability time \( \tau \). The time period before the nutrient concentration change does not have to be explicitly modeled in \( \tau \)-space, as it occurs very early and thus accounts for a minute fraction of the space. Such an expanded model can serve as the basis for process optimizations. It may for instance be possible to optimize the choice between stimulation of cell growth and specific antibody productivity, to obtain the highest final antibody concentration. Such an optimization can perhaps best be done by optimizing the specific antibody productivity in \( \tau \)-space, as the integral of this parameter in \( \tau \)-space is the total antibody amount in the reactor. The aim would then be to optimize the integral \( \int q^{Ab} \, d\tau \), given an objective function. The use of the model as a conceptual basis for process refinement will be further discussed in the chapter on future work.
9. Summary

The objective of this work was to utilize rapid changes in culture conditions of a model mammalian cell fed-batch culture with the goal to improve the protein production, productivity, and quality. This objective was derived from the opportunities that exist to utilize the dynamic response of these cells to environmental stress and changes in culture conditions. Fed-batch culture is the model system of choice for the development of such control strategies, as they are inherently dynamic processes with continuously changing environmental conditions, and because they are likely to be approved by regulatory agencies for the production of biological therapeutics. The research presented in this thesis has shown that it is possible to positively affect the productivity of a mammalian cell fed-batch culture with dynamic nutritional control. The objective was achieved by conducting a series of experiments that searched for stimulatory effects on monoclonal antibody production, by subjecting the hybridoma cells to a period of limitation for glucose or glutamine and then releasing that limitation. The overall result was an improvement of protein production with maintained product quality in dynamically controlled cultures by about 40% over that of a reference batch culture.

9.1 Summary of process performance

The primary conclusion from this work is that dynamic operation of a CRL 1606 hybridoma fed-batch culture can significantly increase the antibody production of the process, as well as both volumetric and specific productivity, compared to a batch culture. The average increase in antibody production seen here is about 40%, compared to the average antibody production for the reference experiments, to a mean antibody
concentration of 119±3 mg/L from a mean of 86±1 mg/L. However, the increased production results from different factors for different types of dynamic nutrient controls. A glutamine concentration change increases specific antibody productivity, while it decreases both growth rate, maximum viable cell number, and the viability time (\([J_{X_v}, dt]\)) of the culture; The increased specific antibody productivity is nevertheless less able to offset the lower viability time, and the end result is an increased antibody production. Glutamine change cultures also have a prolonged growth period and reach peak viable cell number and final antibody concentration about 2 days after a batch or glucose change culture. For a glucose change culture, there is a smaller increase in specific productivity, as well as an increase in both maximum viable cell numbers and the viability time of the culture, resulting in an increased final antibody concentration. A culture with a simultaneous step or ramp change in both glucose plus glutamine concentration behaves as a glucose controlled run in most cases, although in one cases the behavior is similar to that of a glutamine controlled run. This probably reflects the extent to which the cells experience glucose or glutamine limitations in these runs, before the nutrient concentration change occurs.

It is clear from the results that the nutrient concentration changes are responsible for the increased antibody production. There is no difference in the process performance from that of a batch culture in experiments where the concentration change comes just after the start of cell growth, i.e. before the cells experience any significant nutrient limitations as evidenced by growth rate. There further appears to be a window in time during which the nutrient concentration change can stimulate the culture. In experiments where the concentration change is delayed until well into the growth phase, maintaining only a very low feed rate up till the concentration step change, there is no significant increase in either cell growth or antibody production. In contrast, neither the height nor the rate of the
nutrient concentration change seems to have any major effect on the culture within the levels used in this investigation. Nutrient concentration ramp changes, or lower concentration step changes do not behave differently from the step change runs. This is probably because the nutrient concentration difference between limiting and non-limiting conditions are much narrower than those used in this investigation, and the cells will go from limiting to non-limiting conditions within much shorter time frames than their metabolic response times.

The volumetric productivity of a glucose change culture is higher than that of a glutamine change culture. The glucose change culture is therefore the best performing process, as both glucose and glutamine cultures reach similar final antibody productivities. The volumetric productivity of a glucose change culture is significantly higher than that of a batch culture. In this investigation cultures where followed long past the peak in viable cell density to firmly establish the final antibody concentrations. In an industrial production process the medium would be harvested before this point, to avoid excessive cell death and release of cellular proteins and nucleic acids into the medium. This should be accounted for in comparing volumetric productivities, as well as the turnaround time of the reactor before the next culture can be started. For example, in the glucose step-change culture 90/6 cell death commences at 110 h after inoculation. The antibody concentration at this time is 110 mg/L. The corresponding figures for the batch culture 89/5 are 105 h and 66 mg/L.

Assuming a turnaround time of 8 h, the volumetric productivity becomes 0.93 mg/L·h (110 mgL⁻¹/118 h) for the dynamically operated culture, and 0.58 mg/L·h (66 mgL⁻¹/113 h) for the batch process, an improvement of 60%. In addition, judging from the variation in the final antibody concentrations of the different cultures it may be possible to further improve the performance of dynamically operated processes over that of batch cultures. This will be expanded on in the suggestions for future research below.
9.2 Mathematical description of process behavior

The behavior of the batch and dynamically operated processes are summarized in the parameters of a model that was developed from the experimental data. The model describes the culture behavior accurately during the entire process, including the death phase. The model includes parameters for the maximum apparent growth rate and specific antibody productivity as well as decay factors for these parameters. The model can provide a conceptual framework for future experimentation, and a basis for process optimization (see suggested future research).

9.3 Summary of events during dynamically operated cultures

The changes in nutrient concentration that the cells are subjected to during the course of a dynamically operated culture have a remarkable impact on their metabolism, growth rate, size distribution, and production of secreted proteins (antibodies), compared to a batch culture. These effects appear to be initiated during the nutrient limitation period, and then decay with runtime and viability time.

9.3.1 Inoculation, lag and the nutrient limitation phases

The events just after the inoculation of the reactor with cells are the same for a batch and a dynamically operated culture. During the lag phase, the cells shift in size to larger sizes, and start dividing after 12 to 24 h. Shortly thereafter, the first difference between batch and dynamically operated cultures becomes visible. In a batch culture the cell will continue to divide until they reach their maximum viable cell density, and then start to die. In a dynamically operated culture, the cell becomes limited for glucose or glutamine approximately 12 to 24 h after growth started (for the nutrient concentrations used in this
in investigation). As the nutrient limitation becomes more severe the cells shrink in size, down to about 80% of the average volume of cells growing in a batch culture. There is a difference between glucose limited cells and glutamine limited cells during this period. Cells subjected to a glucose limitation appear to be able to compensate by utilizing glutamine quite readily, as evidenced by growth rates and glutamine uptake rates. Cells limited for glutamine appear less able to compensate for the limitation, and they grow slower during this period than glucose limited cells. As a consequence the glucose limited culture will reach the target cell density used to initiate the nutrient concentration change faster than a glutamine limited culture. A glucose change culture reaches the target cell density about 36 h after inoculation, while the corresponding time for a glutamine change culture is about 48 h. During the nutrient limitation period, a culture limited for both glucose and glutamine appears to be more severely limited for one of the nutrients and will behave accordingly. That is, a culture with a simultaneous change in both glucose and glutamine concentration displays a behavior consistent with that of either a glucose change culture, or a glutamine change culture.

9.3.2 After the nutrient concentration change

After a change in nutrient concentration, there is a dramatic shift in the behavior of the culture. Very shortly after the change the cells display a transient in oxygen uptake, which declines back to a value about 10% higher than before the change after less than 6 h. At this time, the cells also increase the specific uptake rate of the limiting nutrient to more than twice the rate before the change. Between 2 to 6 h after this increase, an increase in the specific production rate of the corresponding waste product (i.e. lactate or ammonia) is seen, at levels consistent with the uptake rate of the nutrient. There is no significant difference seen between ramp-changes and step-changes in nutrient concentrations at this time, or later
in the culture (as explained elsewhere). A culture subjected to a ramp-change in the concentration of the limiting nutrient behaves approximately the same as if it was subjected to a step-change in concentration, although a somewhat slower change in culture parameters is sometimes seen. The nutrient uptake and waste product formation rates peaks between 4 to 12 h after the change. However, the cells do not start dividing immediately, instead, they increase in size, up to about 30% of the average cell volume of an actively growing batch culture. This is about a 60% increase in average volume from before the nutrient concentration change, approximately a 35% increase in the surface area of the cells. During this time the cells divide at a rate faster than before the change, but clearly slower than their normal rate. First about 24 h after the change do the cells start dividing at their maximum rate. Also at this time there is a difference between a glucose change culture and a glutamine change culture. A glucose change culture resumes its growth at a rate similar to or higher than that of a batch culture. A glutamine change culture resumes its growth at a rate that is lower than that of a batch culture. Most importantly, after the nutrient concentration change there is an increase of the specific antibody production over that of a batch culture. Again glucose and glutamine change cultures behave differently. For a glucose change culture, there is an increase in specific antibody productivity of about 0.6 mg/10^9 cells·h over the maximum seen in a batch culture (1 mg/10^9 cells·h). For a glutamine change culture, there is a larger increase, up to 1.2 mg/10^9 cells·h higher than the maximum rate seen in a batch culture. The specific antibody productivity peaks about 12 to 24 h after the nutrient concentration change for a glucose change culture, and about 24 to 48 h after the change for a glutamine change culture.
9.3.4 The remainder of the culture

The changes in culture behavior that where induced during the “limitation-limitation release” cycle of a dynamically operated culture continues to impact the culture for a significant time period after the change in nutrient concentration. As before, glucose and glutamine change cultures behave differently. A glucose change culture continues to grow at a rate similar to a batch culture, but displays a somewhat prolonged growth period, and reaches a slightly higher maximum viable cell number. The elevated maximum viable cell number is especially visible if cell size distribution is taken into account. In the 3-dimensional graphs presented the peak representing viable cells a glucose change culture is significantly higher than that of a batch culture. By contrast, a glutamine change culture continues to grow at a rate lower than that of a batch culture. Consequently the growth period of these cultures is longer. The glutamine change cultures reaches its maximum viable cell number almost two days after a batch or a glucose change culture. The same effect is seen in the antibody production of the cultures. A glutamine change culture reaches its final antibody concentration about 2 days after a glucose change culture. The elevated specific antibody production rate is seen to decay over the course of the dynamically operated cultures, approaching that of a batch culture approximately at the time of the peak in viable cell number of the cultures. This is strikingly visible in viability time (explained in the introduction to the result and discussion chapter). Here the rates of glucose change and glutamine change cultures are seen decaying at a similar rate, approaching that of a batch culture. The stimulation of the specific rate induced by the nutrient concentration change influences the cells for a significant time. Also the changes in cell metabolism that were induced after the nutrient concentration persist for some time during the course of the culture. Both glucose and glutamine change cultures have a less aerobic metabolism than a batch culture, and have lower oxygen uptake rates. A glutamine change culture continues to utilize glutamine at a rate higher than a batch culture, and has an
ATP production rate clearly lower than a batch culture, consistent with the reduced growth rate of these cultures. A consequence of the higher glutamine utilization is a higher ammonia formation rate. Glutamine change cultures therefore have higher ammonia concentrations in the media than does a batch culture, both during the main growth period of the culture and at the end. A glucose change culture also utilize more glutamine than a batch culture, but it in addition also has a higher glucose uptake rate. The ATP production rate of these cultures are somewhat lower than that of a batch culture. The higher glucose and glutamine uptake rates of these cultures lead to higher concentrations of both lactate and ammonia in the medium than seen in a batch culture, during the main growth and antibody production period as well as at the end of the culture.

After the peak in viable cell number has been reached in the cultures, the cells starts to die at an accelerated rate. The average cell density in all types of cultures decreases significantly just before the peak, as does the specific nutrient and oxygen uptake rates. However, the cells continue to metabolize nutrients and produce antibodies. The final antibody concentration of the cultures is reached about one third into the death phase.

9.4 Antibody composition and quality

There is no significant difference in the quality of the antibody produced in dynamically operated runs compared to the reference runs, as judged by the limited analysis performed here. While minor differences can be detected, there is no apparent correlation with the nutrient control scheme used during the run. CRL 1606 secretes chimeric antibodies assembled from all possible combinations of the MOPC 21 immunoglobulin chains, and the anti-fibronectin immunoglobulin chains that stem from the spleen cell fusion partner. Moreover, the cells secrete not only complete antibodies (Heavy$_2$Light$_2$), but also
incomplete antibodies (Heavy₂Light) as well as heavy chain dimers (Heavy₂) and free heavy and light chains. The heavy chains of the antibodies are glycosylated as expected, and no fragments of antibodies has been detected in the runs. Probing immunoblots with less restrictive antisera reveals minor, but probably insignificant, differences between different runs.

9.5 Waste product formation and inhibition
All the dynamically operated cultures investigated in this work have higher concentrations of waste products than is normally seen in batch cultures, both at the end of the cultures as well as during the main growth and antibody production phases. Glutamine change cultures have higher concentrations of ammonia, and glucose change cultures have higher concentrations of both lactate and ammonia. No correlation has been found in the data presented here between waste product concentrations and either cell growth or antibody production. In fact, the high producing cultures are all in the higher range in terms of final waste product concentrations. This is in apparent contrast to a number of other investigations where inhibitions of high waste product concentrations have been well documented, especially for ammonia. However, the results presented here do not exclude inhibitory effects of waste products, although it may call into question the level of the inhibitions for the cell line used. Similar indications has also been found in other investigations (Dodge, Ji, and Hu, 1987). Thus, while inhibitory effects by ammonia and lactate no doubt exists, it is clear that the stimulatory effects from the dynamic operation used here outweigh the influence of such inhibitory effects.
9.6 Cellular mechanisms

The intracellular mechanisms responsible for the increase in specific antibody productivity seen in dynamically operated cultures are not known. Neither is the mechanism known that regulates the growth rate at a lower level following a glutamine concentration change. However, considering the nutrient limitation period the cultures experience before the change in nutrient concentration, it can be speculated that the effects seen are the result of a stress response by the cells. This is further supported by the fact that the length of the nutrient limitation period and the type of nutrient limitation (glucose or glutamine) are of importance for the response. Mammalian cells are known to increase their specific protein synthesis under a number of different conditions that are environmentally detrimental to them (see literature review). Several parts of the cellular machinery of a mammalian cell can conceivably cause the increase seen in specific antibody productivity. However, the rate limiting steps in mammalian cell synthesis and secretion is at present believed to be the post translational processing of proteins in the endoplasmic reticulum, and the regulation of export of proteins from the endoplasmic reticulum to the Golgi (see literature review).

There are a number of indications in the literature that the rate of post translational processing can be effected by a number of environmental conditions (see the literature review). A common denominator for these effects is that the components of the post translational network are induced to higher levels under conditions of stress for the cell, such as high temperature or nutrient starvation. For instance, the immunoglobulin binding protein BiP is constitutively present in the endoplasmic reticulum at high concentrations, but is induced to even higher levels by glucose starvation and other conditions known to effect protein folding and glycosylation (Lee, 1987; Kozutsumi et al., 1988). It is possible that the nutrient “limitation-limitation release” that cells are subjected to in dynamically controlled cultures affect the function of the post translational machinery, thereby increasing the rate of protein processing and secretion. This could be achieved by an induction of
parts of the proteinaceous machinery by the low nutrient conditions during lag and early
growth phase, followed by a stimulation by the large nutrient concentration change.
However, there are also a number of other cellular mechanisms that could explain the increase
in antibody production, such as an increased transcription of immunoglobulin genes. Any
attempt to rationalize the increase in specific antibody production therefore remains a
speculation at this stage.

The mechanism(s) behind the increase in the average cells size following a change in
nutrient concentration are not clear. One possible explanation could be that the cells
maintain their internal osmolarity by an increased flux of water along with the increased flux
of nutrients into the cells and the concurrent generation of waste products. Also, the
increased flux in nutrients into the cells and waste products out of the cells may require a
larger membrane surface area. It would be interesting to investigate for example the number
of glucose transporters present on the cell membrane before and after a glucose
concentration change. Further interpretation of this phenomena has to await more detailed
data.
10. Conclusions

This research was conducted to establish a simple proposition: the dynamic response of mammalian cells to environmental stress and to changes in their environment can be utilized to improve the performance processes using such cells. The work has shown that it is possible to take advantage of the increased specific antibody productivity of mammalian cell under conditions of environmental stress, while avoiding some of the disadvantages of those conditions. This was accomplished by inducing dynamic changes in the concentration of the primary nutrients, glucose and glutamine. Dynamic operation of bioreactors offers a way to achieve enhanced process efficiency. In this investigation the average increase in protein production was 40% over that of a reference batch culture, while the volumetric productivity could be approved by about 60%. The results also indicate that there is a potential to increase productivity beyond that achieved here.

10.1 Summary conclusions

The main conclusions from this research are:

1. Dynamic operation of a mammalian cell fed-batch bioreactor can increase antibody concentration and productivity. For the hybridoma model system (CRL 1606) there was an increase in monoclonal antibody production by an average of 40% over that of a reference batch culture.

2. The best performing protocol was a glucose change culture, which has a volumetric productivity about 60% higher than that of a batch culture. A glutamine change culture has
a prolonged growth period but a lower volumetric productivity.

3. The increase in productivity was the result of different factors for different type of nutritional control. A glucose change culture has a somewhat higher viable cell number and specific antibody productivity compared to a batch culture; the result is higher antibody production. A glutamine change culture has a lower viable cell number, but the specific antibody productivity is significantly higher resulting in improved antibody production. Cultures with a simultaneous change in the concentration of both glucose and glutamine can behave similar to either a glucose or a glutamine change culture. One possible explanation for this could be that there is a dominating nutrient limitation for either glucose or glutamine. The results are consistent with a stimulation of the specific antibody productivity in dynamically operated cultures.

4. There was no difference seen in the performance between nutrient concentration step-change or ramp-change cultures. The height of the nutrient concentration change (i.e. the concentration difference before and after the change) also does not appear to be important.

5. There appears to be a window in time during which a change in nutrient concentration can affect the culture. If the change comes too early there is no response. One reason for this could be the culture has not experienced a significant nutrient limitation at this point. If the change comes too late (after the early growth phase) there is no response; this may be because the cells are adversely affected by a too long nutrient limitation period.

6. There was no difference found in antibody quality between batch cultures and dynamically operated cultures, by the western blot and gel electrophoresis analysis performed. The CRL 1606 hybridoma to secretes chimeric antibodies from the MOPC21
and anti-fibronectin antibody chains (H₂L₂) as well as incomplete antibodies (H₂L), heavy chain dimers (H₂) and free light chains (L). There were no significant intracellular stores of antibodies found.

7. The introduction of viability time (∫Xv dt) provides a methodology for comparing cultures with differing growth patterns and is useful for interpreting results.

8. A model was developed that can adequately describe the behavior of the cultures under dynamic conditions. This can serve as a basis for future experimentation and process optimizations.

9. Dynamic operation of a culture has significant effects on the size distribution of the cells. Compared to a batch culture, cells in a dynamically operated culture have a smaller average volume before the nutrient concentration change, and a larger average volume after the change. There is a delay after the concentration change before the cells start dividing, during which the cells increase in size.

10. Dynamically operated cultures have a more anaerobic metabolism after the nutrient concentration change. A glutamine change culture metabolizes glutamine at a higher rate than a comparable batch culture, with a corresponding higher rate of ammonia formation. A glucose change culture metabolizes both glucose and glutamine faster than a batch culture and has a higher rate of both of lactate and ammonia formation.

11. The altered metabolic rates of a dynamically operated culture results in higher concentrations of metabolic waste products in the medium. A glutamine change culture has
higher concentrations of ammonia; a glucose change culture has higher concentrations of both lactate and ammonia. There is no evidence of waste product inhibition in these cultures. Although the results does not exclude inhibitions, it is clear that the stimulation of the culture from dynamic operating protocols outweigh any inhibition.

12. Dynamic oxygen uptake rate measurement correlates well with viable cell density and can serve as an estimation of viable cell density. A control system was constructed that uses a combination of feedback estimation of viable cell density and model predictive feed-forward control to determine nutrient feed rates. This control system was capable of maintaining a nutrient concentration trajectory within 10% of the desired value.

10.2 Significance

The experimental approach used here utilizes a sophisticated control system to obtain desired nutrient concentration trajectories. Having established the basis for the process operation in this work, the results suggest that it may be possible to utilize a simpler controls system to achieve the desired result. By proper design of the conditions of the process, it may be necessary to control only the start phase of the culture and the process can be left to proceed to completion uncontrolled after the change in nutrient concentration. The design of an industrial production process utilizing dynamic operation may therefore not be much more complicated than for a batch culture.

It has been shown here that the dynamics of mammalian cell cultures can be used to advantage in the development of industrial production process. It also has been shown that it is possible to take advantage of the stress response of mammalian cells, while minimizing the disadvantages of environmental stress. Mammalian cells are perhaps not most
productive when they are operated in a steady state, but instead are able to increase their secretory activity under more rapidly changing culture conditions. Fed-batch cultures could be the most convenient way to operate such processes.

In conclusion, the dynamics of mammalian cell cultures can be understood, can be investigated systematically, and can be used to take advantage of the responses of these cells to achieve an improved process performance.
11. Recommendations for Future Research

The possibility to improve process performance by utilizing dynamic changes in culture environment provides a complementary avenue for process development, in addition to the more common use of factorially designed batch cultures. However, as this is a first attempt to investigate such behavior, the research presented here opens up many questions. It is clear that the potential exist to further improve process performance above that seen in the results presented above. But how to accomplish this? The fact that glutamine change cultures exhibit an significantly increased specific antibody productivity raises questions about the cellular mechanisms behind this response. Investigating such mechanism may be a challenging task; are there ways to at least obtain a basic understanding of the cellular events occurring in a dynamically operated culture? Further, in these high producing cultures there was an unusually high concentration of waste products. Is it possible to utilize the experimental approach of this investigation to experimentally verify the level of waste product inhibitions under conditions close to those of a real production process? While this research may have found one way to utilize a stress response of mammalian cells to improve process performance, it raises the possibility that there may be other ways to obtain similar effects. The sections below provide some ideas on how to experimentally pose such questions, and how to obtain the answers.

11.1 Production potential

The potential exists to further improve that productivity of dynamically operated cultures. For instance, if the viable cell density of a glutamine change culture could be maintained at the level of that of a glucose change culture (or even a batch culture), the productivity could
be improves by over 200% over that of the reference batch culture. Fig. 11.1 shows the production that could be achieved in such an ideal culture. In this calculation, the specific productivity of a glutamine change culture has been multiplied by the viable cell number of a glucose change culture. When the resulting volumetric productivity is integrated over runtime, the result is a final antibody concentration of over 200 mg/L, an improvement of more than 200% over the productivity of a batch culture. This can be viewed as a best case scenario. While worst cases are not difficult to obtain, the real question is of course how to design process development strategies that can elicit such productivities. The simple strategy that was attempted in this investigation, combining a glucose plus glutamine concentration change, did not achieve this goal. However, the variation in the level of antibody productivity seen among the dynamically operated cultures implies that it may be possible to further improve process performance, if the operation of the processes can be optimized. Potential avenues for further process refinement are outlined in the section below.

11.2 Process refinement

Process refinement should continue on several parallel tracks. On one hand the research into other methods to induce and utilize changes in process conditions should continue. At the same time, the existing paradigms for design and control of dynamically operated fed-batch reactors should be refined and optimized. The parameters that are of importance for the production of proteins should be elucidated, and the functionality of the culture parameters should be investigated. From the data at hand, it appears that it is mostly the events before the nutrient concentration change that influence process behavior. This is, however, an assumption that should be verified experimentally. Among the parameters that may influence process production and behavior are the concentration of the limiting nutrient
Fig. 11.1. Hypothetical process improvement. The antibody productivity shown is obtained by combining the high specific antibody productivity of a glutamine change culture with the high viable cell number of a glucose change culture.
during the period before the concentration change; the time of the nutrient limitation period; the initial concentration of nutrients at inoculation; and the cell density at inoculation.

Investigating the influence of a set of inter-related parameters like those mentioned above are best done in a factorially designed experiments. In this case these parameters have to be investigated for each of the nutrients, glucose and glutamine, and can require a large set of experiments. The results can be used to develop a mathematical model of the process behavior which can be used to optimize the process given a desired objective function. Suggestions for modeling and optimization are presented separately below.

Designing and testing other methods to utilize dynamic changes in the culture environment and to induce improved production may require more creative approaches. Only a few possibilities will be discussed here. The possibility of combining the high specific productivity of a glutamine change culture with the cell density of a batch or glucose change culture is intriguing. It raises the question if this can be achieved by limiting the cells for both glucose and glutamine. This assumes that the response to limitations of these two nutrients can be separated. In this investigation the combination of simultaneous glucose plus glutamine changes was tried, unsuccessfully. Also, the use of ramp-changes as opposed to step-changes did not further improve process performance. There are, however, a number of possibilities left untried to elicit the desired response. It appears difficult to limit the cells for both nutrients simultaneously. However, the ratio of the nutrients during the limitation period may have been inappropriate. Therefore, different ratios of glucose to glutamine concentration can be investigated. The cells may nevertheless always be more limited for one nutrient that for the other. Another, perhaps more robust method, is to attempt to serially limit the cells for the nutrients. By subjecting the cells to a limitation for one nutrient first, and thereafter for the other, it may be possible to elicit a response of true
dual nutrient limitation. One way to design such a process is to inoculate the culture in a medium where both nutrients have low concentration, but where only one will be limiting. After a period of limitation for this nutrient, the culture can be fed a medium that contains a low concentration of the limiting nutrient only. This will feed a small amount of the limiting nutrient, and at the same dilute the other nutrient to a lower concentration. Now the concentrations of the nutrients will switch to be limiting for the other nutrient. After an appropriate time of limitation for the other nutrient, the culture is then subjected to the nutrient concentration change. Such a process will utilize three different media. A start media, a nutrient limitation change media, and a high nutrient concentration feed medium.

Methodologies to elicit stress responses of mammalian cells other than nutritional control also can be investigated. One possibility is to investigate the effect of limitations for and changes in oxygen concentration in the media. Some work in this direction has already been published. (Ozturk and Palsson, 1990; Meilhoc, Wittrup, and Baily, 1990; Miller, Wilke, and Blanch 1987 and 1991). Temperature is of course a parameter that is known to elicit a stress response in mammalian cells (thereof the name “heat shock”, see literature review). Several other investigations have already been made to utilize temperature and temperature changes to improve process production, not all of them successful as conditions that induce an increased specific production of proteins also in general are detrimental to cell growth (see literature review). An example of such an approach was recently presented by Sureshkumar and Mutharasen (1991). They grew hybridoma cells at 33 °C which maximizes the viable cell density in the reactor. When the culture had reached the maximum viable cell density, the temperature was shifted to 39 °C where the specific antibody productivity is highest (but where cell growth is impaired). However, as the cell viability decreased rapidly at the higher temperature, the resulting final antibody production was not different from that of a normal culture at 37 °C. Nevertheless, the possibilities to
increase process production by temperature manipulations are not exhausted, and it may be feasible to design successful strategies.

11.3 Control strategies for dynamically operated reactors

In this investigation, considerable attention was focused on controlling the concentration trajectory of the nutrient under investigation, especially after the nutrient concentration change. The purpose of this was to establish that the limitation of the nutrient and subsequent change in nutrient concentration was responsible for the process response, and not unintended variations in nutrient concentrations during the culture. The results presented above confirms this assumption. They also indicates that the nutrient concentration trajectory after the concentration change may not be of importance, as long as the concentration is in a clearly non-limiting region. The control system designed as part of this project may therefore have been applied in the wrong phase of the process. Tight and accurate control of the nutrient concentration trajectory may be more important during the nutrient limitation period than during the time after the concentration change. In fact, it may be that the trajectory after the concentration change does not have to be controlled at all. It may perhaps be possible to feed all of the feed medium to the reactor at once, and then let the culture take its natural course. This assumption of course has to be experimentally verified. If the assumption is corroborated, the focus of the control system then shifts from the period after the concentration change to the period of the nutrient limitation.

The exact influence and importance of the limiting nutrient concentration trajectory during the limitation phase is not known, and has to be established. It is possible that the exact concentration is not important, as long as it is well inside the limiting region. In this case it may not be necessary to control the culture environment at all. It would suffice to design
start and feed mediums with appropriate compositions. The only parameter of importance for the control of the culture in this case would be the timing of the nutrient concentration change. In the unlucky event that there is a marked dependence on the trajectory of the limiting nutrient during this period, then a suitable control system has to be employed. Maintaining a controlled nutrient trajectory at the low concentrations desirable before the concentration change can be a difficult task. The main indicator of culture growth used here, estimation of viable cell density from OUR, is inaccurate in this region due to the low cell density. There are, however, other ways to obtain estimates of the nutrient concentration and uptake rates in this culture phase. One way is to directly analyze the medium for the concentration of the controlled nutrient. This can be achieved by using a automatic sampling device that extracts a sample of the medium while maintaining a sterile barrier. The sample can be automatically be assayed in an analyzer such as an HPLC or a FIA system. Alternatively or complementary, an estimate of viable cell density and growth during this period of the culture can be obtained from a redox probe. The redox potential of the medium declines rapidly with the onset of cell growth (Hwang, 1992), and redox potential can therefore provide an accurate signal representative of viable cell density at a time when the oxygen uptake rate is low and its measurement inaccurate. However, feeding fresh feed medium will increase the redox potential and this has to be accounted for. Fig. 11.2 shows an example of the connection between redox potential, oxygen uptake rate, and viable cell density for a fed-batch culture. Fig. 11.3 zooms in on the beginning of the culture, and shows that there is a much larger change in redox potential during this phase than in oxygen uptake rate. The cause of the “hump” in the redox signal at the beginning of the culture at 5 h is not known. It may indicate an onset of growth, followed by a limitation for nutrients. To utilize the redox signal, a possible control strategy could be to maintain a desired trajectory of redox potential during this period, by controlling the flow of the feed medium. With proper design of the feed medium it could be possible to obtain a desired
Fig. 11.2a (upper) and 11b (lower). Example of the connection between redox potential, oxygen uptake rate, and viable cell density for a fed-batch culture with an early glucose plus glutamine step change (run 91/3). Media volume shown for reference. The redox signal has not been compensated for dissolved oxygen potential.
Fig.11.3 a (upper) and 1b (lower). Example of the connection between redox potential, oxygen uptake rate, and viable cell density for a fed-batch culture with an early glucose plus glutamine step change (run 91/3). Zoom in on the beginning of the culture. Media volume shown for reference. The redox signal has not been compensated for dissolved oxygen potential.
concentration trajectory reproducibly in this way. Lastly, accurate control of nutrient concentration during this period may more easily achieved employing a more dilute nutrient feed medium. Such a control system should therefore use three separate media: a start medium in the reactor, a nutrient limitation maintenance media, and a nutrient concentration change feed media.

The success of the control system employed here to a large extent depends on the availability of a feed-back signal proportional to the culture state; the oxygen uptake rate. To take full advantage of this signal, the system used a rule based program to select the appropriate relationship between OUR and viable cell density. Both this relationship and other culture parameters, such as nutrient uptake rates, change with the culture environment. There is a need in such control systems to select appropriate parameters among a large set under complex and changing conditions. One way to design such control systems is to employ artificial intelligence methodologies in the design of the system. Programming technologies such as expert systems and neural networks can facilitate the management of complex information as well as provide a methodology to incorporate qualitative knowledge. However, before such intelligent systems can be used to their full potential for the control of mammalian cell cultures a more complete knowledge about operating strategies for, and behavior of these processes is needed. Contributing to that knowledge base has been part of the motivation for this work.

11.4 Modeling and optimization

To maximize the protein production of a dynamically controlled culture it is desirable to obtain a mathematical model of the culture response to environmental factors. Such a model was suggested above. To use this model for process optimization, the specific antibody
productivity could be modeled as a function of the important process parameters before the nutrient concentration change, for instance the start concentration of the limiting nutrient \( C_o \), the nutrient trajectory during the limitation phase \( C_{\text{lim}} \), the time of the nutrient limitation period \( t_{\text{lim}} \), and the initial cell density \( N_{v0} \), i.e.

\[
q^{\text{Ab}} = f(C_o, C_{\text{lim}}, t_{\text{lim}}, N_{v0})
\]

In such a model it is not necessary to explicitly describe the behavior of the culture before the nutrient concentration change. Only the subsequent behavior has to be accounted for. The total antibody concentration is equal to the integral of the specific antibody concentration in \( \tau \)-space

\[
A^{\text{Ab}} = \int_{0}^{t} q^{\text{Ab}} \, d\tau
\]

The objective function of an optimization can therefore be to maximize this integral, subject to suitable constraints, for example the number of dead cell that can be allowed in the culture before harvest. The result can be numerically transformed to runtime, in order to design the control trajectories for the control system.

### 11.5 Antibody quality

In this thesis only a limited investigation of antibody quality has been presented. A more comprehensive study of antibody quality as a function of culture conditions should be done. There have been indications in this investigation that the process conditions affect product quality. For instance, in some cultures large unspecific binding of antibody was seen in some of the immunoassays used. Product quality is largely defined by the methods used to
measure it. Here immunoprobing of western blots with a set of polyclonal antisera was used, as well as 2-D electrophoresis and glucosidase digestion. There are a number of other methods that can be used to assert the composition and quality of the antibodies produced. To mention some: isoelectric focusing in combination with immunoblots probed with an array of antisera can provide information of charged isomers (Coco-Martín et al., 1991). Lectin column chromatography can provide an indication of heterogeneity of glycosylation, and so on. A thorough investigation of this subject can fill a thesis by itself, and will be left to a graduate student so inclined.

11.6 Waste products

The results obtained in this work have to some extent called into question the level of inhibition exerted by the waste products lactate and ammonia on the CRL 1606 hybridoma. It is possible that they are not as inhibitory as indicated in previous investigations (Glacken, 1987; Adema, 1989), although the existence of inhibition is well documented. This may be due to the methods used to investigate such inhibitions. Cells subjected to high concentrations of waste products during lag phase may be influenced by this for a significant part of the culture. Such a response may therefore be more indicative of the influence of these compounds on cells in lag phase than in actively growing cultures. However, in a production process the start concentration of lactate and ammonia are always close to zero.

The methodology and control system developed in this research may be used to investigate waste product inhibitions under conditions and dynamics that mimic those of a production process. By superimposing ramp-functions in lactate and ammonia concentration on top of the “natural” generation of these compounds during the course of a culture, it may be
possible to obtain a more realistic estimate of the severity of these inhibitions. Fig. 11.4
show an example of the base level of a waste product secreted by the culture, and the
concentration trajectories of the superimposed waste product concentrations. Such
trajectories can be obtained by feeding the culture a medium containing lactate or ammonia
as well as nutrients. It is difficult to obtain an exact value of the inhibition constants, as the
experiments will be done under constantly varying culture conditions. To obtain more exact
values, continuous cultures of actively growing cells can be used as an alternative, although
the results may not be directly applicable to fed-batch cultures. Nevertheless, the approach
suggested above may be able to contribute to an evaluation of the importance of waste
product inhibitions in batch and fed-batch cultures. Similar approaches have already
appeared in the literature. For instance, Omasa et al. (1992) used fed-batch cultures to
maintain a level lactate concentration, and found that reduction of growth rate at high lactate
concentration was as much due to the increased osmotic pressure as to the lactate
concentration itself.

11.6 Cellular mechanisms
It is intriguing to note that the largest increase of specific antibody production is found after
a glutamine limitation, and that this stimulation was larger than that obtained after a glucose
limitation. This fact suggest that there may be a difference in the response to limitations of
these two nutrients, and raises the question what intracellular events occurs during the
limitations. The pattern of heat shock and glucose regulated proteins whose expression is
increased during a glucose limitation is well documented for a number of cells types (see
literature review). However, the corresponding cellular response for glutamine limitation
has not been investigated in detail. It would be interesting to elucidate if the same proteins
that are expressed during glucose limitations are expressed at higher levels during a
Fig. 11.4. Waste product concentration profiles in a series of cultures designed to investigate the influence of waste products. Concentration profiles generated by feeding the cultures medium containing the waste product under investigation.
glutamine limitation, and/or if there are other proteins that are expressed. This could
provide a first answer to the question of whether there is a separate mechanism for response
to glutamine limitation, distinct from that seen for glucose limitation. Such an investigation
could utilize the methodologies outlined in the literature on this subject and reviewed in the
literature chapter. This could be an excellent topic for a PhD thesis.

It should be mentioned that there can be other explanations for the increase in specific
antibody productivity following a glutamine limitation. For instance, the response could be
dependent on the changes in the metabolism seen after a glutamine concentration change.
In this context it should be noted that while both glucose and glutamine are used as energy
sources by mammalian cells, glutamine has been implicated to be preferentially used for
protein formation (Zielke et al., 1980; Wice et al., 1981; Medina and De Castro, 1990).

11.∞  The end

Pity the researcher developing mammalian cell culture processes. These processes are
immensely complex in their behavior, and the level of knowledge about the molecular
biology and cellular regulation of these cells at present prevents predictions of process
behavior based on first principles. Research in the development and control of these
cultures is therefore sometimes as much an art as a science. This work is not the end of
research on process development of mammalian cell cultures. But it is perhaps the
beginning of a new approach to view the behavior and development of these processes. The
data presented here opens up a new avenue for the design of large scale mammalian cell
cultures, although it unfortunately also introduces a whole new set of parameters to be
investigated. The work never ends......sigh.
12. References


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Appendixes

Appendix 1
Definitions, Calculations, and Data Treatment.

Appendix 2.
COMPARISON OF AN ELECTROCHEMILUMINESCENT HOMOGENOUS IMMUNOAASSAY AND AN ELISA FOR MONITORING PRODUCTIVITY IN MAMMALIAN CELL BIOREACTORS

Appendix 3
Additional experimental data.
Appendix 1
Definitions, Calculations, and Data Treatment

Balances are made on the total amounts of a species in the reactor. An implicit assumption in this is that the cells are not affected by the volume changes and dilution of cells that occurs during the course of the run. That requires that growth rates, uptake rates, etc of the cells are not a function of either media volume, or cell density, within the region of interest, which has been verified by the data presented above.

Nomenclature:
The nomenclature used here differs somewhat from that used to describe more traditional culture systems:

\( N \) = density of cells \((10^9 \text{ cells/L})\)

\( V \) = volume \((\text{L})\)

\( X \) = total cells in reactor = cell density \( \times \) medium volume \((10^9 \text{ cells}) = N \cdot V\)

\( C \) = concentration of a species (nutrient, waste product, etc) in the reactor \((\text{mmol/L = mM})\)

\( A \) = Total amount of a species in the reactor \((\text{mmol}) = C \cdot V\)

\( q \) = specific uptake or production rate (for nutrients, waste products, and antibodies)

\( \text{(mg/10^9 \text{ cells/h})} \)

\( Q \) = volumetric uptake/production rates \((\text{mmol/L} \cdot \text{h})\)

\( \mu \) = specific apparent growth rate \((\text{h}^{-1})\). \( \mu = \mu_{\text{real}} - \alpha \); Also called \( \mu_{\text{app}} \) in text.

\( \mu_{\text{real}} \) = real growth rate \((\text{h}^{-1})\).
\( \alpha = \text{specific death rate (h}^{-1}) \)

\( F = \text{feed flow rate (for nutrient feed to reactor) (L/h)} \)

**Sub- and super-scripts:**

Superscripts:  
- GLU = glucose  
- GLN = glutamine  
- LAC = lactate  
- AMM = ammonia  
- Ab = antibody

Subscripts:  
- v = viable (live)  
- d = dead  
- T = total (= live + dead)  
- feed = feed stream concentration or rate (mM or L/h)  
- t = at time t  
- 0 = at time 0  
- t+1 = at the end of the next time period

**Examples:**

Total amount of viable cells in reactor = \( X_v \)

Total amount of glucose in the reactor = \( A^{GLU} \)

Specific antibody production rate = \( q^{Ab} \)

Specific glutamine uptake rate = \( q^{GLN} \) (sum of maintenance and growth related uptake)
End of run data:

Values from the final data of the experiments are calculated from individual measurements as mean values (\( \overline{m} \)), weighted by the inverse of the variance (\( \sigma^2 \)) of the individual measurements (\( m_i \)) (Bevington, and Robinson, 1992).

\[
\overline{m} = \frac{\sum (m_i/\sigma_i^2)}{\sum (1/\sigma_i^2)}
\]

The standard deviation \( \sigma \) is calculated from the inverse sum of the variances of the individual measurements:

Standard deviation = \( \sigma \); and the variance = \( \sigma^2 \).

\[
\sigma_m^2 = \frac{1}{\sum (1/\sigma_i^2)}
\]

The standard error \( \alpha \) of the weighted mean:

\[
\alpha_m = \sqrt{\frac{\sigma_m^2}{N_0}}
\]

where \( N_0 \) is the number of measurements.

Other statistical parameters, such as ordinary means, variances, coefficients of variations, are calculated by standard methods (Bevington and Robinson 1992).

Rate data:

Cells are assumed to go through two processes during their existence in the reactor.

Growth, characterized by a specific growth rate \( \mu \) (1/h). Death, characterized by a specific
death rate $\alpha$ (1/h). Break down of dead cells to cell debris is neglected.

Definitions:
For cells:

Growth rate
$$\mu_{\text{real}} \equiv \frac{1}{X_v} \cdot \frac{d(X_T)}{dt}$$

Death rate
$$\alpha \equiv \frac{1}{X_v} \cdot \frac{d(X_d)}{dt}$$

Apparent growth rate:
$$\mu \equiv \frac{1}{X_v} \cdot \frac{d(X_v)}{dt}$$

$\mu = \text{apparent growth rate} = \mu_{\text{real}} - \alpha$; also called $\mu_{\text{app}}$ in the text.

For nutrients and waste products:
All definitions and calculations of specific rates are based on viable cells. This assumes that only live cells are metabolically active, and that there is no significant release of antibodies by dead cells.

Examples:

Specific glucose uptake rate:
$$q^{\text{GLU}} \equiv \frac{1}{X_v} \cdot \frac{d(A^{\text{GLU}})}{dt}$$

Specific antibody production rate:
$$q^{\text{Ab}} \equiv \frac{1}{X_v} \cdot \frac{d(A^{\text{Ab}})}{dt}$$
For viability time: This parameter is defined in the introduction to the Results and Discussion chapter.

**Balance on cells in the reactor:**

\[
\frac{d(X_T)}{dt} = \mu_{real} X_v - k X_d
\]

\[
\frac{d(X_v)}{dt} = \mu X_v = \mu_{real} X_v - \alpha X_d
\]

\[
\frac{d(X_d)}{dt} = \alpha X_v - k X_d
\]

Here the cell breakdown rate \( k \) is neglected, i.e. assume \( k = 0 \).

**Balance on nutrients, waste products, and products in reactor:**

Use an integral balance between time 0 (start) and time \( t \), and use total amounts fed.

Example for a nutrient, glucose:

\[
A_{0,GLU} + A_{F,GLU}|_t - A_{t,GLU}|_t = A_{Consumed}|_t = \int_0^t q_{GLU} \cdot X_v \, dt
\]

| Total GLU in reactor + Fed into - Total GLU in reactor = Total amount GLU consumed up by the cells |
|-------------------------+------------------------------+-----------------------------+-------------------------------|
| at start of reactor up at time \( t \) up to time \( t \) up to time \( t \) |
| run (measured)          |                              |                            |                               |
Where:

\[ A_0^{\text{GLU}} = \text{total GLU in reactor at start of run (known)}. \]

\[ A_{F}^{\text{GLU}}|_t = \int_0^t F(t) \cdot C_F^{\text{GLU}} \, dt = \]

\[ = \text{total glucose fed to the reactor between time 0 and time t, and } F = \text{feed flow rate (from feed bottle weights)}. \]

\[ A_t^{\text{GLU}}|_t = \text{total GLU in reactor at time t (from sample analysis)}. \]

For a waste product, or the antibody produced, the balance is the same, except that in this case the start and feed terms are zero.

To obtain the time profiles of specific uptake rate, a curve is fitted to the cell growth profile and to the nutrient uptake profile. The specific uptake rate is then calculated from the time derivative of the fitted curve, and the cell growth curve, using the definitions of the specific rate.
Appendix 2.

Excerpts from a paper published in

COMPARISON OF AN ELECTROCHEMILUMINESCENT HOMOGENOUS IMMUNOASSAY AND AN ELISA FOR MONITORING PRODUCTIVITY IN MAMMALIAN CELL BIOREACTORS

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\textbf{SUMMARY}

An Enzyme Linked Immuno Sorbent Assays (ELISA) and an Electro-chemiluminescent Immuno Assay (CIA) are compared for the purpose of monitoring product formation in mammalian cell bioreactors. The ELISA had a relative standard deviation of 10\%, compared to 8\% for the CIA. The CIA was found to be a fast and accurate alternative to the ELISA. The use of more than one immuno assay format was also shown to provide additional insight into process performance.

\textbf{Introduction}

To improve productivity in mammalian cell culture processes, convenient, accurate measurement of the protein product is vital. Methods for selectively measuring the concentration of these products in complex cell culture media has attracted some attention
(Hunter and Bosworth, 1986). Several techniques are available, including Radio Immuno Assays (RIA) (Yalow, 1978), and the Enzyme Linked Immuno Sorbent Assays (ELISA) (Engvall and Perlman, 1971; Voller and Bidwell, 1985). While the ELISA is the most popular assay, for reasons of convenience, speed and accuracy, it frequently has a standard error of over 10% (Schuurs and van Weemen, 1980). To detect the influence of changes in culture conditions on productivity, convenient, faster, and more accurate assays are required.

The Chemiluminescent Immuno Assay (CIA) (Weeks, 1986; Sturgess, and Woodhead, 1986; Wood, 1985) offers a convenient and accurate method that is often more rapid than the ELISA. Instruments for this type of assay, using electrochemiluminescence reactions, are in development (The Origen analyzer, IGEN Inc., Rockville, Maryland). We present here a comparison between the ELISA and CIA, for monitoring antibody production in hybridoma cultures.

**Materials and Methods**

Cultures of the hybridoma line CRL 1606 (Schoen et. al., 1982), producing an anti-fibronectin monoclonal IgG1 antibody, were performed in a Braun Biostat M (B. Braun, Allentown, Pennsylvania) 1.5 liter reactor, in batch, fed-batch or continuous mode. The culture medium was Dulbecco Modified Eagles Medium, containing between 0 and 5% foetal calf serum.

ELISA's for anti-fibronectin monoclonal antibodies where performed by standard procedures (Engvall, 1980). All incubations where done either at 4°C overnight, or at 25 °C for 4 h. All washes where done three times with pH 7.2 phosphate buffered saline (PBS) containing 0.05 % Tween 80 (PBS-Tween). Samples where measured in duplicates, and standards in duplicates or triplicates: Briefly, 96 well micro-titer plates (Falcon EIA plates,
Becton Dickinson, Lincoln Park, New Jersey) where coated with 100 µl/well of a 1µg/ml solution of human fibronectin (Calbiochem, LaJolla, California), in a pH 9.6 carbonate buffer. The plates were washed and after-coated, to reduce non-specific binding, by incubating 200 µl per well of a solution of 1% bovine serum albumin in carbonate buffer. After washing, the plates where stored for up to two months with PBS at 4°C. Assay samples were diluted 1:10 in PBS-Tween, and applied to the first row of each column of the plate. Samples were diluted serially in the columns, with a dilution step of 1:5 in each row. After incubation and washing, 100 µl/well of a Rabbit-anti-Mouse IgG peroxidase conjugate (Sigma, St Louis, Missouri), diluted 1:1000, was incubated with the plates. The plates were washed and then developed by adding 100 µl/well of the substrate, 2,2'-azino-bis(3-ethylbenzthi)azoline-6-sulfonic acid (Sigma, St Louis, Missouri). The reaction was stopped after 5 to 15 min, depending on the reaction speed, by adding 50 µl per well of a 50% H₂O₂ solution. Absorbance at 414 nm was measured in a microtiter plate photometer (Flow Laboratories, McLean, Virginia). Standard curves were obtained from absorbance data of standards of known concentration, that had been treated in the same way as the samples.

The CIA assays were done according to the procedure described in the ORIGEN kit: for quantitating IgG (Catalogue number 402-006-01, IGEN Inc., Rockville, Maryland), see below. All samples were done in duplicates, and all standards in duplicates or triplicates. Samples from the hybridoma cultures were diluted between 1:5 and 1:20 in fresh culture medium. Incubations were done on an orbital shaker for 45 min at 37 °C. The samples and standards were automatically measured in the Origen analyzer. Standard curves of luminescence versus concentration were obtained from the antibody standards provided with the Origen test kit, and the concentration of samples were calculated from the standards.
Results and Discussion

Assay Principles

The ELISA, as used here, is a direct binding assay, Fig.1, for determining the amount of anti-fibronectin monoclonal antibody present in the samples. The antigen (fibronectin) is coated directly onto the plastic of a micro-titer well. Samples containing the analyte, are applied to the well, where it binds to the antigen coated surface. After washing a rabbit-anti-mouse antibody-peroxidase conjugate, is applied to the well in excess. After a final wash, the peroxidase substrate is added, to produce a colored product, in proportion to the amount of analyte.

The CIA utilizes a metal chelate label, ruthenium(II) tris-bipyridine, which emits light when, together with other components in the assay buffer, it is exited by electro-oxidation at an electrode (Bard and Faulkner, 1980). The amount of light emitted is measured by a photo multiplier tube in the instrument. The immunoassay is a homogenous competitive sandwich assay, fig 1b. The solid phase consist of a 2 μm latex bead with a goat-anti-mouse kappa chain bound to it. Mouse monoclonal antibody in the sample will bind to the solid phase. A ruthenium chelate labeled mouse-IgG antibody present in the assay solution competes with the sample monoclonal antibody for the solid phase binding sites. The label will emit significantly less light, in proportion to the amount of analyte present, when the chelate is bound in an immuno-complex to the solid phase; thus the amount of bound monoclonal antibody can be determined.
Figure 1a. The ELISA assay principle.
Standard Curves

An example of an ELISA standard curve, using triplicate standards, is shown in Fig. 2a. The concentration of the sample is estimated from a curve fitted to the standard data (Rodbard et al., 1987), using the sample dilution that gives the smallest relative standard deviation between the duplicate samples. In Fig. 2a the effect of increasing error in the photometer measurement towards lower absorbance values can also be seen. We have limited the absorbance range for measurements to between 0.1 and 1.4 absorbance units. Fig. 2b shows an example of a CIA standard curve, using triplicate standards. The sample concentration was estimated from a curve fitted to the standard data.

Measurement Results

Duplicate samples were measured for both ELISA and CIA and antibody concentrations were calculated from the standard curves as described above. Relative standard deviation (also called coefficient of variation) was calculated for each duplicate sample concentration in both ELISA and CIA by:

\[ s_{rel} = \sqrt{\frac{(\text{Conc}_1 - \text{Conc})^2 + (\text{Conc}_2 - \text{Conc})^2}{\text{Conc}}} \]

where \( \text{Conc}_1 \) is the calculated concentration for the first measurement, \( \text{Conc}_2 \) is the same for the second measurement, and \( \text{Conc} \) is the average concentration of the first and second measurement. Measurements that had \( s_{rel} \) values above 50% were discarded and redone at a later time. The data presented here does not contain any of these measurements, nor data out of range at the high or low end. Less than 15% of the measurements were discarded for the ELISA, and less than 5% for the CIA.
Figure 1b. The Chemiluminescent assay principle.
Figure 2a and b. Standard curves and standard deviations for ELISA and CIA assays.

Example of absorbance or luminescence of standards, in triplicate, and the relative standard deviations are shown for a single ELISA and CIA assay.
Figures 3a and 3b show histograms of the relative standard deviation frequencies for ELISA and CIA assays, respectively. The grand mean $s_{rel}$ value of a total of 115 duplicate measurements was 10.0% for the ELISA; for 79 duplicate CIA measurements the grand mean $s_{rel}$ value was 8.2%. By comparison, the relative standard deviations of RIA's used in our laboratory are about 5% (G. Grampp; Personal communication, data not shown). However, the grand mean $s_{rel}$ values do not tell the whole story about the average expected error. As seen from Figure 3a and 3b, the ELISA has a broader spread of $s_{rel}$ values than the CIA, which is highlighted by the weighted average functions of the frequency distributions, incorporated as solid lines in Fig. 3a and b. This is further verified by the Median Relative Standard Deviation values, which are 8.9% and 6.0% for the ELISA and CIA, respectively. The more narrow distribution of $s_{rel}$ value for the CIA allows us to discard measurements with a $s_{rel}$ value above 30% for the CIA without having to redo too many of our measurements. This brings down the $s_{rel}$ value for the CIA to below 7%. For the purpose of the comparison of the CIA with the ELISA presented here, we have included measurements with $s_{rel}$ up to 50% in the CIA data presented.

**Conclusions**

The CIA provides a convenient and accurate alternative to the ELISA. We measured a standard deviation of 10% for the ELISA, and 8% or less for the CIA. By comparison a similar RIA assay has a standard deviation of about 5%. A CIA takes about 1.25 h to perform, of which 45 min is incubation time, while the ELISA takes about 0 h, of which 4 h is incubation time. The CIA requires less dilutions and washing than the ELISA; this reduces the error introduced by serial dilutions of the samples, and as the manual labor for the assay. The tradeoff is that the CIA requires more expensive reagents and instruments.
Figure 3a and b. Normalized frequency histograms of relative standard deviations for ELISA and CIA assays. $s_{rel}$ frequency values where normalized to the total sample number. Solid lines are weighted average distribution functions of the normalized frequencies.
References


Appendix 3
Additional experimental data

For documentation, analysis data for most of the experiments referred to in the thesis are listed here. The listed data is "raw" data. In the analysis presented in the thesis, some of the data points have been treated as outliers. Analysis methods are outlined in materials and methods. Unless otherwise specified, the immunoassay data shown is from anti-IgG CIA assays (see materials and methods). The serum concentration is 2.5 % in all media except where otherwise stated. For medium composition, see materials and methods.

List of runs included:

Run 89/1. Dynamically operated culture with an early glutamine concentration step change. ............................................................. 328
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Run 89/6. Glutamine limited culture according to a protocol designed by Glacken ................................................................. 335
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Run 90/1. Dynamically operated culture with a glutamine concentration step change. ............................................................. 343
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Run 91/1. Dynamically operated culture with a simultaneous glucose plus glutamine concentration step change. ........................................ 371

Run 91/2. Dynamically operated culture with a late simultaneous glucose plus glutamine concentration step-change. ........................................ 374
Run 89/1. Dynamically operated culture with an early glutamine concentration step change.

Start medium in reactor: 25.0 mM glucose.
                      1.3 mM glutamine.
Start volume:        0.93 L.

Feed medium:        25 mM glucose.
                      33 mM glutamine.
Feed medium volume: 1.07 L.

Setpoint after nutrient concentration change: 10 mM glutamine
Run 89/1.
Run 89/1.
Run 89/5. Batch culture.

Batch medium in reactor: 25 mM glucose.

10 mM glutamine.

Volume: 1.95 L.
Run 89/5.
Run 89/5.
Run 89/5.
Run 89/6. Glutamine limited culture according to a protocol designed by Glacken (1987).

Start medium in reactor:
25 mM glucose.
1.3 mM glutamine.
2.6 % foetal calf serum

Start volume: 0.93 L.

Feed medium:
25 mM glucose.
33 mM glutamine.
Serum free

Feed medium volume: 1.07 L.
Run 89/6.
Run 89/6.
Run 89/7. Glutamine limited culture according to a protocol designed by Glacken (1987).

Start medium in reactor: 25 mM glucose.
1.3 mM glutamine.
2.6 % foetal calf serum

Start volume: 0.93 L.

Feed medium: 25 mM glucose.
33 mM glutamine.
Serum free

Feed medium volume: 1.07 L.
Run 89/7.
Run 89/7.
Run 90/1. Dynamically operated culture with a glutamine concentration step change.

Start medium in reactor: 25.0 mM glucose.
1.3 mM glutamine.
Start volume: 0.93 L.

Feed medium: 25 mM glucose.
33 mM glutamine.
Feed medium volume: 1.07 L.

Setpoint after nutrient concentration change: 10 mM glutamine
Run 90/1.
Run 90/1.
Run 90/1.
Run 90/2. Dynamically operated culture with a glutamine concentration ramp change.

Start medium in reactor: 25.0 mM glucose.
                      1.3 mM glutamine.
Start volume:         0.93 L.

Feed medium:          25 mM glucose.
                      33 mM glutamine.
Feed medium volume:   1.07 L.

Setpoint after nutrient concentration change: 1.3 to 10 mM glutamine linear ramp.
Run 90/2.
Run 90/2.
Run 90/2.
Run 90/5. Dynamically operated culture with a glutamine concentration step change.

Start medium in reactor: 25.0 mM glucose.
                         1.3 mM glutamine.
Start volume:            0.93 L.

Feed medium:            25 mM glucose.
                         33 mM glutamine.
Feed medium volume:     1.07 L.

Setpoint after nutrient concentration change: 10 mM glutamine
Run 90/5.
Run 90/5.
Run 90/5.
Run 90/6. Dynamically operated culture with a glucose concentration step change.

Start medium in reactor: 3.0 mM glucose.
10 mM glutamine.

Start volume: 0.93 L.

Feed medium: 75 mM glucose.
10 mM glutamine.

Feed medium volume: 1.07 L.

Setpoint after nutrient concentration change: 30 mM glucose
Run 90/6.
Run 90/6.
Run 90/6.
Run 90/7. Dynamically operated culture with a simultaneous glucose plus glutamine concentration step change.

Start medium in reactor: 3.0 mM glucose.
1.3 mM glutamine.

Start volume: 0.93 L.

Feed medium: 75 mM glucose.
33 mM glutamine.

Feed medium volume: 1.07 L.

Setpoint after nutrient concentration change: 30 mM glucose
10 mM glutamine
Run 96/7.
Run 90/7.
Run 90/7.
Run 90/8. Dynamically operated culture with a low glucose concentration step change.

Start medium in reactor: 3.0 mM glucose.
10 mM glutamine.

Start volume: 0.93 L.

Feed medium: 25 mM glucose.
10 mM glutamine.

Feed medium volume: 1.07 L.

Setpoint after nutrient concentration change: 10 mM glucose
Run 90/8.
Run 90/8.
Run 90/8.
Run 90/9. Dynamically operated culture with a simultaneous glucose plus glutamine concentration ramp change.

Start medium in reactor: 3.0 mM glucose.
1.3 mM glutamine.

Start volume: 0.93 L.

Feed medium: 75 mM glucose.
33 mM glutamine.

Feed medium volume: 1.07 L.

Setpoint after nutrient concentration change: 3.0 to 30 mM glucose linear ramp.
1.3 to 10 mM glutamine linear ramp.
Run 90/9.
Run 90/9.
Run 90/9.
Run 91/1. Dynamically operated culture with a simultaneous glucose plus glutamine concentration step change.

Start medium in reactor: 3.0 mM glucose.
1.3 mM glutamine.

Start volume: 0.93 L.

Feed medium: 75 mM glucose.
33 mM glutamine.

Feed medium volume: 1.07 L.

Setpoint after nutrient concentration change: 30 mM glucose
10 mM glutamine
Run 91/1.
Run 91/1.
Run 91/2. Dynamically operated culture with a late simultaneous glucose plus glutamine concentration step change.

Start medium in reactor: 3.0 mM glucose.
1.3 mM glutamine.

Start volume: 0.93 L.

Feed medium: 75 mM glucose.
33 mM glutamine.

Feed medium volume: 1.07 L.

Setpoint after nutrient concentration change: 30 mM glucose
10 mM glutamine
Run 91/2.