THE EFFECT OF PROCESS VARIABLES ON THE
GLYCOSYLATION OF INTERFERON-GAMMA PRODUCED IN
CHIO (CHINESE HAMSTER OVARY) CELLS

A thesis submitted for the degree of
DOCTOR OF PHILOSOPHY
by
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For my father
Abstract

As regulatory requirements of human therapeutics become more stringent the ability to monitor product authenticity is given greater importance. The demands for batch-to-batch consistency must now be demonstrated in ever greater detail. Industry must develop the techniques to rapidly monitor the quality of their product to meet these strict guidelines. In addition, the ability to influence the quality of a product could offer significant improvements to the efficacy of a new drug. To investigate these proposals a common industrial cell line, which had been genetically engineered to express a human protein of therapeutic use, was chosen as a suitable model system. The Chinese hamster ovary cells (CHO 320) produced recombinant human interferon-gamma (IFN-γ), a glycoprotein with two N-glycosylation sites at low titres. About half of all therapeutic proteins are glycosylated, a state which confers critical implications to the activity of a drug. This additional factor offered a further degree of detail required to fully appreciate the influence of process factors on cell physiology, product titre and quality.

The first improvements in product monitoring involved studies which demonstrated the separation of purified IFN-γ from culture supernatant into its three site-occupancy variants (glycoforms): doubly, singly and non-glycosylated. A micellar electrokinetic capillary electrophoresis (MECE) technique was used to separate these glycoforms in a rapid and quantitative manner not previously achieved by conventional SDS-PAGE. The first
process parameter investigated was time of culture using a typical small scale industrial bioreactor system. The use capillary electrophoresis demonstrated that during batch culture, the most common industrial culture system, there was a decrease in the proportion of doubly glycosylated species with culture time.

The CHO 320 cell line was batch cultured in a 15 l suspension bioreactor under controlled environmental conditions for the first time in this laboratory. It was demonstrated that by sustaining a constant power to volume ratio during culture a stable culture environment was maintained with typical CHO 320 growth cycles observed. By using mass spectrometry in combination with exoglycosidase sequencing the detailed mapping of recombinant human IFN-γ was monitored during culture for the first time. It was observed that a biantennary structure was the dominant glycosylation variant and that the proportion of high mannose and truncated IFN-γ glycoforms increased with time of culture. In addition, by the quantitative labelling of released sialylated glycans the sialylation of the molecule could be monitored for the first time as well. Using the fluorescent 2-aminobenzamide (2-AB) label there was a suggested decrease in the amount of sialylated species. Analysis of IFN-γ's C-terminal by mass spectrometry indicated that the IFN–γ polypeptide secreted by the CHO cell line was truncated by at least ten amino acids and additional proteolytic cleavage occurred in the latter part of culture.
The second process parameter investigated was that of bioreactor system itself. The second bioreactor system was a perfused 2 l fluidised bed reactor. These systems are the only credible and challenging alternative to the simple batch suspension systems used in industry. Using this system a 4-fold increase in cell density was achieved as well as an improved cellular productivity. As a result a high product titre perfusate was achieved. Recombinant IFN-γ was analysed by MECE and demonstrated a constant distribution of site-occupancy variants over the greatly extended culture time. The same biantennary structure was observed but no increase in truncated or high mannose structures was observed. However, 2-AB labelling of released N-glycans demonstrated an increase in tri- and tetra-sialylated species after 210 hours. A novel capillary electrophoresis technique was used in the determination and quantitation of the isoelectric point variants of IFN-γ. Capillary isoelectrofocusing demonstrated at least 11 pI variants of IFN-γ with an increase in the number of the acidic species after 210 hours. This technique was shown to superior to conventional gel isoelectrofocusing in both terms of time and quantitation.

This study shows that by the utilisation of advanced analytical techniques rapid monitoring of a model protein can be achieved. By these techniques it was demonstrated that both culture time and a novel bioreactor can influence the production of a model therapeutic protein to a level of detail not available before.
Acknowledgements

I would like to thank all those who have provided support during my Ph.D. studies. These include my supervisors: Nigel Jenkins, Andrew Ison and in particular, Alan Bull for his enthusiastic and relentless encouragement. I would like to thank those people who contributed to an enjoyable and knowledgeable working environment - Andrew Thomson, Nicola Green, Alison Hovey, Malcolm Gould, Kirit Patel, Andrew Hooker, Pauline Kemp, John O'Hara, Mark Rendall and especially David James, to whom I am indebted for the success of much of the work presented in this thesis. In addition, I am very grateful to Mohamed Al-Rubeai for his early encouragement of my work in this field.

I owe a great debt of thanks to all members of the Department of Biosciences for their friendliness and support during my time spent in the department. In addition, the ACB Management Group provided continual assistance and assessment over the course of the project, Ray Newsam for photographic work as well as UCL for their assistance. I would also like to thank Pharmacia Biotech for their part in the collaboration work and specifically Steve Gibson, Steve Clerk, Audrey Long and also Gerald Blüml of the AIM in Austria for his technical assistance. Endless gratitude is due to my friends and family for their emotional and often financial support during my endeavours. The completion of this thesis would have been devalued without their assistance.
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Abbreviations

AIDS: acquired immune deficiency syndrome
2-AB: 2-aminobenzamide
AMPI: anti-mouse polyvalent immunoglobulins
Asn: asparagine
Asp: aspartate
ATPase: adenosine triphosphatase
BHK: baby hamster kidney
BME: Eagle’s basal media
CE: capillary electrophoresis
CH₃CN: acetonitrile
CHO: Chinese hamster ovary
CIEF: capillary isoelectrofocusing
CO₂: carbon dioxide
Cys: cysteine
2,5-DHB: 2,5-dihydroxybenzoate
DHFR: dihydrofolate reductase
dO₂: dissolved oxygen tension
EGF: epidermal growth factor
ELISA: enzyme-linked immunosorbent assay
EPO: erythropoietin
ESI: electrospray ionisation
FAB: fast atom bombardment
GalNac: N-acetylgalactosamine
GlcNac: N-acetylglucosamine
Gln: Glutamine
Glu: Glutamate
His: Histidine
HPLC: High performance liquid chromatography
Ig: immunoglobulin
IFN-γ: interferon-gamma
IL-2: interleukin-2
Iso: isoleucine
$k_{L,a}$: mass transfer coefficient
LDH: lactate dehydrogenase
Lys: lysine
MALDI: matrix-assisted laser desorption-ionisation
MECE: micellar electrokinetic capillary electrophoresis
MES: 2-[N-morpholino]ethanesulfonic acid
Met: methionine
mRNA: messenger ribonucleic acid
MS: mass spectrometry
MTX: methotrexate
N-: asparagine-linked
NMR: nuclear magnetic resonance
OPA: o-phthalaldehyde
OUR: oxygen uptake rate
$pO_2$: partial pressure of oxygen
PAGCE: polyacrylamide gel capillary electrophoresis
PAGE: polyacrylamide gel electrophoresis
Phe: phenylalanine
PDGF: platelet-derived growth factor
PNGaseF: peptide-N-glycosidase F
Ppm: parts per million
Pro: proline
RPMI: Roswell Park Memorial Institute
Rp: reversed-phase
Rpm: revolutions per minute
Rps: revolutions per second
s: second
SDS: sodium dodecyl sulphate
Ser: serine
TFA: trifluoroacetic acid
Thr: threonine
Thy: thymidine
TIMP: tissue inhibitor of metalloproteinases
Trp: tryptophan
Tyr: tyrosine
TOF: time-of-flight
tPA: tissue-plasminogen activator
Val: valine
V/v: volume per volume
W/v: weight per volume
Chapter 1 Introduction

1. Introduction

1.1 General Introduction

The birth of animal cell biotechnology occurred at the beginning of the twentieth century when Rous and Jones (1916) were able to propagate cells dispersed from tissue. However, it did not begin in earnest until Earle and co-workers developed culture media and growth substrates for the \textit{in vitro} growth of large numbers of primary cells (Earle, 1943, Earle \textit{et al.} 1951). Through the next ten years, media were developed which could support \textit{in vitro} cultivation of a variety of animal cells (Eagle \textit{et al.}, 1955). Improvements in media design led to large increases in the propagation life of animal cells. However, it became clear that human cells only had a limited replicative potential in culture with the work of those such as Haylflick and Moorhead (1961) with human fibroblast cells. Despite this limitation, which was common to all primary cell lines, their therapeutic potential was realised with the production of vaccines for human and veterinary use. Cells were propagated for a number of population doublings to act as a substrate for virus multiplication. Later, it was observed that cells in culture could spontaneously give rise to permanent cell lines. These transformed cell lines often exhibited different metabolic and morphological characteristics that offered new opportunities for improved production capabilities.
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In the 1970s new processes using prokaryotic bacterial cells or eukaryotic yeast cells were developed which were expected to overtake animal cell systems. These microbial systems were genetically engineered to produce recombinant proteins, many of which were of pharmaceutical importance (Adamson and Schmidli, 1986). These new techniques were then applied to transformed animal cell lines to produce similar or even the same proteins but at much lower production levels. However, several biologically active proteins could only be produced by their natural producers or very familiar species. Poor product secretion and incomplete post-translational modifications (complex glycosylation, phosphorylation, carboxylation and sulphation) made the use of most prokaryotes and even yeasts impossible. Therefore some industrial microbial production systems were adapted to the requirements of animal cell cultures. Further improvements in cell line development and successful large scale cultivation have meant that an increasing number of new pharmaceuticals are produced in animal cell systems (Leist et al., 1990).

1.1.1 Properties of animal cells

Animal cells require a narrow range of environmental growth conditions, are sensitive to shear forces, have complex nutritional requirements, slow growth rates and relatively low product yields. Animal cells are usually at least 10 times larger than microorganisms, have no cell wall, more numerous organelles and a susceptible cytoskeleton. They have a
Chapter 1.2 General Introduction

bigger and more complicated genome with unstable genotypes. Their biochemistry is more complex and the cell cycle is 10-100 times longer. Despite these obstacles the continued application of genetic engineering to animal cells has improved their capabilities and presented new and potential products in abundance.

1.1.2 Products from animal cells

Animal cell culture has long been used for the production of a number of important biological products. The first was polio vaccine produced in monkey kidney cells in 1954. Other vaccines followed and these are still some of the largest processes exploiting animal cell cultures. The main categories of animal cell products with some examples are listed in Table 1-1.
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Table 1-1 Industrial scale animal cell production systems.

<table>
<thead>
<tr>
<th>Product</th>
<th>Example</th>
<th>Cell line</th>
<th>Reference</th>
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<tr>
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<td>Foot-and-mouth vaccine</td>
<td>Baby hamster</td>
<td>Spier (1983)</td>
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<td></td>
<td></td>
<td>kidney (BHK)</td>
<td></td>
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<tr>
<td>Antibodies</td>
<td>Anti-mouse</td>
<td>Mouse hybridoma</td>
<td>Kohler and Milstein (1975)</td>
</tr>
<tr>
<td></td>
<td>Immunoglobulins (Ig)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-viral</td>
<td>Interferons</td>
<td>Human fibroblasts</td>
<td>Lazer (1983)</td>
</tr>
<tr>
<td>Enzymes</td>
<td>Tissue-plasminogen</td>
<td>Myeloma Myeloma</td>
<td>Birch and Rhodes (1988)</td>
</tr>
<tr>
<td></td>
<td>activator (tPA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immunoregulators</td>
<td>Interleukin-2 (IL-2)</td>
<td>BHK BHK</td>
<td>Wagner et al. (1989)</td>
</tr>
<tr>
<td>Hormones</td>
<td>Human growth hormone</td>
<td>Chinese hamster ovary (CHO)</td>
<td>Friedman et al. (1989)</td>
</tr>
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</table>

Attention soon became focused on increasing productivity and production lifetime in animal cell systems. Through developments in cell biology and process intensification these goals are being met.
1.2 Cultivation Methods

1.2.1 Culture methods

Animal cells can be grown adherently or in suspension culture. Some cell types must attach to a suitable surface for growth such as endothelial or fibroblast cells. Some grow in suspension such as lymphocytes. Others can grow in either mode, such as CHO cells, which are usually transformed cells derived from anchorage-dependent cell types.

To cultivate anchorage-dependent cells, a suitable wettable surface is required for attachment. Historically, glass has been used as the growth surface. It is both wettable and contains negatively charged silicate or borate groups which aid attachment. Presently, plastic culture vessels that have been irradiated to produce negatively charged groups or coated to encourage attachment are more common. The most commonly used devices are flat flasks and roller bottles. Roller bottles that have a large surface area for attachment are laid on rollers in an incubator and rotated slowly. For large scale operation scale up becomes a labour intensive task with monitoring and control of cells difficult. The introduction of robotic culturing has improved their economic feasibility. Several attempts have been made to develop new methods for large scale cultivation. These have included hollow-fibre systems (Ku et al., 1981), stacked-plate (Weiss and Schleicher, 1968) and rotating multiple tubes.
Chapter 1.2 Cultivation Methods

(Girard, 1980). However, these still have many of the disadvantages of roller bottle systems.

The development of microcarriers in 1967 by Van Wezel provided answers to some of these problems. Microcarriers are small spheres (50-20,000 μm) made of a variety of materials which can be kept in suspension. Many have their surface treated to improve cell attachment and growth. These have the advantages of providing a high degree of surface area in a homogeneous environment while allowing efficient process monitoring. Scale-up is simpler since one vessel can replace many roller bottles. Giard demonstrated their industrial application to viral production in 1977. However, from a production standpoint the cost of microcarriers is prohibitively expensive in batch culture systems even with their continual reuse. An alternative approach is to continuously perfuse microcarrier cultures with fresh medium. Kluft et al. (1983) described the production of tPA from Bowes melanoma cells in a 40 l perfused microcarrier culture.

Of all cultivation methods, only suspension cultures can be said to be truly homogeneous. It then follows that culture conditions can become defined, reproducible with reliable on-line monitoring and control. Most industrially used cell lines such as CHO, BHK or hybridomas are able to grow in suspension culture. Cell damage can become a concern in these systems from a variety of sources: agitation, mass transfer limitations and foaming are typical concerns. Shake flasks and magnetically stirred vessels are the
Chapter 1.2 Cultivation Methods

simplest and most frequently used systems for small scale work. However, for larger scale production a single-unit process in the form of a well-designed bioreactor is required. Most bioreactors are based on agitated microbial fermenters with some modifications. These have usually been to the impeller system where a much lower degree of agitation is required. Recently, interest has grown in the use of airlift fermenters to provide a low shear cultivation method for hybridoma cell growth (Katinger and Scheirer, 1982). These utilise gas to lift and mix the cell suspension and provide oxygen. However, still the most common bioreactors in industrial use are those based on successful systems for the growth of microorganisms.

Like microbial fermentations, stirred tanks are the most commonly used industrial production systems. They have been modified to suit animal cells and maintain the premise that the stabilisation of the nutritional environment is a requirement. Their simplicity of operation and the versatility for use both for batch, fed-batch and continuous operation have assured their lasting popularity. In the cases where product formation is followed by cell death, such as the production of some viruses, production is carried out in batch mode. In fed-batch mode, a portion of the cell suspension is replaced with fresh media and may be run for up to months at a time. In continuous systems, media is constantly fed and cells and media are constantly removed although during continuously perfused systems the cells are retained. Under these steady state conditions the cells are maintained in exponential growth. Changes to feed rate and nutrient compositions allow for manipulation of steady state cell
Chapter 1.2 Cultivation Methods

density. Since this system allows for greater manipulation of cell physiology there is more scope for process optimisation than batch culture.
Chapter 1.3 Culture Environment

1.3 Culture Environment

1.3.1 Bioreactor

Reactors for animal cells can be divided into three main types: membrane, column and stirred tank. Materials used for animal cell cultivation must be inert, nontoxic, have smooth surfaces and corrosion resistance. Stainless steel is the material of choice in the bioprocess industries; although glass, plastics and ceramics have also been used. A proficient bioreactor must provide a homogeneous environment, aseptic operation, on-line monitoring and meet safety requirements.

Membrane reactors were developed to provide a tissue-like environment for cell growth. Hollow fibre cell culture systems, for instance, were modelled closely on the environment of cells in living animals (Knazeck et al., 1972). These units are composed of a packed bundle of hollow fibres in a solid shell. Cells are grown on the shell side of the fibres and growth medium is perfused through the lumen. Pores in the fibre walls allow the free passage of small molecular molecules. This allows the passage of nutrients to the cells and the removal of metabolic toxins. In 1980, Lim and Sum reported a technique for microencapsulating pancreatic islets that prolonged their survival in vivo. Cells are suspended in an insoluble but semi-permeable calcium alginate gel that allows the passage of low molecular weight nutrients and waste products.
Chapter 1.3 Culture Environment

The pore size of the microcapsules can be varied to allow, or disallow, the passage of product through the capsular wall.

Column-type reactors are pneumatically agitated and are used for adherent cell lines with the exception of airlift reactors. They include packed and fluidized bed reactors that are used to achieve high cell densities on support surfaces such as microcarriers. An airlift system consists of suspended, concentric draught tube enclosed by a tall, cylindrical vessel. Gas bubbles fed through a sparger at the bottom of the vessel enrich the medium in the lumenal side of the draught tube. This makes the medium less dense than the air lean medium on the annular side. A convection current is set-up in which cell suspension is gently carried up the lumen of the draught tube and down the annular space. This system has proved particularly suitable to shear sensitive cell lines and is readily scaleable. Celltech Biologics (Slough, U.K.) has produced monoclonal antibodies for many years in airlift reactors. However, heterogeneity of environment in larger production systems still requires careful consideration.

Stirred tanks are still the most commonly used industrial reactors because of their simplicity, scale-up potential and proven track record. They differ most significantly in their means of cell suspension. The axial type of flow is the preferred pattern for animal cells and all kinds of axial mixers have been proposed (Prokop and Rosenberg, 1989). Stirred tank systems lend themselves equally well to batch, fed-batch and continuous operation. The requirement to retain cells in the bioreactor during medium perfusion lead
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Himmelfarb et al. (1969) to the development of a spin filter device. These are often installed on the impeller whose rotational motion prevents cells from attaching to the filter while allowing cell-free medium to be withdrawn from the cell-free environment within the filter. Tolbert et al. (1983) demonstrated that such a system could generate almost a thirty-fold increase in cell densities over conventional suspension culture.

1.3.2 Aeration and agitation

Animal cells are more sensitive to mechanical and hydrodynamic shear forces than microorganisms because of their size and lack of a cell wall. Papoutsakis and Kunas (1989) demonstrated that hybridoma cells when exposed to increased levels of agitation in a controlled sparged bioreactor environment suffered a substantial reduction in growth rate. However, Oh et al. (1989) demonstrated that high agitation alone was not sufficient to cause cell damage under conditions of surface aeration. When Handa-Corrigan and co-workers investigated (1989) cell-bubble interactions in a sparged bioreactor they deduced that cell damage occurred mainly at the point of bubble disengagement. Hua et al. (1993) also concluded that under normal operating conditions, agitation alone is insufficient to cause cell damage. The interfacial effects associated with bubble formation, bubble break-up/coalescence and bubble bursting appeared to be the major sources of cell damage.
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Oxygen demand of animal cells is much less than other microbial systems. Kilburn and Webb (1968) found that mouse LS cells grew well in the range 40-100 mmHg. When medium used for growing cells was equilibrated with an oxygen/nitrogen mixture in which the partial pressure of oxygen (pO$_2$) was between about 5-20% of the total pressure, the resulting concentration was found to be optimal for a wide range of cells (Spier and Griffiths, 1984). The oxygen uptake of most cells falls within the range 0.05-0.5 mmol l$^{-1}$ h$^{-1}$ oxygen at 10$^6$ cells ml$^{-1}$ (Thomas, 1986).

Oxygen is usually supplied as compressed air that may be delivered to the surface of a culture, through semi-permeable tubing submerged in the medium or as sparged bubbles. Surface aeration is the simplest form but can result in dissolved oxygen gradients that may become critical at larger scales. Air sparging beneath the impeller arrangement is the most common aeration method. However, this does introduce bubbles and the potential for harmful cell-bubble interactions. Reuveny et al. (1986) demonstrated the use of a caged aeration system to separate cells from the aerated space to prevent cell damage. They also demonstrated that the optimal pO$_2$ for antibody production is different from that for cell growth. Applegate and Stephanopoulos (1992) demonstrated the use of semi-permeable silicone tubing for smaller systems but which is generally impractical for large vessels. Takagi et al. (1993) developed an oxygen supply strategy for the production of tPA by microcarrier culture. Above the minimum agitation speed to maintain microcarrier suspension and thus avoid oxygen depletion tPA productivity was higher at
low agitation speeds while the cell concentration was unaffected by agitation speed. It is a necessity to ascertain the correct $pO_2$ level for a cell line and therefore the method of aeration suitable for operation scales. The agitation system should be chosen only after these considerations have been met.

1.3.3 Temperature and pH

Animal cells require a narrow range of temperature and pH conditions which vary between cell lines. The normal temperature for cell cultivation is 37°C. Strategies to improve product yields by extending cell viability have focused on several physicochemical parameters such as temperature. Reuveny et al. (1985) demonstrated that growing murine hybridoma cells at temperatures three to nine degrees below optimum (37°C) increased cell viability but lowered monoclonal antibody production. Bloemkolk et al. (1992) investigated the kinetics of growth and antibody formation of an anti-interleukin-2 producing hybridoma cell line. Lower temperatures during suspension culture were found to increase cell densities but without significant changes in monoclonal antibody production.

Different cell types exhibit widely different pH optima (Eagle, 1973). The optimum pH is often sharp but there is usually a wide workable range. Miller et al. (1988) investigated the influence of pH on the growth and productivity of a mouse hybridoma. There was an optimal plateau for cell growth and viability between pH 7.1 and 7.4. They also demonstrated lower glucose
Chapter 1.3 Culture Environment

cConsumption rates suggesting inhibition of glycolysis. At low or high pH the antibody metabolic quotient was higher indicating that antibody formation was not growth-associated. McQueen and Bailey (1990) found that there was no change in the specific antibody production rate during batch culture of a hybridoma cell line. Cell growth was approximately constant over the pH range, 6.8 to 7.6. These results underline the cell line dependence of pH effects.
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1.3.4 Measurement and control

The increasing demand for products produced by animal cell systems has put pressure on strategies to be developed to maximise productivity and efficiency. Accurate on-line measurements of process states such as temperature, pH and dissolved oxygen are necessary for efficient direct control. These three variables are usually regulated by standard control loops. In situ probes for these parameters as well as several others are common. They are steam sterilisable and have low response times. Temperature is usually maintained by utilising the outside surface area of the bioreactor to transfer heat. This large surface area minimises the temperature difference between the heat transfer medium and the culture fluid. The three most common heat transfer media are water, air and a solid electric heating pad. The most simple and efficient form of pH control is a CO\textsubscript{2} controlling one. CO\textsubscript{2} is added to the culture medium containing a sodium bicarbonate buffer to maintain the pH via the following reactions:

\[ \text{CO}_2(aq) + \text{H}_2\text{O} = \text{H}^+ + \text{HCO}_3^- \]

As cells begin to grow, lactate is produced which is neutralised by bicarbonate ions. As the bicarbonate concentration decreases, the CO\textsubscript{2} partial pressure required to maintain a given pH also decreases. Eventually, there is no requirement for CO\textsubscript{2} and eventually bicarbonate ions will be exhausted. The pH may then decrease but can be counteracted by the addition of liquid base.
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For a sparingly soluble gas, such as oxygen, the rate of transfer from the gas phase to the liquid phase is proportional to the liquid driving force; i.e.,

\[ N_0 = k_{L a} (C_G - C^*_L) \]  

(1)

where

- \( N_0 \) = oxygen transfer rate (mmol O\(_2\) l\(^{-1}\) h\(^{-1}\))
- \( k_{L a} \) = mass transfer coefficient (h\(^{-1}\))
- \( C_G \) = oxygen concentration in the gas phase (mmol O\(_2\) l\(^{-1}\)); and
- \( C^*_L \) = oxygen concentration in the gas phase that would be in equilibrium with the actual concentration of oxygen in the liquid phase (mmol O\(_2\) l\(^{-1}\)).

The mass transfer coefficient reflects both molecular diffusion and ‘turbulent’ mass transfer and area for mass transfer. It follows from equation (1) that the magnitude of \( k_{L a} \) is of considerable importance in the design of fermenters.

The mass transfer coefficient can be determined by several methods including static and dynamic gassing-out methods. In the former, the air dissolved in the medium of the bioreactor is purged with nitrogen gas and when aeration is restarted the increase in the dissolved oxygen concentration is measured:
Chapter 1.3 Culture Environment

\[
d\frac{C_L^*}{dt} = k_{L,a} (C_L^* - C_L) \quad (2)
\]

where
\[C_L = \text{oxygen concentration in the liquid phase (mmol O}_2 \text{ l}^{-1}).\]

The \(k_{L,a}\) can be determined from the slope of the \(\ln(C_L^* - C_L)\) versus \(t\) graph.

In the dynamic gassing-out method, the aeration of an actively respiring system is stopped and the decrease in dissolved oxygen due to respiration is monitored (3). Aeration is resumed and the increasing oxygen concentration is followed (4).

\[- \frac{dC_L}{dt} = q_{o_2}x = \text{constant} \quad (3)\]

\[
C_L = -\frac{1}{k_{L,a}} \left( \frac{dc}{dt} + q_{o_2}x \right) + C_L^* \quad (4)
\]

where
\[q_{o_2} = \text{specific rate of cellular oxygen utilisation (mmol O}_2 \text{ cell}^{-1} \text{ h}^{-1})\]

\[x = \text{cell concentration (cells l}^{-1})\]
Chapter 1.3 Culture Environment

The $k_{La}$ can be determined from the slope of the $C_L$ versus $\left(\frac{dC}{dt} + q_{o_2}x\right)$ graph.

At steady state conditions, the rate of transfer of oxygen from the gas phase equals the rate of cellular consumption:

$$k_{La} (C^* - C_L) = q_{o_2}x$$

If the cell line has a known $q_{o_2}$ the bioreactor can be optimised to provide sufficient aeration capacity to supply the cultures at the highest densities. The engineering of the bioreactor system is often one of the last steps in the development of the correct culture environment.
Chapter 1.4 Medium Composition

1.4 Medium Composition

The first step in the formation of suitable conditions for animal cell growth and production is the choice of medium. The media used for the growth of animal cells were originally based on biological fluids such as plasma and embryonic extracts. However, these had the disadvantages of being particularly vulnerable to contamination and exhibiting batch variation. The first chemically defined media were based on analyses of plasma that led to complex formulations such as Medium 199 which contains over 60 synthetic ingredients. In 1955 Eagle designed a basal media (BME) which reduced the number of components to a minimum necessary for cell growth. Several more basal media were designed based on this formulation with minor variations that found applications with different cell lines. This included Roswell Park Memorial Institute (RPMI) 1640 which was found to be particular suitable for the culture of cells in suspension.

Serum is still used for the growth of some cell lines but it is expensive, ill defined, complicates product recovery and is a potential source of adventitious agents such as mycoplasma viruses (Keay, 1979). There has been a move to serum free and even protein free media in recent years. In the case of serum free growth, the required growth factors, transport proteins and other supplements are added to the medium or are produced by the cells themselves. Broad et al. (1991) illustrated the use of serum free media for the suspension culture of myeloma cells for the production of tPA. Despite the drive to
optimise media for the convenience of downstream processing rather than improvements in cell growth or production the monitoring of cell physiology is still of great necessity particularly in the development of new cell lines for commercial purposes.
Chapter 1.5 Cell Physiology

1.5 Cell Physiology

The nutritional requirements of cells in vitro differ from the nutritional requirements of intact animals. These differences can reflect alternative physiological requirements of transformed cell lines.

1.5.1 Growth requirements

1.5.1.1 Carbohydrates

Glucose is the most common carbohydrate found in animal cell culture media. Indeed, the best alternatives are the di-, tri-, and polysaccharides that are composed of glucose monomers. Eagle (1958) found several compounds that supported growth in a similar fashion to glucose; these included the hexoses D-fructose, D-galactose and D-mannose. The concentration of glucose in the culture medium is critical in determining the rate of its disappearance. For most cell lines, the higher the concentration, the greater its rate of utilisation. Reitzer et al. (1979) found that although the growth of HeLa cells was similar on either glucose, galactose or fructose the metabolic pathways used were different for each substrate. With glucose 80% of it was used to derive energy via the glycolytic pathway but with fructose the pathway was inactive and with galactose somewhere in-between. It was suggested that the energy requirement was being met by glutamine, an amino acid.
Later, Eagle modified his BME medium to include glutamine which brought the total number of amino acids he found beneficial for cell growth to 13. The other 12 were arginine, cysteine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, and valine. With some exceptions, these amino acids are considered essential for all cell lines although their rate of utilisation may vary widely. The amino acid utilised to the greatest extent is glutamine which is related to its use as an energy source. The oxidative breakdown of amino acids typically accounts for only 10 to 15% of the metabolic requirement of animals whereas in transformed cell lines glutamine alone can account for 50%. Glutamine may be completely converted to CO$_2$ which generates the most energy but it is also involved in the synthesis of other amino acids and lipids. Branched-chain amino acids which include isoleucine, leucine and valine are also well utilised, but at much slower rates than glutamine. The following are also used to a similar extent: histidine, lysine, methionine, phenylalanine, threonine and tyrosine. Alanine, glutamate, serine, glycine, proline, and aspartic acid tend to be unused or may actually increase in concentration (Thomas, 1986). The change in concentrations of other amino acids is much less predictable.


Chapter 1.5 Cell Physiology

1.5.1.3 Growth factors

Growth factors are often required as supplements in serum free media. These are either substances of known physiological importance or those known to promote cell growth in some unknown manner. Insulin is a small protein that is included in most serum free media formulations at relatively high concentrations. Its involvement in glucose metabolism is thought to be its most important role for in vitro work. Transferrin is another common additive due to its role in the transport of iron into the cell.

1.5.1.4 Trace elements

The selection and concentration of trace elements for optimum cell growth is a complex and difficult procedure. Nielsen (1981) found at least 15 trace elements that were beneficial for the growth of some vertebrate cells. This list includes: cobalt, copper, iodine, iron, manganese, molybdenum, zinc, selenium, chromium, nickel, vanadium, arsenic, silicon, fluorine, and tin. The selection of suitable trace elements is hampered by their presence as contaminants in other medium components. The move to serum and protein free media makes their selection particularly more important.
1.5.1.5 Vitamins

When in 1955 Eagle designed BME he included seven vitamins: choline, folic acid, nicotinamide, pantothenate, pyroxidal, riboflavin and thiamine. The omission of any of these led to eventual cell death in mouse L cells and HeLa cells. Most modern cell culture media contain at least one or more of these vitamins. Others added include ascorbate, vitamin B\textsubscript{12}, biotin and inositol.

1.5.1.6 Lipids

Cells cultured \textit{in vitro} generally do better with a source of lipids than without although their mode of action is largely unknown. Serum is a rich source of lipids but in its absence approximately 90\% of cellular lipids can be synthesised from glucose (Bailey, 1972). Several lipids, in the form of lipoproteins, liposomes and lipids complexed to albumin have been shown to stimulate growth of a variety of cell types. Darfler (1990) found that the preparation of water-soluble lipid emulsions enabled the delivery of a variety of lipid mixtures. The inclusion of phosphatidylcholines and cholesterol oleate improved the growth of hybridomas and CHO cells significantly.
As the need for increasing amounts of therapeutic agents has grown so has the requirement for increasingly larger cultivation systems for animal cells. The bioreactor environment has imposed its own demands on cell physiology which are often far removed from those found in tissue flask cultures. Extended culture times, higher glucose and glutamine concentrations to support higher cell densities, and accumulation of by-products are common to industrial production. The requirements for accurate models that describe the kinetics of cell metabolism are necessary for the design and operation of bioreactors. Only from these can responses to bioreactor perturbations on cell growth and productivity be predicted and external responses applied where required.

The mechanisms of metabolic control are poorly understood for mammalian cells even for the very common pathways. Glucose and glutamine are the major sources of energy and carbon in cell culture media. Glucose is required for nucleoside synthesis and glutamine for purine and pyrimidine synthesis. Glucose and glutamine are used in competition for the production of aspartate and for energy production. Transformed cells display higher rates of aerobic glycolysis than quiescent cells. This increase in glucose uptake is mediated by an elevation in the number of transport proteins. They also exhibit a decoupling of the respiratory chain and thereby an elevated production of lactate. Glucose deprivation leads to repression of most other cellular proteins.
Chapter 1.5 Cell Physiology

Transformed cell lines display higher rates of glutamine consumption most of which is converted to CO\(_2\) (Zielke, 1984). Other major products include lactate and glutamate. Most of the derived carbon skeletons enter the tricarboxylic acid cycle as \(\alpha\)-ketoglutarate for the biosynthesis of amino acids. Other amino acids are used less extensively and vary in their consumption between cell lines. Some are used in the production of energy, particularly branched chain amino acids. Even less is known about the pathways involved in the utilisation of growth factors, vitamins, trace elements and lipids in cultured cells.

The cell cycle of higher eukaryotic cells is illustrated in Figure 1-1. The normal cell cycle: M to G\(_1\) to S to G\(_2\) to M can be interrupted by entering a quiescent stage (G\(_0\)). This stage is usually reversible. This cycle can be interrupted by depletion of key nutrients and growth factors or environmental factors. The M phase represents mitosis and S the period of DNA synthesis; the precise role of the G\(_2\) phase is less clear. Growth regulation of normal, primary cells consists of co-operation between positive and negative regulatory factors. Their synthesis is controlled by regulator genes. Positive regulatory factors such as the growth factors epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) are often added to serum free media of \textit{in vitro} animal cell cultivations. However, transformed cells differ from primary cells by unlimited proliferation.
During batch cultivation cells spend most of their growth cycle in the $G_1$ phase. If product synthesis is growth related then specific production rates will be highest during this period. When exponential growth has ended cells begin to re-enter the $G_1$ phase. If protein production is not linked to growth then product synthesis may continue through the decline phase of the cell population. In the former situation the choice for industrial scale production is usually batch-type cultures. Glaxo-Wellcome grow cells in a fed-batch manner where a portion of the cell suspension is replaced every 2-3 days by fresh medium. This process can be run for several weeks and may be useful for labile products. Alternatively if the product is a hybridoma-produced antibody then production may be linear during exponential and decline phases of growth. Celltech Biologics (U.K.) allow these types of cultures to out-grow in order to produce maximum levels of antibody. These cultures can take between 5-10 days depending on the hybridoma employed.
Chapter 1.6 Protein Expression

1.6 Protein Expression

During and following protein translation, polypeptides can be subject to a number of different modifications. Translation of both cytoplasmic and membrane proteins is initiated on mRNA in the cytoplasm. Subsequently, the nascent polypeptide chain becomes associated with the membrane of the endoplasmic reticulum. Following translocation of the chain across the membrane, processing such as proteolytic cleavage and glycosylation can occur. Proteolysis is an unpredictable and poorly understood process in mammalian cell culture systems. This modification may occur intra- or extracellularly, and its extent will depend on the protein substrate, host cell and process conditions. Proteolysis may itself be desirable; for example, cleavage of inactive recombinant protein precursors by pro-protein endoproteases may be necessary to generate bioactive products. To this end, a soluble, recombinant form of the mammalian endoprotease furin can now be synthesised in CHO cells, and may facilitate the production of mature proteins which have been incompletely processed by the host cell (Nakayama, 1994).

In other cases, unwanted proteolysis may be deleterious to recombinant protein function, and has prompted the construction of protease-negative strains of \textit{E. coli}.

In eukaryotic production systems, proteolytic activity can occur extracellularly due to the release of proteases from lysed cells, although the enzymes responsible remain largely uncharacterised. Environmental factors may affect
Chapter 1.6 Protein Expression

proteolysis - Teige et al. (1994) demonstrated that higher proteolytic activity was associated both with a switch from serum to serum free production and the culture lifetime. By the end of fermentation the product, antithrombin III, suffered considerable product degradation probably due to the loss of protease inhibitors present in serum. In addition, recombinant human IL-2 was shown to undergo N-terminal truncation under glucose limiting conditions during perfusion culture (Gawlitzek et al., 1995a).

Many animal cell proteins are modified by the covalent addition of oligosaccharides. These modifications may form a significant mass addition to the protein as well as covering a significant proportion of their surface. There are three common forms of oligosaccharides: mucin, proteoglycan and N-linked types. Mucin type oligosaccharides are found on epithelial cell surfaces in the body linked by a N-acetylgalactosamine (GalNac) to a threonine or serine residue. The oligosaccharides are generally small and contain high levels of sialic acid, galactose, GalNac and fucose residues. Proteoglycans, which are connective tissue polysaccharides linked to a small protein core, contain a unique O-glycosidic linkage between D-xylose and serine as well as GalNac to serine and threonine. The N-linked oligosaccharides are linked to polypeptide chains through an N-glycosidic bond between an N-acetylglucosamine (GlcNac) residue and an asparagine residue (Kornfeld and Kornfeld, 1980).
Chapter 1.6 Protein Expression

The N-linked oligosaccharide chains in mammalian cells fall broadly into two classes: high mannose and complex types although hybrid structures also exist (Figure 1-2). In most cases, both carbohydrate chains are composed of a pentasaccharide core consisting of three branched mannose residues sequentially linked to two GlcNac residues that are attached through an amide linkage to asparagine. The common core structure occurs because they are all formed from the same precursor lipid-linked oligosaccharide which is transferred to nascent peptides and then processed to form various structures. The high mannose glycoproteins contain up to an additional six mannose residues; the complex glycoproteins are further derivitized with a number of sugars including GlcNac, galactose, sialic acid and fucose. The complex types may be further modified by the addition of extra branches on the mannose residues. The majority contain two, three, or four outer branches.
Chapter 1.6 Protein Expression

Figure 1-2 Structures of the three main types of asparagine-linked (N) oligosaccharides. The boxed area encloses the pentasaccharide core common to all N-linked structures. Redrawn from Kornfeld and Kornfeld (1990).

Individual glycosylation sites may contain mixtures of structurally related oligosaccharides. Swiedler et al. (1985) studied a murine lymphoma cell line which simultaneously produced two glycoproteins with a high degree of amino acid sequence homology. Analysis of attached oligosaccharides revealed a unique distribution of structures present at each glycosylation site. This heterogeneity is thought to be derived by competition among similar glycotransferases that add monosaccharides as the protein moves through cellular compartments. The reason for this heterogeneity is not known but under constant and stable conditions in vivo, the heterogeneity is maintained. The precise heterogeneity may be of biological significance in addition to the presence or absence of particular oligosaccharides.
Glycosylation is thought to serve many different biological roles. These include protein stability, biological activity, pharmokinetic and immunological properties. The presence of oligosaccharides may protect the protein from proteolysis, improve thermal lability and discourage precipitation. As a consequence of the effects on protein folding and other interactions, glycosylation may influence the resultant antigenicity of a protein. They may influence the enzyme activity of a protein: Hansen et al. (1988) found that the fibrinolytic activity of tPA was lower in glycosylated forms. The oligosaccharides present may influence the half-life of a protein. There are mannose receptors in the endothelial cells of the liver which remove from the bloodstream glycoproteins with terminal mannose residues (Smedsrod et al., 1988). Cole et al. (1993) found a direct correlation between the amount of sialic acid present on a molecule and its plasma half-life.

1.6.1 Factors influencing protein glycosylation

1.6.1.1 Protein structure

Most levels of protein structure can influence protein glycosylation. Mutations that result in an alteration within the glycosylation site will prohibit oligosaccharide additions which may alter biological properties. Likewise, changes in other parts of the protein may create new glycosylation sites.
Chapter 1.6 Protein Expression

Schwartz and Klenk (1981) demonstrated that minor differences in amino acid sequence of influenza hemagglutins led to differences in oligosaccharide structures. The importance of tertiary structure was suggested by the finding that denatured proteins with high mannose oligosaccharides were often susceptible to digestion whereas the native protein was not.

1.6.1.2 Cell type and location

The cell line or tissue in which a glycoprotein is produced has dramatic effects on the oligosaccharides attached. Differences in the relative activities and complement of glycosyltransferases among species and tissues can explain the many variations in oligosaccharide structures that are found. For instance, CHO and BHK cells lack a functional copy of the gene encoding α2,6-sialyltransferase, which is one of the enzymes required to add sialic acids to outer oligosaccharide structures in glycoproteins. Tsuda et al. (1988) made comparative studies of the N-linked oligosaccharides of urinary erythropoietin (u-EPO) and those from recombinant erythropoietin (r-EPO) produced in BHK cells. They found that the distribution and type of oligosaccharide structures present in r-EPO were different from those in u-EPO. Tissue specific glycosylation is another influence on oligosaccharide synthesis. Parekh et al. (1987) found that Thy 1 from rat brain contains a different complement of oligosaccharides than does Thy 1 from rat thymus. These alterations may influence the activity of Thy 1 within the organ in which it is produced.
Interferon-gamma (IFN-γ) is one of a group of three interferons, the other two being IFN-α and IFN-β. The interferons are secreted polypeptides which have immunoregulatory roles as well as antiviral and antitumour properties. Human IFN-γ is known to enhance killer cell activity and increased antibody production in B cells (Lengyl, 1982). The gene was cloned and expressed in *E. coli* and found to be expressed as a 166-amino acid polypeptide with properties of authentic human IFN-γ (Gray *et al.*, 1982a). The same group determined the gene sequence of IFN-γ and concluded that there was only a single gene for IFN-γ. It coded for a 146-amino acid protein with a 20-amino acid signal sequence and two potential N-glycosylation sites (Gray and Goeddel, 1982b). The amino acid sequence of human IFN-γ, including the signal sequence is illustrated in Figure 1-3.

The amino acid sequence shows a considerable number of basic residues which results in the high pI value of the molecule (Yip *et al.*, 1981). The complete amino acid sequence of IFN-γ from peripheral blood lymphocytes was determined by Rinderknecht *et al.* (1984). They observed that the first three amino acids predicted by the recombinant *E. coli*-derived IFN-γ were missing as well a different C-terminus. They purified both a dimeric form, 45,000 kDa, and two monomeric forms of differing molecular weights, 20,000 and 25,000 kDa. Kelker *et al.* (1984) resolved an additional variant of 15,500 and concluded that these were glycosylation site-occupancy variants. The

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Chapter 1.6 Protein Expression

highest molecular weight variant was the most abundant and the low molecular weight variant, considered to be non-glycosylated, the least abundant.

MKYTSYILAF QLCIVLGSGLG
CYCQDPYVKAEENLKKYFNAGHSDVADNGTLFLGILKNW
EESDRKIMQSQIVSFYFKLFKNFKDDQSIQKSVETIKEDM
NVKFFNSNKKKRDDFEKLTNYSVTDLNVQRAIHHELIQVM
AELSPAAKTGKRKRSQMLFRGRRASQ

Figure 1-3 The amino acid sequence of recombinant IFN-γ expressed in E. coli. The first 20 amino acids form the signal sequence. Amino acids in bold, underlined or italic typescript indicate potential glycosylation sites, basic and acidic amino acid residues respectively.

Curling et al. (1990) discovered up to 12 molecular weight variants of recombinant IFN-γ expressed by CHO cells. Three variants were collected after removal of all N-linked oligosaccharides which suggested the additional heterogeneity was probably due to proteolytic cleavage. This has been confirmed recently by Goldman et al. (1997) who demonstrated an increase in the heterogeneity of truncated IFN-γ polypeptides during batch culture. During glucose-limited continuous culture the glycosylation of IFN-γ was found to be influenced by the concentration of glucose present in the culture (Hayter et al., 1992). This has made IFN-γ an ideal tool for the monitoring of recombinant protein heterogeneity during culture (Hooker et al., 1995, Harmon et al., 1996).
1.6.3 Cell metabolism and environment

It is known that changes in cell metabolism that occur in vivo lead to radical alterations in oligosaccharide structures (Jenkins, 1996). This has also been found to be the case with cells grown in culture. However, it is not known how well the structural alterations in glycosylation during in vitro cultivation may be influenced. Cultured cells are maintained in a tightly controlled environment which has leant itself to the evaluation of individual components of a system. Some of the conditions that have effects on glycosylation are listed in Table 1-2.

Table 1-2 Culture conditions that affect glycosylation.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cell type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of culture</td>
<td>CHO</td>
<td>Hooker et al. (1995)</td>
</tr>
<tr>
<td>Glucose limitation</td>
<td>CHO</td>
<td>Hayter et al. (1991)</td>
</tr>
<tr>
<td>Glucose starvation</td>
<td>CHO</td>
<td>Rearick et al. (1981)</td>
</tr>
<tr>
<td>Oxygen starvation</td>
<td>Rat</td>
<td>Regoecki et al. (1990)</td>
</tr>
<tr>
<td>Energy depletion</td>
<td>Chick embryo</td>
<td>Datema et al. (1981)</td>
</tr>
<tr>
<td>Orotic acid</td>
<td>Rat liver</td>
<td>Narashimhan et al. (1988)</td>
</tr>
</tbody>
</table>

Clearly many external parameters may influence the structure of glycoproteins. Robinson et al. (1994) found that during fed-batch culture of a hybridoma cell
Chapter 1.6 Protein Expression

line the glycosylation of monoclonal antibodies produced changed with the time in culture. They found an increase in the proportion of high mannose structures present in the culture supernatant at the end of the culture. Glycosylation of recombinant placental lactogen in CHO cells was found to be consistent only within the pH range of 6.9-8.3, outside this range it was underglycosylated (Borys et al., 1993). An increase in the concentration of ammonium ions in the culture medium resulted in reduced sialylation of granulocyte-colony-stimulating factor expressed in CHO cells (Andersen et al., 1994).
1.7 Analysis

1.7.1 Cell physiology

Monitoring of cell physiology is often a subjective matter. There are few on-line measurements of cell physiology and off-line measurements may prove contradictory. Meilhoc et al. (1990) found that hybridomas grown under conditions of high dissolved oxygen tension had a reduced specific growth rate but normal cell viability. Cell viability is the most common indicator of the health of a cell culture. Trypan blue and erythrosin B are two dyes which differentially stain viable and non viable cells. The leakage of dye into the cell is indicative of a porous membrane which suggests a necrotic state (Hoffstein et al., 1975). There is good evidence for net potassium loss and sodium entry at an early stage in the lethal process but this is thought to not be sufficient to kill the cell: more significant is the uncontrolled entry of calcium (Shanne et al., 1979). Calcium is known to inhibit the membrane sodium-potassium ATPase but it appears more likely that the primary site of action of the incoming calcium is on membrane lipids, activating phospholipase and thus initiating the dissolution of membranes which is observed morphologically.

Another indicator of cell physiology is the determination of the cell populations' growth rate which gives a measure of the cells' proliferative
Chapter 1.7 Analysis

abilities. The specific growth rate (μ) is the rate of increase in cell concentration per cell per hour:

\[ \mu = \frac{1}{x} \frac{dx}{dt} \]

The specific growth rate has units of reciprocal time (h^{-1}), and is related to the cell doubling time (t_d) by the following equation:

\[ t_d = \frac{\ln 2}{\mu} \]

During exponential growth the increase in cell concentration over a time period (t) can be described by the following equation:

\[ x_t = x_0 e^{\mu t} \]

where

- \( x_t \) is the cell number at time = t;
- \( x_0 \) is the cell number at time = 0.

Substituting natural logs and rearranging gives:

\[ \mu = \frac{\ln x_t - \ln x_0}{t} \]
Chapter 1.7 Analysis

When the natural log of the viable cell number is plotted against time, the exponential period of cell growth (logarithmic growth) is linear and the slope of this line is equal to the specific growth rate.

The determination of the cells’ ability to consume nutrients and produce waste by-products into culture are often used as indirect measures of the health of a cell population. Since the rate of consumption or production of a metabolite will change with increasing cell number it is usual to calculate specific rates of consumption or production (also known as metabolic quotients).

\[ q_m = \frac{1}{x} \frac{dm}{dt} \]

where

\( q_m \) is the metabolic quotient for a metabolite (m).

During steady state conditions, where all the concentrations within the vessel are independent of time e.g. during chemostat culture the specific rate of utilisation may be easily evaluated based upon measurement of the inlet (\( C_o \)) and outlet concentrations (\( C_i \)):

\[ q_m = \frac{F (C_o - C_i)}{V_R x} \]

where

\( F = \) volumetric flow rate of feed and effluent liquid streams

\( V_R = \) total volume of culture within the vessel
Chapter 1.7 Analysis

Glucose and its by-product are the most common metabolites monitored during culture. Glutamine, glutamate and ammonia are also used as indicators of the state of the cell culture. Most of the common metabolites can be rapidly determined by enzyme assays or by high resolution liquid chromatography (HPLC) techniques. Stoll \textit{et al.} (1994) developed an HPLC technique for the accurate determination of glucose, lactate, glutamine and pyrrolidone carboxylic acid (the by-product of spontaneous glutamine breakdown).

The oxygen uptake rate (OUR) is now considered to be one of the most important indicators of cell physiology during culture. The methods for monitoring OUR are based on measurement of the oxygen concentration in the gas or liquid phase. The availability of dissolved oxygen in culture can have profound effects on cell physiology. Shi \textit{et al.} (1993) found that when oxygen became limiting during perfusion culture the specific glucose and glutamine uptake rates increased as did the lactate production rate. It was also noted that the lactate dehydrogenase (LDH) activity was also higher which suggested that oxygen had become a limiting factor. The measurement of LDH has often been used to monitor cell lysis in perfusion systems where direct determination of cell viability is difficult. The OUR of porcine epithelial cells during perfusion culture for virus production was determined on-line by Kussow \textit{et al.} (1995). They used the measurement to estimate the glucose consumption rate for perfusion rate adjustment and to optimise the virus infection process. Yoon and Konstantinov (1994) used a novel OUR monitoring technique to
Chapter 1.7 Analysis

observe a decrease in the specific OUR during the progress of a perfusion fermentation. The decrease was attributed to a general reduction in cellular metabolism.

Flow cytometry is not a common method in cell culture. However, its ability to quantitate any cell-associated property for which there is a fluorescent probe makes it an extremely flexible tool (Al-Rubeai and Emery, 1993). In flow cytometry, cells pass in single file through a laser beam with which they each interact (Figure 1-4). This gives information about cell size, shape, density and surface morphology for each cell that passes through the system. Labelling of one or more cellular constituents allows quantitative determination and the potential to physically select individual cells based on these determinations. More commonly, flow cytometry is used to monitor parameters which are indicators of cell physiology. Staining cells with DNA fluorophores allows measurement of cell ploidy or cell cycle. During culture the proportion of the cell population in each cell-cycle phase changes. During the lag phase DNA synthesis increases and this is seen as an increase in the proportion of cells in S phase. During exponential growth the proportion of cells in G1 increase and those in S phase decrease. During the death phase G1 cells are observed to continue to increase in relation to cells in S and G2/M phases. Several studies have demonstrated the impact of bioreactor environment on the cell cycle of cultures animal cells (Papoutsakis and Kunas, 1989, Al-Rubeai et al., 1993).
Chapter 1.7 Analysis

1.7.2 Protein quality

The characterisation of a particular glycoprotein can involve the elucidation of a great deal of available information. Sometimes it may be sufficient to ascertain only whether a glycosylation event has occurred. Alternatively, the quantities of individual sugar units, their location on different glycosylation sites and their sequence and structural attachment to the polypeptide chain may also be desired. However, the importance of both the types and proportions of oligosaccharides present on a glycoprotein has lead to the requirement for highly sophisticated analytical techniques. Jenkins and Curling (1994) stated that the availability and improvement of methods for complex glycan analysis may lead to increased regulatory pressure for more detailed batch to batch quality control. Although several recombinant glycoproteins have been approved for therapeutic use in humans there are no
strict requirements yet for details of glycan content. But the increasing
acknowledgement of the influence of oligosaccharide content on biological
activity will only increase the requirement for improved analysis.

The availability of various characterisation methods for recombinant
glycoproteins has been reviewed by Spellman (1990). Basic information on
the presence or absence of oligosaccharides (macroheterogeneity) can be
obtained by the use of polyacrylamide gel electrophoresis, glycosidase
treatment, and Western blotting using glycan-binding agents. The gel
approach was used successfully to monitor the changes in the
macroheterogeneity of a glycoprotein during batch culture in CHO cells
(Castro et al., 1995). The analysis of immunopurified IFN- γ revealed a
decrease in the fully glycosylated form with time in culture.

Another technique for rapid elucidation of glycosylation macroheterogeneity is
capillary electrophoresis (CE). This method involves separation of glycoforms
by electrophoretic flow through narrow-bore capillaries which may be coated.
The technique can be used in a variety of ways to perform separations based
on net charge, micellar association and molecular weight. Frenz and Hancock
(1991) demonstrated the charge separation of the glycosylation variants of
CD4, a potential drug for AIDS therapy. When sialic acids were removed
from the carbohydrate groups of the protein by neuraminidase, its charge
heterogeneity was reduced and efficient separation was lost. Micellar
electrokinetic capillary electrophoresis (MECE) is closely related but a
micellar solution is used as the running buffer. Separation is achieved according to the partition coefficients of the sample components between the micellar and bulk phases. This technique is particularly suited to species of similar charge. Methods to mimic the selectivity and molecular weight-based separations of SDS-PAGE chemistry have lead to the development of polyacrylamide gel-filled capillaries. These require higher sample quantities but give rapid, high efficiency separations and allow more accurate quantification of components of a mixture than can be obtained by conventional staining protocols employed for slab gels.

The most common methods for analysis of monosaccharide content (microheterogeneity) include lectin affinity chromatography, exoglycosidase digestion, mass spectrometry (MS) and nuclear magnetic resonance (NMR). Green and Baenziger (1989) demonstrated that lectin affinity HPLC could be used to obtain detailed information for glycoproteins. Lectins are carbohydrate-binding proteins which are specific for particular oligosaccharide structures. When covalently immobilised on silica, individual oligosaccharides can be separated rapidly from a heterogeneous population. HPLC analyses reveal a characteristic 'fingerprint' for each oligosaccharide. However, several lectin columns are required and structural detail is limited. But with a glycoprotein available in limited quantities lectins provide a powerful tool in the analysis of an oligosaccharide structure not previously described.
Chapter 1.7 Analysis

The general availability of large quantities of these pharmaceuticals lends their analysis to methods of high accuracy but low sensitivity. NMR is one such technique and can, by itself, identify a complete structure. A $^1$H NMR spectrum of an oligosaccharide or glycopeptide can be interpreted by knowledge of the spectra of known structures such as the H-2 and H-3 of mannose, H-3 of sialic acid and the methyl protons of N-acetyl groups. Tsuda et al. (1988) demonstrated the ability of NMR to identify specific oligosaccharide linkages and therefore suggest biological roles for particular sequences. Some techniques require the glycoprotein to be treated before analysis. A common approach is enzymic digestion of the glycoprotein and separation of the peptides by HPLC to form a peptide map. Treatment with the endoglycosidase peptide-N-glycosidase F (PNGaseF), which cleaves the β-aspartylglucosaminyl bond of all known types of N-linked oligosaccharides, causes the glycopeptide peaks to shift thus identifying them. The glycopeptides can then be routinely collected and analysed further. Alternatively, the oligosaccharides can be collected and used for analysis. However, this does not give information on site heterogeneity when a glycoprotein has more than one glycosylation site the.

The most sensitive approach to characterising glycosylation site heterogeneity is probably mass spectrometry (James, 1996b). There are several types of MS each with subtle differences influencing mass accuracy, cost and ease of use. Fast-atom bombardment (FAB) has the highest mass accuracy of the spectrometric methods. However, FAB is expensive and requires a large
amount of sample. Two methods of ionisation, developed relatively recently, are particularly suitable for analysis of peptides and proteins: matrix-assisted laser desorption/ionisation (MALDI) and electrospray ionisation (ESI) MS (Siuzdak, 1994). These techniques have proved to be a relatively rapid means of confirming the primary structure of recombinant proteins and analysis of post-translational modifications such as glycosylation which contribute to recombinant protein heterogeneity (Aebersold 1993, Wang and Chait, 1994, Burlingame, 1996).

When using MALDI analysis the sample is dispersed with an excess of matrix material, e.g. sinapinic acid, which strongly absorbs laser light. When a laser beam is fired at the mixture the sample is vaporised into ions which can be measured by a time-of-flight (TOF) analyser. MALDI has an effective mass range of 0.5 to 200 kDa and requires less sample. Sutton et al. (1993) characterised the recombinant human tissue inhibitor of metalloproteinases (TIMP) using this method. Glycopeptides were generated by trypsin digestion and separated by reversed phase (rp) HPLC. The composition and sequence were then elucidated by digestion with specific glycosidases in a reagent-array analysis method demonstrated by Edge et al. (1992). They were able not only to obtain molecular weight but also structural details of oligosaccharides at different glycosylation sites. In this laboratory we have previously demonstrated the use of MALDI MS in combination with exoglycosidase array sequencing to examine site-specific N–glycosylation of recombinant human IFN-γ produced by eukaryotic expression systems (James et al., 1995). This technique was subsequently employed by the laboratory to monitor changes in
Chapter 1.7 Analysis

the N–glycosylation of IFN–γ during the batch culture of CHO cells (Hooker et al., 1995). Further analyses of intact IFN–γ proteins by ESI MS described the heterogeneity of recombinant IFN–γ populations derived from different eukaryotic host cells in detail (James et al., 1996a).
Chapter 2 Materials and Methods

2. Materials and Methods

2.1 Cell Culture System

2.1.1 Cell line

The cell line used throughout this work was a recombinant CHO cell line (clone 320) donated by Wellcome Biotechnology (Beckenham, Kent, U.K.). It was developed from a dihydrofolate reductase (DHFR) deficient CHO-K1 mutant by co-transfecting with a plasmid vector containing the DHFR and human IFN-γ genes under the control of the SV40 early promoter. The IFN-γ gene was amplified by methotrexate selection. The CHO 320 cell line was adapted to suspension culture and for growth in a serum free medium based on RPMI 1640 (Hayter et al., 1989 and Hayter et al., 1991).

2.1.2 Medium preparation

The preparation of all growth media was performed under sterile conditions in a laminar flow cabinet model Gelaire BSB (Flow Laboratories, High Wycombe, Buckinghamshire, U.K.). Sterile disposable cell culture equipment was obtained from Falcon (Becton-Dickinson, Oxford, U.K.) or Gelman Sciences (Ann Arbor, Michigan, U.S.A.) unless otherwise stated.
Chapter 2.2 Cell Culture System

Filtration units were acquired from Gelman Sciences or Sartorius Ltd. (Epsom, Surrey, U.K.).

2.1.3 Medium

The culture medium was a serum-free based on RPMI 1640 (Imperial Laboratories, Andover, U.K.) supplemented with BSA, bovine insulin, human transferrin, L-glutamine, trace elements and methotrexate (MTX) and developed by the Animal Cell Culture group at the University of Kent (Hayter et al., 1989). The concentrations of the trace elements were based on those described by Hamilton and Ham (1977). All chemicals were supplied from Sigma, Poole, U.K. except BSA which was obtained from Miles Biochemicals, Slough, U.K. The stock and final concentrations of each medium component are presented in no particular order in Table 2-1.
### Table 2-1 Cell culture medium supplements with concentrations.

<table>
<thead>
<tr>
<th>Component</th>
<th>Stock concentration</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSA (Pentex Fraction V)</td>
<td>100 g l⁻¹</td>
<td>5 g l⁻¹</td>
</tr>
<tr>
<td>Human transferrin</td>
<td>100 mg l⁻¹</td>
<td>5 mg l⁻¹</td>
</tr>
<tr>
<td>Bovine insulin</td>
<td>100 mg l⁻¹</td>
<td>5 mg l⁻¹</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>200 mM</td>
<td>2 mM</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>100 mM</td>
<td>1 mM</td>
</tr>
<tr>
<td>L-alanine</td>
<td>10 mM</td>
<td>0.1 mM</td>
</tr>
<tr>
<td>Putrescence</td>
<td>0.1 mM</td>
<td>1 μM</td>
</tr>
<tr>
<td><strong>Trace elements</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iron sulphate (FeSO₄)</td>
<td>0.3 mM</td>
<td>3 μM</td>
</tr>
<tr>
<td>Zinc sulphate (ZnSO₄)</td>
<td>0.3 mM</td>
<td>3 μM</td>
</tr>
<tr>
<td>Sodium selenite (Na₂SeO₃)</td>
<td>1 mM</td>
<td>10 nM</td>
</tr>
<tr>
<td>Copper sulphate (CuSO₄)</td>
<td>1 mM</td>
<td>10 nM</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methotrexate</td>
<td>0.1 mM</td>
<td>0.1 μM</td>
</tr>
</tbody>
</table>

The base RPMI 1640 medium was prepared using ultra pure water from a Milli-Q Plus system (Millipore, Watford, U.K.) and filtered through a 0.22 μm Micro Capsule filter unit and stored at 4°C in 1 l aliquots. Medium prepared for fermentation cultures was prepared with base RPMI 1640 already containing L-glutamine at the required concentration. This was prepared as above but filtered through a Sartorius filter into an inoculation vessel. Protein
Chapter 2.2 Cell Culture System

components were filtered separately and added aseptically with the addition of 1 ppm Antifoam C for suspension cultures and 10 ppm Antifoam C/0.1 % w/v pluronic F68 for fluidised bed perfusion cultures.
The sterilisation of glass- and plasticware and inoculation vessels was performed in a Rodwell autoclave model Ensign (Hornchurch, U.K.) for 30 minutes at 120°C. Vessels below 2 l and some plasticware were also sterilised in a Series 2100 media autoclave (Prestige Medical, Blackburn, U.K.). Batch suspension cultures were performed in 25, 125 and 250 ml Falcon shaker flasks (Becton Dickinson Labware, Plymouth, U.K.); 0.5 and 1 l spinner vessels by Techne (Cambridge, U.K.) and 0.5, 1, 2.5 and 5 l spinner vessels by Bellco (V.A. Howe & Co., Oxon, U.K.). All microcarrier cultures were performed in 0.5 l Bellco vessels which were precoated with Sigmacote (Sigma) to prevent attachment of microcarriers to internal surfaces. All microcarriers were supplied by Pharmacia Biotech (St. Albans, U.K.) and prepared according to manufacturer’s instructions. The Cytodex 3 and Cytopore spinner cultures were agitated at 40 rpm and the Cytoline 1 cultures were agitated at 60 rpm. The individual properties of the microcarriers used are listed in Table 2-2.
Table 2-2 Physical characteristics of three different microcarriers.

<table>
<thead>
<tr>
<th>Microcarrier</th>
<th>Matrix</th>
<th>Size</th>
<th>Density</th>
<th>Pore size</th>
<th>Surface area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(mm)</td>
<td>(g mL⁻¹)</td>
<td>(µm)</td>
<td>(m² g⁻¹)</td>
</tr>
<tr>
<td>Cytodex 3</td>
<td>Dextran/collagen</td>
<td>0.18</td>
<td>1.04*</td>
<td>N/A</td>
<td>0.46</td>
</tr>
<tr>
<td>Cytopore</td>
<td>Cellulose</td>
<td>0.20-0.28</td>
<td>1.03*</td>
<td>30</td>
<td>1.1</td>
</tr>
<tr>
<td>Cytoline 1</td>
<td>Polyethylene/silica</td>
<td>length 17-25,</td>
<td>1.32</td>
<td>1-400</td>
<td>?</td>
</tr>
</tbody>
</table>

* in 0.9% NaCl, N/A = not applicable because microporous, ? = unknown.

The fermentation system included a 15 l LH (Inceltech U.K. Ltd., Reading, U.K.) glass-jacketed, single impeller bioreactor with a 2:1 aspect ratio. It has setpoint control of temperature by an LH controller and dO₂ and pH by an Anglicon controller (Brighton Systems, Brighton, U.K.). The temperature was maintained at 37°C (± 0.2°C), dO₂ was maintained at 40% of air saturation (± 1%) and pH at 7.2 (± 0.05). Oxygen and pH probes were supplied by Mettler-Toledo (Greifensee, Switzerland) and the temperature probe by Brighton Systems. Oxygen was supplied by gassing the medium with air below the impeller with a 1 µm sintered glass sparger. Alkali addition to control pH was made using a 0.1 M NaOH solution delivered onto the surface of the medium. Data-logging was supplied by Biotechnology Computer Systems, South Bank Technopark, U.K. run on a 386 DX personal computer (Viglen Ltd., Alperton, U.K.). The bioreactor was autoclaved in a BMM Weston/Drayton Castle ‘S’
Chapter 2.2 Cell Culture Methods

Series autoclave for 40 minutes at 120°C. The bioreactor system was used for batch experiments with daily sample volumes of 0.5-2.0 l resulting in a 50% reduction of the working volume from 12 to 6 l. Larger samples were taken early in the experiment when the product titre was lowest; the agitation speed was stepped down in parallel with the working volume so that a constant power-to-volume ratio of 0.02 W l\(^{-1}\) was maintained.

The fermentation system for fluidised bed culture was a 2 l bioreactor (Cytopilot-Mini) obtained from Pharmacia Biotech. It was operated with setpoint control of temperature and \(dO_2\) and pH by two independent Anglicon controllers (Brighton Systems). The temperature was maintained at 37°C using an external Techna water bath (± 0.1°C), \(dO_2\) was maintained at 40% of air saturation (± 2%) and pH at 7.2 (± 0.1). Ingold oxygen and pH probes were supplied by Mettler-Toledo AG. Data-logging was supplied by Biotechnology Computer Systems run on a 386 DX personal computer (Viglen Ltd.). The bioreactor was autoclaved in a Rodwell autoclave model Ensign for 40 minutes at 118°C. The methods employed with the fluidised bed reactor as well as a schematic of the bioreactor are covered in more detail in Section 3.7.

2.2.1 Static Gassing-Out Method

The theory behind this method is outlined in Section 1.3.4. Nitrogen gas was used to purge air from the medium by sparging beneath the moving
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impeller. When an anoxic condition was achieved air was sparged into the medium at a constant rate of 300 ml min\(^{-1}\). The increase in dissolved oxygen concentration \((C_L)\) was recorded every 3 minutes for 15 minutes or until the dissolved oxygen concentration had reached 40% of saturation. The temperature of the medium was maintained at 37°C.

2.2.2 Cell inoculum

All cell inoculums for 15 l fermentation cultures were prepared in two identical 5 l Bellco spinner vessels (Bellco). In each spinner vessel 2.5 l of cells were cultured for 3 days or until the viable cell density had reached a mid-exponential density of approximately \(5 \times 10^5\) cells ml\(^{-1}\). Two approaches were then employed to prepare the final inoculum. In most cases, the cells were allowed to settle for at least 30 minutes at 37°C and the top 2.0 l of cell culture removed. The remaining 0.5 l of ‘concentrated’ cell culture (containing at least 60% of the original cell number) was then transferred to a 2 l inoculum vessel containing 1 l of prewarmed medium. Alternatively, after cell settling had been completed, the cell slurry was centrifuged at 1000 rpm for 5 minutes in 200 ml aliquots. The cell pellets were resuspended in prewarmed medium and transferred to a 2 l inoculum vessel. For small scale microcarrier cultures cells were also seeded from cultures in the mid-exponential phase of growth. The total cell inoculum was added to half the final volume of medium which had been preincubated with the microcarriers overnight. For a period of 4 h the microcarrier cultures were agitated
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intermittently: 45 min on, 15 min off for each one hour period. After the 4 h the rest of the medium was added and the spinner vessels agitated continuously at their respective agitation rates. Cell inoculation of the Cytopilot-Mini is detailed in Section 3.7 but was based on the second approach given above.

2.2.3 Cell banks

All working cell stocks were created from a master cell bank maintained at the University of Kent. Cultures were tested every 8 months for the presence of mycoplasma. Cells were grown for a minimum of 6 passages because during early passages the viability of the cell line was too poor for experimental use. The cells were also not cultured for longer than 20 passages because the CHO 320 cell line did not maintain a constant expression level (personal communication, P. Hayter).
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Analytical grade reagents were purchased from Sigma (Poole, U.K.) unless otherwise stated. Monoclonal antibody 20B8 raised against recombinant IFN-γ from *Escherichia coli* as well as 20B8 coupled to Sepharose (Reselute) was provided by Celltech Biologics Ltd. (Slough, U.K.). Neuraminidase (*ex. Arthrobacter ureafaciens*), β-galactosidase (*ex. Streptococcus pneumoniae*), β-hexosaminidase (from chicken liver), β-hexosaminidase (*ex. Streptococcus pneumoniae*), α-mannosidase (*ex. jack bean*), β-mannosidase (*ex. Helix pomatia*), PNGaseF (recombinant *Flavobacterium meningosepticum* produced in *E.coli*), Endoglycosidase H (recombinant *Streptomyces plicates* produced in *E.coli*) and asialo, galactosylated triantennary and β-mannosyl, α-fucosyl chitobiose oligosaccharides and the 2-aminobenzamide (2-AB) labelling kit were all purchased from Oxford GlycoSystems Ltd., Abingdon, U.K.

Trypsin (porcine sequencing grade) was purchased from Promega, Southampton, U.K. Endoprotease Asp-N (mutant *Pseudomonas fragi*) was obtained from Boehringer Mannheim, East Sussex, U.K. Anti-IFN-γ polyvalent immunoglobulins and anti-mouse monoclonal antibodies were obtained from Genzyme, Cambridge, U.K. Recombinant human IFN-γ (produced in *E. coli*) was obtained from NIBSC, Potters Bar, U.K. The polyacrylamide gel capillary electrophoresis (PAGCE), eCAP capillary
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isoelectrofocusing (CIEF) (pI 3-9) kits and both neutral and coated capillaries were supplied from Beckman Instruments, High Wycombe, U.K.
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2.4.1 Enumeration of viable cell density in suspension culture

In an Eppendorf tube, 100 μl of cell culture fluid was mixed with 100 μl of erythrosin B and cell counts made in a Neubauer counting chamber (well depth 0.1 mm), using a light microscope (Model ID 02, Zeiss, Oberkochen Germany). Cell viability was determined by erythrosin dye exclusion, non-viable cells staining pink. A minimum of 500 cells were counted.

2.4.2 Enumeration of total cell density on microcarriers

Determination of cell number was based on the nuclei exclusion method of Sanford et al. (1950). From a well-mixed suspension a 1 ml sample of microcarriers were removed and transferred into a 15 ml centrifuge tube. The microcarriers were allowed to settle and the supernatant was removed by pipette. The microcarriers were washed twice with PBS and the supernatant discarded. A 1 ml solution of 0.1 M citric acid/0.1% crystal violet/1.0% Triton-X 100 was added to the microcarriers and the suspension was rotated on a whirlywheel for 2 h at 37°C or overnight at room temperature. This achieved lysis of the attached cells and the release and staining of the nuclei. The mixture was then vortexed for 1 minute and the nuclei, stained dark blue, were counted using a Neubauer counting chamber as above. In addition,
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Cytopore microcarriers were passed through an injection needle 10 times to generate a homogenous cell/microcarrier suspension.

2.4.3 Visualisation of cell growth on microcarriers using light, fluorescence and confocal microscopy

From a well-mixed culture a 0.5-1.0 ml sample of microcarriers was removed. The microcarriers were allowed to settle and the supernatant was removed by pipette. The microcarriers were then washed twice with PBS and the supernatant discarded. The growth of CHO cells and their progress to confluence was visible with Cytodex 3 microcarriers at a magnification of 400x using a Zeiss light microscope. The presence of cells on all three microcarriers listed in Table 2-2 could be visualised using a fluorescence microscope (Zeiss) after a 5 min incubation at room temperature with a 1% w/v solution of acridine orange. However, the resolution of individual cells (stained orange) on both the Cytopore and Cytoline 1 microcarriers at a 100x magnification was poor. A qualitative determination of cell viability was used with Cytoline 1 microcarriers. To a sample of washed Cytoline 1 microcarriers a 1:1 mixture of fluorescein diacetate (5 µg FDA ml⁻¹ PBS) and ethidium-bromide (1 µg EB ml⁻¹). Both components were stored separately in 1 ml aliquots at -20°C. Viable cells stained green and dead cells stained red under a fluorescence (Zeiss) or confocal microscope (Leica U.K. Ltd., Milton Keynes, U.K.)
2.4.4 Visualisation of cell growth on Cytoline 1 microcarriers by scanning electron microscopy

From a fluidised bed reactor sample a 1 ml sample of Cytoline 1 microcarriers was removed and transferred into a 15 ml centrifuge tube. The microcarriers were allowed to settle and the supernatant was removed by pipette. The microcarriers were then washed in 1 ml of PBS and 1.0 ml of 2% glutaraldehyde/PBS solution added and stored at 4°C overnight. The carriers were washed twice with PBS and dehydrated in ethanol. The ethanol was then substituted with acetone in increasing proportions: 25%, 50%, 75% and finally 100% v/v. The microcarriers were treated by critical point drying with liquefied carbon dioxide and then coated with gold in an argon atmosphere. Cell adhesion was observed with a 525M scanning electron microscope (Philips, Eindhoven, Holland).
2.4.5 Metabolite and by-product determination

2.4.5.1 Enzymatic determination of glucose

Glucose was assayed by the o-dianisidine method (Sigma procedure number 510) which is based on the following coupled enzymatic reactions:

\[
\text{Glucose oxidase} \\
\text{Glucose} + \text{H}_2\text{O}_2 + \text{O}_2 \rightarrow \text{Gluconic acid} + 2\text{H}_2\text{O}_2
\]

\[
\text{Peroxidase} \\
\text{H}_2\text{O}_2 + \text{o-dianisidine} \rightarrow \text{Oxidised o-dianisidine}
\]

All the reagents for the assay were supplied with the kit unless otherwise indicated.
2.4.5.1.1 Reagent preparation

**Enzyme solution:** The contents of one capsule of PGO (peroxidase and glucose oxidase) enzymes were added to 100 ml of Milli-Q water and dissolved by very gentle agitation.

**Colour reagent solution:** One vial of o-dianisidine dihydrochloride was reconstituted with 20 ml Milli-Q water. The solution was prepared fresh for each assay.

**Combined enzyme-colour reagent solution:** 100 ml enzyme solution was mixed with 1.6 ml of o-dianisidine dihydrochloride solution and mixed with very gentle shaking. The solution was prepared fresh for each assay.

**Standards and samples**

Standards were prepared in the range 0.375-12 mM using D-glucose prepared in Milli-Q water by 5 doubling dilutions. Then both standards and samples were diluted 1:10 with Milli-Q water.

2.4.5.1.2 Assay procedure

The assay was performed in duplicate in disposable 1 ml plastic cuvettes, to which 100 μl of each standard, sample and a blank (Milli-Q water) were added. To these mixtures were added 1 ml of combined enzyme-colour reagent and the mixture well mixed. The cuvettes were incubated at room temperature for 45 minutes avoiding exposure to bright light. The absorbance
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was read at 450 nm using a UV/VIS spectrometer Model UNICAM 8625 (Unicam Ltd., Cambridge, U.K.) using the blank as reference. The sample concentration was calculated by construction of a standard curve.

2.4.5.2 Enzymatic determination of lactate

Lactate was determined by a lactate dehydrogenase procedure (Sigma procedure number 735). This assay relies on the action of lactate oxidase on lactate which produces pyruvate and hydrogen peroxide. The hydrogen peroxide catalyses the oxidative condensation of chromagen precursors to produce a coloured dye with an absorption maximum at 540 nm. All the reagents for the assay were supplied with the kit unless otherwise indicated.

2.4.5.2.1 Reagent preparation

Lactate reagent: 10 ml of Milli-Q water was added to each vial of lactate reagent. The solution was mixed by gentle inversion.

Standards and samples

Lactate standards were made at 20, 15, 10, 5 and 1 mM in Milli-Q water. These were aliquoted into 1 ml vials and stored at -20°C.
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2.4.5.2.2 Assay procedure

The assay was performed in disposable 1 ml cuvettes, to which 10 μl of each standard, sample and a blank (Milli-Q water) were added in duplicate. To these were added 1 ml of lactate reagent and the contents mixed and incubated for 5-10 minutes at room temperature. The absorbances were read at 540 nm and the concentrations of samples determined from the construction of the standard curve.

2.4.5.3 Determination of ammonia

Ammonia was determined by the indophenol method (Fawcett and Scott, 1960), which involves the reaction of ammonia with hypochlorite and phenol to form indophenol blue.

2.4.5.3.1 Reagent preparation

The individual components (not added in any particular order) are listed in Table 2-3.
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Table 2-3 Reagents for the ammonia assay.

<table>
<thead>
<tr>
<th>Assay Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Phenate</td>
<td></td>
</tr>
<tr>
<td>Phenol (BDH)</td>
<td>2.5 g</td>
</tr>
<tr>
<td>5M NaOH</td>
<td>6.24 ml</td>
</tr>
<tr>
<td>Milli-Q water</td>
<td>94 ml</td>
</tr>
<tr>
<td><strong>Note:</strong> stable for one month at 4°C.</td>
<td></td>
</tr>
<tr>
<td>Sodium Nitroprusside Stock</td>
<td></td>
</tr>
<tr>
<td>Sodium nitroprusside (Sigma)</td>
<td>0.3 g</td>
</tr>
<tr>
<td>Milli-Q water</td>
<td>30 ml</td>
</tr>
<tr>
<td><strong>Note:</strong> aliquoted into 1 ml Eppendorfs and stored at -20°C.</td>
<td></td>
</tr>
<tr>
<td>Sodium Nitroprusside Working Solution</td>
<td></td>
</tr>
<tr>
<td>Sodium nitroprusside stock</td>
<td>1 ml</td>
</tr>
<tr>
<td>Milli-Q Water</td>
<td>100 ml</td>
</tr>
<tr>
<td><strong>Note:</strong> prepared immediately prior to use.</td>
<td></td>
</tr>
<tr>
<td>Sodium Hypochlorite</td>
<td></td>
</tr>
<tr>
<td>Sodium hypochlorite solution (BDH)</td>
<td>0.71 ml</td>
</tr>
<tr>
<td>Milli-Q water</td>
<td>100 ml</td>
</tr>
<tr>
<td><strong>Note:</strong> prepared immediately prior to use.</td>
<td></td>
</tr>
</tbody>
</table>

Standards and samples

An ammonia standard was created using a 5 mM ammonium chloride (Fisons) solution which was aliquotted in 1 ml vials and stored at -20°C. A standard
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concentration range was prepared by performing 5 serial dilutions to give a 5-0.1625 mM array.

2.4.5.3.2 Assay procedure

To 3 ml disposable cuvettes were added 50 µl of standard, sample or blank (Milli-Q water) in duplicate. Then the assay reagents were added in the following order:

1. sodium phenate 0.5 ml
2. sodium nitroprusside working solution 0.75 ml
3. sodium hypochlorite 0.75 ml

The contents were mixed thoroughly and incubated at room temperature for 30 minutes. The absorbance was measured at 630 nm in a spectrometer. The sample concentrations were determined from the construction of a standard curve.

2.4.5.4 Automated metabolite determination

A Biolyzer (Kodak IBI Ltd., Cambridge, U.K.) was used to monitor glucose, ammonia and lactate by the use of dry enzyme slides. The system was calibrated every 6 months according to manufacturers instructions. The
Biolyzer dry enzyme slides gave equivalent readings for samples previously analysed by the Sigma procedures.

2.4.5.5 Determination of amino acids

Amino acids were analysed by their reaction with o-phthalaldehyde (OPA) to form a fluorescent product. Imines such as proline and hydroxyproline as well as cysteine and cystine are not derivatised using this method. The amino acids which can be detected by the assay were aspartate, glutamate, asparagine, serine, glutamine, histidine, glycine, threonine, arginine, tyrosine, methionine, phenylalanine, valine, isoleucine, leucine and lysine. Amino acids were resolved on a reverse-phase (C18) column (Hichrom, Reading, Berks., U.K.) using a gradient from phosphate/acetate buffer to 70% methanol (v/v). The method is based on one described by Seaver et al. (1984). The original HPLC system consisted of a Model SIL 9A auto-injector (Shimadzu, Hetton, U.K.), a Model Series 4 Liquid Chromatography solvent delivery system (Perkin-Elmer, Norwalk, CT, U.S.A.), a luminescence spectrometer (Perkin-Elmer) and a laboratory computing integrator Model LCI-100 (Perkin Elmer). An excitation wavelength of 334 nm and a detection wavelength of 425 nm was employed. The column’s resolution was monitored using a standard amino acid mixture (Sigma AA-S-18) and calibrated with freshly prepared RPMI with L-glutamine.
2.4.5.5.1 Sample and buffer preparation

The buffers were prepared as listed Table 2-4. Samples and standard (serum free media frozen at -20°C in 500 μl aliquots) were thawed and 400 μl aliquots were centrifuged through 10 kDa exclusion limit ultrafiltration membranes (Millipore) for approximately 20 minutes at 10,000 rpm in a microcentrifuge. The filtrate was then diluted 1:30 with Milli-Q water and 200 μl of each dilution was placed in the auto-injector vials and capped with septum in place.

2.4.5.5.2 Auto-injector preparation

The sample vials were loaded into the auto-injector rack. A 10 ml reagent vial was filled with fluoraldehyde (Pierce Chemical Co., Rockford, U.S.A.). The auto-injector was programmed to add 200 μl of fluoraldehyde to each sample vial, mix 3 times, wait 2 minutes and then make a single injection of 20 μl of each sample preparation onto the column. The sample line was automatically rinsed with buffer A between samples.
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Table 2-4 Buffers for HPLC determination of derivitised amino acids.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Buffer A: Phosphate Buffer</strong></td>
<td></td>
</tr>
<tr>
<td>Disodium hydrogen Phosphate (12 H₂O)*</td>
<td>0.05M</td>
</tr>
<tr>
<td>Sodium dihydrogen phosphate*</td>
<td>0.05M</td>
</tr>
<tr>
<td>Sodium acetate*</td>
<td>0.05M</td>
</tr>
<tr>
<td>Methanol†</td>
<td>2%</td>
</tr>
<tr>
<td>Tetrahydrofuran†</td>
<td>2%</td>
</tr>
<tr>
<td><strong>Buffer B: 70% Methanol</strong></td>
<td>70 %</td>
</tr>
<tr>
<td>Methanol</td>
<td></td>
</tr>
<tr>
<td><strong>Buffer C: 100% Methanol</strong></td>
<td>100 %</td>
</tr>
<tr>
<td>Methanol</td>
<td></td>
</tr>
<tr>
<td><strong>Buffer D: 100% Milli-Q Water</strong></td>
<td></td>
</tr>
</tbody>
</table>

*Fisons, †Aldrich. Buffers A and B were prepared with Milli-Q water.

2.4.5.5.3 Computing integrator preparation

The column was run without sample injection to calculate area and baseline sensitivities with the buffers to be used for the method. The integrator was set to run for 45 minutes, use a peak width of -5 and to reject peaks with an area to height ratio less than 50,000. The plotter was set to have an attenuation of 64 and an offset of 5.
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2.4.5.5 HPLC assay

One litre of each buffer was placed in their respective buffer reservoirs and the system degassed for 5 minutes. The reservoir lines were purged with buffers C, B, D and A in that order. The column was washed with buffer B for 15 minutes before beginning the equilibration section of the chromatography program (Table 2-5). The fluorescent detector was auto-zeroed when a stable reading had been achieved with buffer A running through the system. The auto-injector and computing integrator were programmed for the quantity of samples to be analysed.

<table>
<thead>
<tr>
<th>Sector</th>
<th>Time (min)</th>
<th>Flow (ml min⁻¹)</th>
<th>A (%)</th>
<th>B (%)</th>
<th>C (%)</th>
<th>D (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equilibration</td>
<td>0</td>
<td>1.0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>1.0</td>
<td>85</td>
<td>0</td>
<td>10.5</td>
<td>4.5</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>1.0</td>
<td>60</td>
<td>0</td>
<td>28</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>1.0</td>
<td>0</td>
<td>0</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>1.0</td>
<td>0</td>
<td>0</td>
<td>70</td>
<td>30</td>
</tr>
</tbody>
</table>

The run was completed by washing the column with buffer B for 10 minutes and buffer C for 5 minutes, in which the column was stored. Samples from the perfusion bed bioreactor (Section 3.7) were analysed using a different HPLC system: a Waters 626 Millenium system (Millipore Ltd, Watford, U.K.)
although the same auto-injector was employed. An extended gradient programme (each sector was increased by 150%) and a flow rate of 0.75 ml min$^{-1}$ were used. The fluorescence detector was set with an emission gain of 10 and attenuation of 32. Otherwise the assay was run in an identical manner to that outlined above.

2.4.6 Cell cycle analysis

Cell pellets of at least $1 \times 10^6$ cells ml$^{-1}$ were fixed in 70% ethanol (v/v) and stored at -20°C until required. The fixed cells were centrifuged at 1000 rpm for 5 minutes and resuspended in PBS. The cells were lysed and the nuclei stained with propidium iodide using an Epics Leukocyte Preparation Workstation (Coulter Electronics Ltd., Luton, U.K.). The nuclei were stained for 15 minutes before subsequent cell cycle cytometric analysis on an Epics II flow cytometer (Coulter Electronics Ltd.). Proportions of cells in stages of the cell cycle were determined using Epics Multicycle software (Coulter Electronics Ltd.). Default settings were used. A typical histogram is displayed in Figure 2-1.
Figure 2-1 A typical distribution of cells through the cell cycle during the exponential growth stage of a CHO 320 batch suspension culture. Subsequent analysis using a Gaussian-based curve fitting algorithms gave the proportions: $G_1=57.9\%$, $S=32.3\%$, $G_2/M=9.8\%$ and $G_2/G_1=1.94$.

2.4.7 IFN-γ titre determinations

2.4.7.1 ELISA assay for IFN-γ concentration

Concentrations of IFN-γ were determined by a sandwich enzyme-linked immunosorbent assay (ELISA). The buffers used in the assay are listed in Table 2-6 and the steps are illustrated in Table 2-7. The assay was performed on 96-well assay plates (Dynatech, West Sussex, U.K.) coated with
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a rabbit polyclonal antibody raised against a mixed population of CHO-derived IFN-γ. Recombinant IFN-γ was further detected using a mouse monoclonal 20B8 antibody (Celltech Biologics), recognising a different epitope on the IFN-γ molecule but not associated with the glycosylation sites (Hooker et al., 1996).

In the second ELISA, based on Byron et al. (1992), the 96-well plate was coated with a rabbit polyclonal antibody raised against recombinant IFN-γ from *E. coli* (Genzyme) and further detected with a mouse monoclonal antibody raised against the same recombinant IFN-γ (Genzyme) (Table 2-8). Buffer solutions used were identical except that the polyclonal was diluted 1/3000 (Table 2-6).

In both ELISAs recombinant IFN-γ was determined using anti-mouse polyvalent immunoglobulins (AMPI) conjugated to peroxidase and the colour developed using a peroxidase substrate. A stock of IFN-γ standards at 200 IU ml⁻¹ was kept at 4°C and 1/2 serial dilutions performed in wash buffer to obtain a standard concentration range of 3.125 to 200 IU ml⁻¹. Samples were thawed and appropriate dilutions made and applied with the standards in triplicate to the plate. When the colour reaction was stopped the plates were read at 490 nm with a MR5000 plate reader (Dynatech). A calibration curve was constructed from the absorbance readings of the standards and the concentration of each sample was determined by interpolation.
### Table 2-6 Buffer chemicals used in the IFN-γ ELISA assay.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Coating buffer</strong></td>
<td></td>
</tr>
<tr>
<td>Sodium bicarbonate*</td>
<td>10 mM</td>
</tr>
<tr>
<td>Anti-IFN-γ polyclonal antibody</td>
<td>1/1000 or 1/3000</td>
</tr>
<tr>
<td><strong>Wash buffer</strong></td>
<td></td>
</tr>
<tr>
<td>PBS Tablets†</td>
<td>10</td>
</tr>
<tr>
<td>Tween 20‡</td>
<td>0.1%</td>
</tr>
<tr>
<td>Casein*</td>
<td>0.05%</td>
</tr>
<tr>
<td><strong>Block buffer</strong></td>
<td></td>
</tr>
<tr>
<td>PBS Tablets</td>
<td>1</td>
</tr>
<tr>
<td>Casein*</td>
<td>0.5%</td>
</tr>
<tr>
<td>Tween 20*</td>
<td>0.1%</td>
</tr>
<tr>
<td><strong>Substrate buffer</strong></td>
<td></td>
</tr>
<tr>
<td>Tri-sodium citrate†</td>
<td>0.1 M</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$†</td>
<td>0.2 M</td>
</tr>
</tbody>
</table>

**Note:** Wash and block buffers were warmed on a hotplate to dissolve the casein. Substrate buffer was adjusted to pH 5.0 using 5M HCl. Immediately prior to use, one OPD tablet was added per 25 ml buffer. Once dissolved 3 μl of 30% H$_2$O$_2$ (stored at 4°C) was added. All the buffers were prepared using Milli-Q water. †Fisons; ‡Oxoid (Unipath, Basingstoke, U.K.); *Sigma.
Table 2-7 Procedure for the Kent IFN-γ ELISA assay.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Concentration</th>
<th>Incubation conditions</th>
<th>Volume per well</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coating buffer</td>
<td>1/100</td>
<td>o/n, 4°C</td>
<td>50 μl</td>
</tr>
<tr>
<td>Wash buffer x 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blocking buffer</td>
<td></td>
<td>1 h, R.T.</td>
<td>100 μl</td>
</tr>
<tr>
<td>Wash buffer x 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Samples or Standards</td>
<td>200-3 IU ml⁻¹</td>
<td>1 h, R.T.</td>
<td>50 μl</td>
</tr>
<tr>
<td>20B8 antibody*</td>
<td>10 μgL⁻¹</td>
<td>1 h, R.T.</td>
<td>50 μl</td>
</tr>
<tr>
<td>Wash buffer x 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMPI- peroxidase*</td>
<td>1/750</td>
<td>1 h, R.T.</td>
<td>50 μl</td>
</tr>
<tr>
<td>Wash buffer x 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Substrate</td>
<td></td>
<td>15 min, R.T.</td>
<td>100 μl</td>
</tr>
<tr>
<td>Stop - H₂SO₄</td>
<td>20%</td>
<td></td>
<td>10 μl</td>
</tr>
</tbody>
</table>

R.T. = room temperature; *solutions prepared using wash buffer; o/n = overnight.
## Table 2-8 Procedure for the alternative IFN-γ ELISA assay.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Concentration</th>
<th>Incubation conditions</th>
<th>Volume per well</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coating buffer</td>
<td>1/100</td>
<td>48 h, 4°C</td>
<td>50 μl</td>
</tr>
<tr>
<td>Wash buffer x 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blocking buffer</td>
<td>optional, R.T.</td>
<td></td>
<td>100 μl</td>
</tr>
<tr>
<td>Wash buffer x 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Samples or standards</td>
<td>200-3 IU ml⁻¹</td>
<td>1.5 h, R.T.</td>
<td>50 μl</td>
</tr>
<tr>
<td>Wash buffer x 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monoclonal antibody*</td>
<td>10 μgL⁻¹</td>
<td>1 h, R.T.</td>
<td>50 μl</td>
</tr>
<tr>
<td>Wash buffer x 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMPl- peroxidase*</td>
<td>1/750</td>
<td>1 h, R.T.</td>
<td>50 μl</td>
</tr>
<tr>
<td>Wash buffer x 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Substrate</td>
<td></td>
<td>15 min, R.T.</td>
<td>100 μl</td>
</tr>
<tr>
<td>Stop - H₂SO₄</td>
<td>20%</td>
<td></td>
<td>10 μl</td>
</tr>
</tbody>
</table>

R.T. = room temperature; *solutions prepared using wash buffer.

### 2.4.7.2 Protein determinations

Protein assays were performed with an assay kit supplied by Bio-Rad Laboratories Ltd., Hemel Hempstead, U.K. BSA was used as standard.
stock of BSA standards at 1 mg ml\(^{-1}\) was kept at 4°C and diluted to 200 µl to obtain a standard concentration range: 30, 20, 15, 10, 5, 2.5 µg ml\(^{-1}\). Samples were thawed and appropriate dilutions made and applied with the standards in triplicate to the plate. To each well 50 µl of protein determination solution was added and mixed thoroughly. When the colour reaction was stopped the plates were read at 595 nm with a MR5000 plate reader (Dynatech). A calibration curve was constructed from the absorbance readings of the standards and the concentration of each sample was determined by interpolation.

2.4.8 Purification of IFN-γ

2.4.8.1 Immunoprecipitation of IFN

For each determination a 5 ml sample was immunoprecipitated using Reselute, a suspension of Sepharose beads with covalently bound monoclonal antibody 20B8 (Celltech Biologics). The buffers used for this procedure are listed in Table 2-9.
### Table 2-9 Buffers for immunoprecipitation of IFN-γ.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stock</strong></td>
<td></td>
</tr>
<tr>
<td>5x PBS</td>
<td></td>
</tr>
<tr>
<td><strong>Equilibration or Wash 1</strong></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td></td>
</tr>
<tr>
<td>Tween 20</td>
<td>0.1%</td>
</tr>
<tr>
<td><strong>Wash 2</strong></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td></td>
</tr>
<tr>
<td>Nonidet-P40*</td>
<td>0.5%</td>
</tr>
<tr>
<td>EDTA*</td>
<td>5 mM</td>
</tr>
<tr>
<td><strong>Wash 3</strong></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td></td>
</tr>
</tbody>
</table>

*Sigma.

Samples were buffered using the equilibration buffer. Prepacked Reselute, was added at a concentration of 2 µg ml⁻¹ to each sample, filtered through a 0.20 µm Minisart filter (Sartorius), and incubated on a rotating wheel at 4°C overnight. The samples were centrifuged at 3000 rpm for 3 minutes and the supernatant-buffer mixture removed by aspiration. The beads were resuspended in 1 ml Wash Buffer 1 and washed twice by sedimenting the beads at 8000 rpm for 3 minutes and aspirating the supernatant. The beads were washed twice with Wash Buffer 2 and then twice with Wash Buffer 3. The washed pellets were resuspended in 30 µl 50 mM sodium borate/50 mM
SDS pH 8.5 to dissociate IFN-γ from the Sepharose beads. In the previous version of this method the pellets were boiled for several minutes to encourage dissociation (Castro, 1994). However, the author found this step to be detrimental to the method: SDS-PAGE of released material produced a smear of proteins of varying molecular weights, so the boiling step was removed. The suspension was spun through a 0.20 μm Eppendorf filter unit (Millipore Ltd.) to remove the Sepharose beads. Samples were stored at -20°C until required.

2.4.8.2 Two-step

Supernatant from fermentations was harvested by cell centrifugation, filtered through a 0.2 μm Minisart filter (Sartorius) and adjusted to pH 6.5 with 50 mM 2-[N-morpholino]ethanesulfonic acid (MES) and 0.02% (w/v) NaN₃ (buffer 1). Samples were loaded onto a 1 x 5 cm column of SP-Sepharose fast flow attached to an FPLC system (Pharmacia Biotech Ltd, Milton Keynes, U.K.) at a flow rate of 1.0 ml min⁻¹ and unbound material was washed off with 40 ml of buffer 1. The bound protein was eluted with 50 mM MES + 1 M NaCl, pH 6.5, (buffer 2) and directly loaded under gravity onto a 1 x 0.5 cm immunoaffinity column containing the monoclonal antibody anti-IFN-γ 20B8, cyanogen bromide coupled to Sepharose beads. Therefore, purification of IFN-γ was not influenced by changes in glycosylation. Unbound material was washed off the matrix with 10 ml of buffer 2. Bound
Chapter 2.4 Analytical Methods

IFN-\(\gamma\) was eluted in 4.5 ml of 0.1 M glycine-HCl, pH 2.5 and neutralised with 0.5 ml of 1 M Tris-HCl (pH 8.0).

2.4.8.3 One-step

Samples were loaded onto a 1 x 10 cm immunoaffinity column containing Reselute at a flow rate of < 2.0 ml min\(^{-1}\) at 4°C. Unbound material was washed off the matrix with 100 ml of 50 mM MES + 1 M NaCl + 0.05% Tween 20, pH 6.5 followed by 100 ml of buffer 2 (Section 2.4.8.2). Bound IFN-\(\gamma\) was eluted in 4.5 ml of 0.1 M glycine-HCl, pH 2.5 and neutralised with 0.5 ml of 1 M Tris-HCl (pH 8.0). Purified IFN-\(\gamma\) was concentrated and desalted by ultrafiltration with Centricon\textsuperscript{\textregistered}-10 concentrators (Amicon Ltd, Stonehouse, U.K.) and then buffer-exchanged with 0.1 M ammonium bicarbonate (pH 7.8) or sodium phosphate (pH 8.0) buffer containing 0.02% (w/v) NaN\(_3\) depending on subsequent preparative steps, and stored at 4°C.

2.4.9 Analysis of IFN-\(\gamma\) glycosylation

2.4.9.1 SDS-PAGE

The separation of IFN-\(\gamma\) was achieved using 14% polyacrylamide mini-gels assembled into a Novex Model Xcell gel apparatus kit (British
Chapter 2.4 Analytical Methods

Biotechnology, Oxford, U.K.). The electrode reservoirs were filled with a buffer containing 0.025 M TrisHCl, 0.192 M glycine and 0.1% SDS. Samples were mixed 1:1 v/v with sample buffer consisting of 62.5 mM TrisHCl, 2.5% SDS, 0.1% bromophenol blue and 10% glycerol and 20μl loaded into each well using gel-loading pipette tips. A Bio-Rad SDS-PAGE protein standard, with molecular weights in the range 14.5 to 97.4 kD, was applied to one well to serve as a marker. The gels were run for approximately 2 hours at 125V.

2.4.9.1.1 Silver stain

The SDS-PAGE gels were silver stained using the Silver Stain Plus kit (Bio-Rad) and unless otherwise stated, all the reagents were from Bio-Rad. The gels were treated in three stages: fixing, washing and staining (Table 2-10). Milli-Q water was used throughout the procedure.
Table 2-10 Reagents used in the Bio-Rad Silver Staining assay.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixative</td>
<td></td>
</tr>
<tr>
<td>Methanol</td>
<td>100</td>
</tr>
<tr>
<td>Acetic acid†</td>
<td>20</td>
</tr>
<tr>
<td>Milli-Q water</td>
<td>70</td>
</tr>
<tr>
<td>Fixative enhancer solution</td>
<td>10</td>
</tr>
<tr>
<td>Stain</td>
<td></td>
</tr>
<tr>
<td>Milli-Q water</td>
<td>35</td>
</tr>
<tr>
<td>Silver complex</td>
<td>5</td>
</tr>
<tr>
<td>Reaction moderator</td>
<td>5</td>
</tr>
<tr>
<td>Image development</td>
<td>5</td>
</tr>
<tr>
<td>Development accelerator</td>
<td>50</td>
</tr>
</tbody>
</table>

Note: The solution was prepared while rapidly stirring the contents with a Teflon-coated stirring bar. The development accelerator solution was warmed before addition. †Fisons. The components were prepared in the specified order. The amounts shown are sufficient for 2 gels.

After completion of electrophoresis the gels were immersed in 100 ml of fixative solution in a clean box and agitated gently. After this step the procedure may be interrupted and the gel stored at 4°C overnight if desired. The staining procedure was followed as described in Table 2-11.
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Table 2-11 Silver staining procedure for mini-gels.

<table>
<thead>
<tr>
<th>Step</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixative</td>
<td>30</td>
</tr>
<tr>
<td>Milli-Q water wash</td>
<td>3 x 15</td>
</tr>
<tr>
<td>Staining</td>
<td>10-20</td>
</tr>
<tr>
<td>Stop</td>
<td>10</td>
</tr>
</tbody>
</table>

2.4.9.1.2 Densitometry

After the reaction was stopped, each band on the silver-stained gels could be quantified by conversion into absorbance units with a Bio-Rad 1050 scanning densitometer connected to an Amstrad 1640 personal computer. From the scans obtained, the area under each peak was integrated to determine the relative proportions of each IFN-γ site-occupancy variant (glycoform).

2.4.10 Micellar electrophoretic capillary electrophoresis

The separation of IFN-γ glycoforms was achieved using a P/ACE 2000 capillary electrophoresis system (Beckman Instruments Inc.) linked to a computer running Beckman System Gold. The electrode reservoirs were filled with a degassed SDS-boric acid electrode running buffer containing 50 mM SDS and 50 mM boric acid adjusted to pH 8.5 with 5 M sodium hydroxide. Higher concentrations (200-400 mM) of SDS and boric acid were used to
Chapter 2.4 Analytical Methods

achieve higher resolutions. Purified IFN-γ was loaded onto a 75 μm x 50 cm uncoated capillary with a 10 second injection. New capillaries were prepared as indicated in Table 2-12. Between each sample injection the capillary was washed for 3 minutes (Table 2-13).

Table 2-12 Capillary preparation.

<table>
<thead>
<tr>
<th>Step</th>
<th>Concentration</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium hydroxide</td>
<td>0.1 M</td>
<td>15</td>
</tr>
<tr>
<td>Milli-Q water</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>Electrode buffer</td>
<td>†</td>
<td>10</td>
</tr>
</tbody>
</table>

Note: All the solutions were degassed. †Concentration dependent on resolution desired.

Table 2-13 Wash solutions for capillary zone electrophoresis.

<table>
<thead>
<tr>
<th>Step</th>
<th>Concentration</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium hydroxide</td>
<td>0.1 M</td>
<td>3</td>
</tr>
<tr>
<td>Electrode buffer</td>
<td>†</td>
<td>3</td>
</tr>
</tbody>
</table>

Note: All the solutions were degassed. †Concentration depends on resolution desired.

The separation was run for 20 minutes at a constant voltage of 25 kV and temperature of 25°C. The IFN-γ glycoforms were quantified by their
absorbance at 200 nm. The subsequent absorbance peaks were integrated to determine the proportions of each IFN-γ glycoform present.

2.4.11 Capillary isoelectrofocusing

The separation of IFN-γ was achieved using the same capillary electrophoresis system used for MECE. The system was run using chemicals provided with an eCAP cIEF 3-10 kit (Beckman Instruments Inc.) and protocols based on manufacturers instructions. New capillaries were prepared as illustrated Table 2-14. Purified IFN-γ was loaded onto a 50 μm x 45 cm neutral capillary with a 10 second pressure injection. Between each sample injection the capillary was washed for 3 minutes as illustrated in Table 2-15.

Table 2-14 Capillary preparation.

<table>
<thead>
<tr>
<th>Step</th>
<th>Concentration</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphoric acid</td>
<td>10 mM</td>
<td>15</td>
</tr>
<tr>
<td>Milli-Q water</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>cIEF gel</td>
<td></td>
<td>10</td>
</tr>
</tbody>
</table>

Note: degassed and filtered.
Table 2-15 Wash solutions for capillary isoelectrofocusing.

<table>
<thead>
<tr>
<th>Step</th>
<th>Concentration</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphoric acid</td>
<td>10 mM</td>
<td>1</td>
</tr>
<tr>
<td>Milli-Q water wash</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>cIEF gel</td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

Note: degassed and filtered.

The separation was run until all peaks were eluted (approximately 50 min) at a constant voltage of 13.5 kV and temperature of 20°C. The IFN-γ molecules were quantified by their absorbance at 280 nm (0.04 AU, negative offset: 20%). A pI calibration plot was constructed using markers of known pI and their recorded retention times. The retention times of IFN-γ absorbance peaks were used to determine pI and integrated to determine the proportions of each IFN-γ species present.

2.4.12 Trypsin digestion and peptide separation by reverse-phase HPLC

Acetic acid (25 μl of a 1% v/v solution) was mixed with 25 μg trypsin (Promega) which had been lyophilised from 50 mM Tris-HCl (pH 7.6). Trypsin (1 μg) was added to 50 μg of purified IFN-γ in 50 ml of 0.1 M ammonium bicarbonate containing 0.02% (w/v) NaN₃ (pH 7.8). Digestions were performed over 16 hours at 30°C, followed by a further addition of
Chapter 2.4 Analytical Methods

0.5 μg trypsin and subsequent incubation for 8 hours under the same conditions.

Rp-HPLC separation of trypsin-digested IFN-γ samples was performed using a Vydac 218TP52 column (C18, 2.1 x 250 mm, Western Analytical Products Inc., Temecula, U.S.A.), linked to a Waters 626 Millenium system (Millipore Ltd). Samples were applied in 0.06% (v/v) trifluoroacetic acid (TFA) and the peptides separated with a linear gradient (0-70%) of 80% (v/v) aqueous acetonitrile (CH3CN) with 0.052% TFA. The gradient was run for 100 minutes at a flow rate of 0.1 ml min⁻¹. Peptide peaks were detected at a wavelength of 210 nm and collected individually. The fractions were centrifuged under vacuum for 2 hours at room temperature, lyophilised overnight and stored at -20°C, until analysed by mass spectrometry.

2.4.13 Digestion by endoprotease Asp-N and separation of peptides by reverse-phase HPLC

Purified IFN-γ (50 μg) was incubated with 8 M guanidine hydrochloride for 2h at 50°C. The denatured samples were adjusted to 1 M guanidinium hydrochloride and mixed with 2.5 μg of Asp-N for 18 h at 37°C. Samples were applied in 0.06% (v/v) trifluoroacetic acid (TFA) to a Vydac 218TP52 reverse-phase column (Western Analytical Products Inc.) (C18, 2.1 x 250 mm) connected to a Waters 626 Millenium system (Millipore Ltd.).
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Peptides were separated with a linear gradient (0-70%) of 80% (v/v) aqueous CH$_3$CN with 0.052% TFA in 100 min at a flow rate of 0.1 ml min$^{-1}$. Individual peptide peaks were detected at 210 nm and collected manually. All peaks were collected and centrifuged at room temperature under vacuum to remove CH$_3$CN and lyophilised overnight, then stored at -20°C.

2.4.14 Preparation of released N-glycans

Prior to digestion with PNGaseF approximately 200-400 µg of IFN-γ protein was denatured by addition of 8 µl 10% (w/v) SDS and boiling for 2 min. Proteins were then digested at 30°C for 18 h in a final volume of 200 µl, containing 0.5% (v/v) Nonidet P-40 (BDH, Poole, U.K.), 0.1% (w/v) SDS, 20 units of PNGase F and 50 mM ammonium carbonate, pH 8.1. Released N-glycans were separated from deglycosylated IFN-γ polypeptide and detergents by rp-HPLC with a Waters 616 system (Millipore Ltd.). The entire digestion mixture was loaded onto a Vydac 218TP52 reverse-phase column (C18, 2.1 x 250 mm, Western Analytical Products Inc.) at a flow rate of 0.2 ml min$^{-1}$. N-glycans were collected in the first 8 ml of column flow-through in solvent A (0.05% TFA in H$_2$O) then concentrated and desalted by lyophilization. Deglycosylated IFN-γ protein was eluted with a linear gradient of 100% solvent A to 100% solvent B (0.05% TFA, 80% acetonitrile in H$_2$O) over 30 min, monitored at 210 nm with a Waters 486 detector (Millipore Ltd.). By this procedure, IFN-γ polypeptide co-eluted with Nonidet-P40 detergent after 25 min, followed by elution of excess SDS after 40 min.
2.4.14.1 Analysis of released, sialylated N-glycans by 2-aminobenzamide labelling and reversed-phase HPLC

Sialylated N-glycans released from recombinant IFN-γ proteins were reductively aminated at the reducing terminus with the fluorophore 2-aminobenzamide (2-AB; excitation 330 nm, emission 420 nm) using a kit supplied by Oxford Glycosystems Ltd. All procedures were carried out according to manufacturer’s instructions. 2-AB labelled N-glycans were separated into neutral, mono-, di-, tri-, or tetra-sialylated structures by HPLC with a Glycosep™ C cation-exchange column (4.6 x 100 mm; Oxford Glycosystems Ltd.). By this procedure, neutral oligosaccharides eluted in the void volume whilst charged N-glycans progressively eluted using a linear gradient of 100% solvent A (20% acetonitrile, 80% H₂O) to 100% solvent B (20% acetonitrile, 80% 250 mM ammonium formate, pH 4.5) at a flow rate of 0.3 ml min⁻¹ over 45 min. Oligosaccharide elution was monitored with a Waters 474 scanning fluorescent detector (Millipore Ltd.).

2.4.15 Glycosidase digests and oligosaccharide quantitation

Non-glycosylated T4 (Asn₂₅) and T19 (Asn₉₇) peptides with predicted mass values of 2252.5 Da and 1522.7 Da respectively and their glycosylated counterparts were identified by MALDI-MS. Glycosidase digests were
performed by the addition of 0.5 ml of sample to 0.5 ml of glycosidase (or a combination of glycosidases) and incubated at 30°C for 18-24 h. Aliquots (0.5 μl) were removed for MALDI-MS analysis. Glycopeptide spectra possessed (M+H)^ signals differing by the mass of one or more monosaccharide residues and their identity was confirmed by digestion of glycopeptides with PNGaseF to give a mass corresponding to the free peptide plus 1.0 Da (on conversion of Asn to Asp by this enzyme reaction). For each Asn glycosylation site, glycan structures were determined using linkage-specific exoglycosidase arrays for the sequential removal of oligosaccharides (Edge et al., 1992; Sutton et al., 1994). N-glycans at each glycosylation site were quantified by MALDI-MS after digestion with neuraminidase to remove terminal sialic acids. The integrated area of each identified (M+H)^ signal was calculated and expressed as the percentage of all identified peaks.

2.4.16 Matrix assisted laser desorption-ionisation mass spectrometry

All MALDI-MS spectra were obtained with a VG TofSpec (VG Organic, Manchester, U.K). Aqueous solutions of desialylated N-glycans released by PNGase F were analysed using a saturated solution of α-cyano-4-hydroxy cinnaminic acid in 60% (v/v) CH₃CN or 2,5-dihydroxybenzoate (2,5-DHB) as matrix by the method of Harvey (1993). Spectra were calibrated externally using an equimolar (25 mM) mixture of asialo, galactosylated triantennary ([M+Na]^ = 2029.8 Da) and β-mannosyl, α-fucosyl chitobiose
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([M+Na]^+ = 755.7 Da) oligosaccharides (Oxford Glycosystems Ltd.). Deglycosylated IFN-γ polypeptides were analysed using ‘super-DHB’ as matrix by the method of Tsarbopoulos et al. (1994). Polypeptides were adjusted to a concentration of ±20 pmol µl^-1 and mixed 1:1 with matrix solution on a stainless steel sample disc prior to analysis. In all cases, ions desorbed by pulses (1 Hz repetition rate) of light from a N₂ laser at 337 nm were accelerated either at 20,000 V potential (N-glycans) or 30,000 V potential (deglycosylated IFN-γ) in positive ion mode.

After a linear flight path of 0.65 m, ions were detected by a micro-channel plate detector (2000 V applied voltage) and digitised at 250 MHz. Observed signals were adjusted so as to obtain maximum peak resolution (optimum signal to noise ratio at minimum laser energy) and the spectra from twenty laser pulses were averaged. Spectra were calibrated externally using either horse heart myoglobin (16951.5 Da; Zaia et al., 1992) or peptide residues 1-12 of vasoactive intestinal peptide (1425.5 Da).
Chapter 3 Results and Discussion

3. RESULTS AND DISCUSSION

3.1 A Comparison of Polyacrylamide Gel and Capillary Electrophoretic Methods for Analysing IFN-γ

In order to determine the validity of capillary electrophoresis as a suitable alternative to conventional SDS-PAGE for monitoring the macroheterogeneity of IFN-γ, replicate samples were resolved by both techniques. Two batch cultures of CHO 320 cells in spinner flasks were sampled daily. Each culture displayed similar and typical growth characteristics as reported by Hayter et al. (1991) (Figure 1-1). The maximum viable cell densities which occurred after ~100 h were 0.45 and 0.5 cells ml⁻¹ for cultures A and B, respectively. The cell viability in both cultures dropped gradually from 90% to 60% until ~140 h when it dropped more quickly to zero after ~180 h.

Recombinant IFN-γ was purified from 15 ml of culture supernatant by immunoprecipitation. Purified samples (10 μl per well) were separated on non-reducing SDS-polyacrylamide gels and IFN-γ variants visualised by silver staining (a typical mini-gel is illustrated in Section 3.3.2.1). The non-glycosylated form (0N) elutes first, then the singly (1N) and finally the doubly glycosylated form (2N). The peak areas of each site-occupancy variants (or glycoform) were determined by a scanning densitometer and the
proportions of each site-occupancy variant determined. Replicate samples were resolved by MECE using uncoated capillaries. The IFN-γ variants were measured by their absorbance of ultraviolet light at 200 nm and the proportions of the variants determined. A typical electropherogram obtained by MECE is illustrated in Figure 1-2b. Three major glycosylated species (glycoforms) are evident. These species elute as the doubly (2N), singly (1N) and non-glycosylated (0N) site occupancy variants in that order. Some additional heterogeneity is also visible within each major glycoform by MECE which is likely to correspond to differences in sialic content.

![Cell growth and viability curves from two batch spinner cultures.](image)

**Figure 3-1** Cell growth and viability curves from two batch spinner cultures.
Chapter 3.1 A Comparison of Polyacrylamide Gel and Capillary Electrophoretic Methods for Analysing IFN-γ

Figure 3-2 Separation of immunopurified IFN-γ by (a) polyacrylamide gel capillary electrophoresis and (b) micellar electrokinetic capillary electrophoresis. 2N = doubly glycosylated, 1N = singly glycosylated and 0N = non-glycosylated.

A comparison of the proportions of each site-occupancy variant determined by the two analytical methods is illustrated in Figure 1-3. The correlation of 0N proportions between methods is the closest of the three variants; the 1N and 2N variants showing much less correspondence. Both the 0N and 2N forms fall above the equivalence line, the 1N form below the line. This result suggests that either SDS-PAGE overestimates the 0N and 2N form and underestimates the 1N form or MECE underestimates the 0N and 2N form and
Chapter 3.1 A Comparison of Polyacrylamide Gel and Capillary Electrophoretic Methods for Analysing IFN-γ

overestimates the 1N form. In addition, the 2N values demonstrate a much greater degree of scatter compared to the 1N and 0N variants.

Figure 3-3 Scatter plot of the proportions of IFN-γ site-occupancy variants determined by SDS-PAGE and capillary zone electrophoresis. 2N is glycosylated at Asn\textsubscript{25} and Asn\textsubscript{97}, 1N is glycosylated at Asn\textsubscript{25} and 0N is non-glycosylated.

The reasons for the discrepancy are unclear. It may be due to differences in the separation chemistry employed by the two electrophoretic techniques. MECE separates molecules according to the amount of their association with negatively charged hydrophobic SDS micelles, the least glycosylated species have the least affinity and are retained. SDS-PAGE separates proteins
Chapter 3.1 A Comparison of Polyacrylamide Gel and Capillary Electrophoretic Methods for Analysing IFN-γ

according to molecular size, the smaller species migrate further. Alternatively, it has been suggested that the silver staining technique may stain carbohydrates preferentially (Kobata, 1994). However, although the 2N variant is "overestimated" by SDS-PAGE, the 1N form is "underestimated". This would suggest that the silver ions bind disproportionally to molecules with a higher carbohydrate content.

The following work has been directed towards using SDS polyacrylamide gel-filled capillaries. Samples of purified IFN-γ from a batch culture were analysed both by SDS-PAGE and by polyacrylamide gel capillary electrophoresis (PAGCE). A typical separation by PAGCE is shown in Figure 3-2a. The additional heterogeneity observed by MECE was not apparent by PAGCE which repeatedly gave less resolution, reduced sensitivity and longer elution times than MECE. In addition, molecular weight determination by PAGCE gave values in excess of known glycoform molecular weights. This was assumed to be due to the influence of glycosylation. When the proportions of IFN-γ glycoforms were compared there was a far greater correlation between PAGCE and SDS-PAGE (R = 0.89 compared to R = 0.68) despite fewer sample points (Figure 1-4). This result could be because the separation chemistries in both these methods are identical. Therefore the poorer correlation between SDS-PAGE and MECE may be due to the principle of separation. However, this does not rule out the possibility of an unequal staining phenomenon produced by the silver stain procedure.
Chapter 3.1 A Comparison of Polyacrylamide Gel and Capillary Electrophoretic Methods for Analysing IFN-γ

Regression line
\[ y = 1.05x + 0.76 \]
\[ r = 0.89 \]

Equivalence line
\[ y = x \]

Figure 3-4 Scatter plot of IFN-γ site-occupancy variants determined by SDS-PAGE and gel-filled capillary zone electrophoresis.

Table 1-1 summarises the range of typical glycoform proportions determined by the three separation methods. It is clear that the two SDS-PAGE techniques show the most similar ranges for batch culture samples. In Figure 3-4 it was noticeable that all the 1N and 2 of the 2N species fall above the equivalence line. Since SDS-PAGE has identical separation chemistry to PAGCE the discrepancy might be due to the silver staining technique mentioned earlier. If the binding of silver ions encouraged further binding of silver ions this might explain the higher relative proportion of 2N variants compared to 1N variants in Figure 3-3. However, there is no evidence of this phenomenon in the second comparison.
MECE produced the narrowest ranges of values. This suggests that this technique is either the most accurate, assuming that IFN-γ has a consistent distribution of glycoform proportions during a batch culture. Alternatively, if the resolving power of MECE is poor then variations in the population would be missed. However, the high resolving power of capillary electrophoresis is consistently demonstrated in the literature and later Sections in this thesis will demonstrate this characteristic further. It is for this reason, as well as its ability to produce quantitative, accurate and fast separations without further derivitisation of the IFN-γ molecule, that it was used for all future determinations of IFN-γ macroheterogeneity.

**Table 3-1** Comparison of the range of proportions of IFN-γ site-occupancy variants when determined by three different electrophoretic separation methods. Values are expressed as percentages.

<table>
<thead>
<tr>
<th>Glycoform</th>
<th>MECE</th>
<th>SDS-PAGE</th>
<th>PAGCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0N</td>
<td>5-15</td>
<td>10-20</td>
<td>10-25</td>
</tr>
<tr>
<td>1N</td>
<td>35-45</td>
<td>10-30</td>
<td>20-30</td>
</tr>
<tr>
<td>2N</td>
<td>40-55</td>
<td>50-75</td>
<td>50-55</td>
</tr>
</tbody>
</table>
3.2 Set-up of a Suspension Bioreactor

3.2.1 $K_{La}$ determination

The purchase of a new bioreactor instigated studies on the mass transfer coefficient ($k_{La}$) of oxygen through it before its use for animal cell culture. The particular importance of determining this characteristic is explained as follows. Since the CHO 320 cell line is a low producing cell line and the product amounts required for detailed analysis were large it was necessary to obtain a sizable laboratory fermentation system. Even with a 15 L system it was calculated that half the working volume would be removed during the course of the culture. Therefore a 2:1 aspect ratio system (a 1.5:1 is more typical for mammalian cell fermentation) was purchased so as to maintain a working volume higher than the impeller. This would avoid culture being dispersed to violently. However, despite these considerations it was decided that the large reduction in working volume might induce physical stresses on the cells if a constant impeller speed was maintained. A suitable speed of agitation at large volumes to maintain appropriate mixing and suspension might produce a vortex or foam formation at lower volumes. Therefore, to achieve a ‘stepping down’ of the impeller speed in a calculated manner it was necessary to characterise the bioreactor system in detail to determine a suitable method of calculating the correct agitation speed for every possible volume.
Chapter 3.2 Set-up of a Suspension Bioreactor

The \( k_{La} \) of oxygen through medium was determined by the static gassing-out method (Section 1.3.4 and 2.2.1). A typical trace of the increase in \( dO_2 \) is shown in Figure 3-5a. The temperature of the medium was maintained at 37°C, both the liquid volume, sparger and the agitation rate were varied. The liquid volumes tested were those which would be present at the periods of highest cell densities and therefore highest oxygen utilisation. Ideally, \( k_{La} \) determinations should be performed with the cell population included. CHO cells were not included in the culture medium because their influence on \( k_{La} \) was assumed to be negligible, however, all medium additives (such as BSA, insulin, transferrin) were included. The agitation rates were chosen based on manufacturer’s suggestions. The mass transfer coefficient was calculated from the equation:

\[
\frac{dO_2}{t} = \frac{-\ln(C^*-C_L)}{t}
\]

where

\( t \) is the time in minutes;

\( C = 100\% \) of air saturation.

The probe response time, less than 1 s according to the manufacturer’s data sheet, was assumed to be negligible when compared to the experimental time of between 20 and 40 min. The units of \( k_{La} \) are usually expressed as \( h^{-1} \) or \( s^{-1} \) but are quoted in units of \( \text{mmol O}_2 \cdot \text{l}^{-1} \cdot \text{atm}^{-1} \cdot \text{min}^{-1} \) for convenience during subsequent calculations. A typical determination is illustrated in Figure 3-5b.
3.2.1.1 Influence of sparger

The bioreactor was delivered with a L-shaped tubular sparger with 5 holes of about 2 mm in diameter through which gas escaped beneath the impeller. The influence of a 1 μm sintered sparger replacement, which generated smaller bubbles was investigated. When the non-sintered sparger was replaced with a 1 μm glass sintered sparger the value of $k_La$ was increased at all agitation speeds tested (Table 3-2). Since the 'a' term in the $k_La$
expression refers to the available area for mass transfer the presence of smaller bubbles with a larger surface to volume is likely to improve the mass transfer coefficient significantly.

Table 3-2 Comparison of $k_{La}$ with different sparger systems at different agitation rates with 10 l of medium.

<table>
<thead>
<tr>
<th>Agitation rate(rpm)</th>
<th>Mass transfer coefficient, $k_{La}$ (mmol O$_2$·1$^{-1}$ atm$^{-1}$ min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-sintered sparger</td>
</tr>
<tr>
<td>80</td>
<td>0.0157</td>
</tr>
<tr>
<td>90</td>
<td>0.0161</td>
</tr>
<tr>
<td>100</td>
<td>0.0203</td>
</tr>
</tbody>
</table>

The significant improvement in mass transfer with the sintered sparger led to its permanent use in all subsequent experiments.

3.2.1.2 Influence of agitation rate and volume on the mass transfer coefficient

Experiments were performed with the sintered sparger at three different agitation rates and two culture medium volumes. The $k_{La}$ for each set-up was determined and its relationship to these two variables investigated (Figure 3-6).
Figure 3-6 Dependence of the mass transfer coefficient ($k_{L,a}$) of oxygen ($O_2$) through the medium of a 15 l bioreactor upon agitation speed and volume.

An increase in agitation rate or decrease in culture volume corresponded to an increase in $k_{L,a}$ due to improved mixing. When the agitation speed is compared to the values of $k_{L,a}$ at the two bioreactor volumes a linear relationship was found ($R = 0.98$ at both volumes). Fleischaker and Sinskey (1981) found a similar relationship when determining the $k_{L,a}$ across the air-liquid interface of a bioreactor. From the available data it was possible to determine the influence of each parameter on $k_{L,a}$ (Table 3-3). Only values within each linear plot were used and an equal decrease in volume (10 l to 8 l, a 25% decrease) was compared with equal increases in agitation rate (mean of 100 rpm to 125 rpm and 125 rpm to 150 rpm, both 25% increases). From this correlation it was clear that the influence of agitation rate on oxygen transfer
was far greater than that of volume. More volumes should be tested before any practical applications can be suggested for this data.

**Table 3.3** Influence of volume and agitation rate on $k_{La}$.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Increase or decrease</th>
<th>Mean increase in $k_{La}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (l)</td>
<td>25% decrease</td>
<td>4.3</td>
</tr>
<tr>
<td>Agitation rate (rpm)</td>
<td>25% increase</td>
<td>30.6</td>
</tr>
</tbody>
</table>
3.2.2 Agitation rate determination

From calculations of the mass transfer coefficient of our system suitable agitation rates (N) at different working volumes was determined. The oxygen uptake rate of the CHO 320 cell line is approximately 0.3-0.4 \( \mu \text{mol} \ 10^6 \text{cells}^{-1} \text{hr}^{-1} \) (personal communication, P Hayter). Therefore the cellular oxygen rate at a theoretical maximum cell density of \( 1.0 \times 10^6 \text{cells} \text{ml}^{-1} \) will be:

\[
q_{O_2} x = 0.4 \mu \text{mol} \ 10^6 \text{cells}^{-1} \text{hr}^{-1} \times 1.0 \times 10^6 \text{cells} \text{ml}^{-1}
\]

\[\Rightarrow q_{O_2} x = 0.4 \mu \text{mol} \ \text{ml}^{-1} \ \text{hr}^{-1}\]

\[\Rightarrow q_{O_2} x = 0.007 \mu \text{mol} \ \text{ml}^{-1} \ \text{min}^{-1}\]

\[\Rightarrow q_{O_2} x = 0.007 \text{mmol l}^{-1} \ \text{min}^{-1}\]

At steady state conditions, the rate of transfer of oxygen from the gas phase equals the rate of cellular consumption:

\[k_{L,a} (C^* - C_L) = q_{O_2} x\]
The maximum solubility of oxygen in cell culture medium under similar conditions was determined by Fleischaker and Sinskey (1981) to be 0.130 mmol l\(^{-1}\), equivalent to \(C^*\). The oxygen tension of this system was maintained at 40% of saturation which is equivalent to 0.052 mmol l\(^{-1}\), equivalent to \(C_L\). Therefore the value of \(k_La\) required to meet the theoretical maximum oxygen demand is stated as:

\[
k_La = \frac{q_{O_2}X}{(C - C_L)}
\]

\[
\Rightarrow k_La = \frac{0.007}{(0.130 - 0.052)}
\]

\[
\Rightarrow k_La = \frac{0.007}{0.078}
\]

\[
\Rightarrow k_La = 0.090 \text{ mmol O}_2 \text{ atm}^{-1} \text{ min}^{-1} \text{ l}^{-1}
\]
Linear regression analysis of the plots illustrated in Figure 3-6 gave a means to calculate suitable impeller speeds which would theoretically deliver sufficient oxygen delivery at the highest cell densities. For an 8 litre volume:

\[
N = \frac{k_\text{i} \cdot a + 0.01739}{0.00088}
\]

\[\Rightarrow \quad N = \frac{0.090 + 0.01739}{0.00088}\]

\[\Rightarrow \quad N = 122.0 \text{ rpm}\]

For a 10 litre volume:

\[\Rightarrow \quad N = \frac{0.090 + 0.01395}{0.00082}\]

\[\Rightarrow \quad N = 126.7 \text{ rpm}\]
Chapter 3.2 Set-up of a Suspension Bioreactor

The large sampling regime means a large decrease in the working volume over the course of a batch run. To maintain a constant energy input and therefore theoretically keep shear stresses similar, the power to volume to ratio must be kept constant. The power input to a system can be calculated from the following equation (Bailey and Ollis, 1986):

\[ P = P_0 \rho N^3 D^5 \]  \hspace{1cm} (5)

where \( P = \text{Power (W)} \)

\( P_0 = \text{Power number} \)

\( \rho = \text{fluid density (kg m}^{-3}\text{)} \)

\( N = \text{impeller speed (min}^{-1}\text{)} \)

\( D = \text{impeller diameter (m)} \)
Chapter 3.2 Set-up of a Suspension Bioreactor

For the single pitched blade impeller an approximate value for $P_0$ is 5.5 (Inceltech, personal communication). The fluid density of cell culture medium was assumed to be equivalent to water which is 1000 kg m$^{-3}$. The impeller diameter was 8 cm (= 0.08 m). At an agitation speed of 127 min$^{-1}$ (= 2.12 s$^{-1}$) at a 10 l volume the power input is:

$$P = 5.5 \times 1000 \times (2.12)^3 \times (0.08)^5$$

$$\Rightarrow P = 0.172 \text{ W}$$

$$\Rightarrow \frac{P}{V} = 0.017 \text{ W l}^{-1}$$

For 8 l:

$$P = 5.5 \times 1000 \times (2.03)^3 \times (0.08)^5$$

$$\Rightarrow P = 0.151 \text{ W}$$

$$\Rightarrow \frac{P}{V} = 0.019 \text{ W l}^{-1}$$

To overrate the system the larger of the two values was used. Therefore to calculate the required agitation rate at a given volume equation (5) was rearranged as below:
Chapter 3.2 Set-up of a Suspension Bioreactor

\[ N = \sqrt[3]{\frac{P}{P_pD^5}} \]

\[ \Rightarrow N = 3 \left( \frac{0.019WL^{-1} \times \text{working volume}}{0.018} \right) \text{ rps} \]

This final equation was used to determine the correct impeller speed at different volumes for all 15 l batch suspension cultures. Following Sections will demonstrate the successful bioreactor cultivation of the CHO 320 cell line using this derived formula.
3.3 Time Course Study of IFN-γ Glycosylation

3.3.1 Batch suspension culture

Two batch fermentations were performed under identical operating conditions. The aim was to use matrix-assisted laser desorption mass spectrometry (MALDI-MS) to monitor site-specific glycosylation changes during batch cultivation of recombinant CHO 320 cells producing IFN-γ. The CHO cells in Culture 1 showed typical growth characteristics and reached a maximum viable cell density of $0.5 \times 10^6$ cells ml$^{-1}$ after 72 h (Figure 3-7a). Culture 2 exhibited an extended lag phase before reaching a maximum cell density after 120 h of $0.8 \times 10^6$ cells ml$^{-1}$ (Figure 3-7e). Cell viability dropped sharply in both cultures after peak cell densities had been reached and was particularly noticeable in Culture 1 (Figure 3-7b and f). Recombinant IFN-γ accumulated during the growth phase to concentrations of $10-13 \times 10^3$ IU ml$^{-1}$ (Figure 3-7f). This corresponds to $\sim 0.5 \mu$g ml$^{-1}$ based on the specific activity of IFN-γ: $2.2 \times 10^7$ IU mg$^{-1}$ (NIBSC). Both cultures exhibited similar specific growth rates ($\mu$) which occurred during the exponential phase of growth (Table 3-4). Specific IFN-γ production rates ($q_{IFN}$) also were associated with this period of growth and each culture had a $q_{IFN}$ of approximately 270 IU (10$^6$ cells)$^{-1}$h$^{-1}$. The extended lag phase of Culture 2 appeared to have no effect on the rate of IFN-γ production.
Chapter 3.3 Time Course Study of IFN-γ Glycosylation

Figure 3-7 Viable cell counts and percentage viability (a, e), IFN-γ concentrations (b, f), metabolite concentrations (c, g), and percentage of cells in each phase of the cell cycle (d, h) during two batch fermentations of recombinant CHO 320 cells.
Chapter 3.3 Time Course Study of IFN-γ Glycosylation

Glucose was utilised rapidly and depleted by 110 h in Culture 1 and by 144 h in Culture 2 (Figure 3-7c and g). However, both cultures showed similar specific glucose rates ($q_{\text{Glucose}}$) which suggested that the extended lag phase does not inhibit glucose uptake (Table 3-4). Lactate and ammonia both accumulated during the cultures (Figure 3-7c and g). Lactate showed a rapid initial increase reaching final concentrations of 16 and 7 mM in Cultures 1 and 2 respectively. Accordingly, the specific lactate production rate ($q_{\text{Lactate}}$) of Culture 1 was approximately double that of Culture 2. Ammonia levels increased slowly during both cultures and reached 5 and 1 mM in Cultures 1 and 2 respectively (Figure 3-7c and g). However, Culture 1 exhibited a 12-fold higher rate of specific ammonia production ($q_{\text{Ammonia}}$) compared to Culture 2 (Table 3-4). These values for final levels of lactate and ammonia are below those found to be inhibitory for this cell line (Hayter, 1991). Thus the utilisation of glucose is related to the growth phase whereas lactate and ammonia production are more related to the complete profile of growth.

Cell cycle distributions of the cells are shown in Figure 3-7h for Cultures 1 and 2 respectively. Results are not provided for the whole culture lifetime due to the build up of cell debris which prevents accurate determinations of the state of the cell cycle. Culture 1 displayed a higher proportion of $G_1$ and lower proportion of $S$ phase cells than Culture 2. Neither culture showed much change initially in the percentage of cells in the $G_1$ phase which is probably due to cells responding to an arrest of cells in $G_1$ following inoculation. When the late-exponential growth period had been reached an increase in the
percentage of cells in the G\textsubscript{1} phase occurred. G\textsubscript{1} phase cells formed about 64% of the total cells after 140 h of growth in both experiments. The proportion of cells in S phase exhibited a decrease which occurred conversely to the increase in the proportion of cells in G\textsubscript{1}. Both cultures exhibited little change in the quantities of cells in G\textsubscript{2}/M phase throughout the culture. Proportions of cells in the G\textsubscript{2}/M phase never rose above 15%. The longer lag phase in Culture 2 appeared to have prolonged the arrest of cells in G\textsubscript{1}. Subsequent batch fermentations in this system demonstrated growth and IFN-\gamma production characteristics typical of Culture 1.

### Table 3-4 Growth, productivity and metabolic quotients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Culture 1</th>
<th>Culture 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\mu) (h(^{-1}))</td>
<td>0.016</td>
<td>0.018</td>
</tr>
<tr>
<td>(q_{\text{IFN}}) (IU (10(^6) cells(^{-1}) h(^{-1}))</td>
<td>273</td>
<td>269</td>
</tr>
<tr>
<td>(q_{\text{Glucose}}) (mM (10(^6) cells(^{-1}) h(^{-1}))</td>
<td>0.311</td>
<td>0.300</td>
</tr>
<tr>
<td>(q_{\text{Lactate}}) (mM (10(^6) cells(^{-1}) h(^{-1}))</td>
<td>0.435</td>
<td>0.192</td>
</tr>
<tr>
<td>(q_{\text{Ammonia}}) (mM (10(^6) cells(^{-1}) h(^{-1}))</td>
<td>0.087</td>
<td>0.007</td>
</tr>
</tbody>
</table>

#### 3.3.2 Analysis of amino acid metabolism during Culture 1

The utilisation and production of amino acids was monitored during fermentation Culture 1. The amino acids were derivitised by o-phthalaldehyde, separated by rp-HPLC and quantified with a fluorescence detector (Seaver et al., 1984). The separation of calibrating amino acid
standards (AA-S-18, Sigma) and amino acids from cell culture medium on the second system (Millipore) are illustrated in Figure 3-8 and Figure 3-9. Identical gradient profiles, elution rates and an identical column were employed for the separations. However, the resolution was not as good as that achieved with the original system which was able to separate glycine from threonine.

Figure 3-8 HPLC separation of derivitised amino acids from standard solution AA-S-18.
Chapter 3.3 Time Course Study of IFN-γ Glycosylation

Figure 3-9 HPLC separation of derivitised amino acids from complete culture medium.

The integration of each amino acid peak from Figure 3-9 and the known quantities present in RPMI 1640 allowed the creation of a calibration curve (Figure 3-10). Peak area emission data from Culture 1 were then converted to concentration values (Table 3-5). During the initial period all the amino acids except glutamate display an initial utilisation by the CHO cells. Many of the amino acids also returned to approximately their original concentration by the end of the culture: isoleucine, leucine, lysine, methionine, phenylalanine threonine, tyrosine, valine and to some extent aspartate. This increase generally occurred during the period of increased cell non-viability which is likely to result in leakage of small molecules such as amino acids.
Glutamine is perhaps the most critical amino acid in the metabolism of transformed cell lines because of its utilisation as an energy source (Zielke et al., 1984). The change in the glutamine concentration during Culture 1 is illustrated in Figure 3-11. The profile of glutamine utilisation was similar in profile to the decrease in glucose - complete disappearance occurred at the point of maximum cell density (Figure 3-7c and g). Glutamine can be metabolised by either of two routes: mitochondrial deamination by glutaminase to produce glutamate and ammonia or by cytosolic amidotransferases to form glutamate and nitrogenous biosynthetic intermediates (Figure 3-12). Glutamate not converted to α-ketoglutarate (ammonia being the mitochondrial by-product) might be released into the culture medium. Both the increase in ammonia and glutamate concentrations are also illustrated in Figure 3-11. These two profiles show close agreement and would suggest that glutamine degradation is predominately by the glutaminase pathway for energy production.
Chapter 3.3 Time Course Study of IFN-γ Glycosylation

Figure 3-10 Calibration curve of OPA-derivitised amino acids from RPMI-1640 and their quantitation by a fluorescence detector.
### Table 3-5 Changes in the extracellular levels of amino acids during batch fermentation Culture 1.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Asp (mg l⁻¹)</th>
<th>Glu (mg l⁻¹)</th>
<th>Asn (mg l⁻¹)</th>
<th>Ser (mg l⁻¹)</th>
<th>Gln (mg l⁻¹)</th>
<th>His (mg l⁻¹)</th>
<th>Gly (mg l⁻¹)</th>
<th>Thr (mg l⁻¹)</th>
<th>Arg (mg l⁻¹)</th>
<th>Tyr (mg l⁻¹)</th>
<th>Met (mg l⁻¹)</th>
<th>Phe (mg l⁻¹)</th>
<th>Val (mg l⁻¹)</th>
<th>Iso (mg l⁻¹)</th>
<th>Leu (mg l⁻¹)</th>
<th>Lys (mg l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>14.9</td>
<td>16.4</td>
<td>36.8</td>
<td>27.0</td>
<td>166.2</td>
<td>3.7</td>
<td>8.2</td>
<td>6.4</td>
<td>78.9</td>
<td>15.8</td>
<td>8.1</td>
<td>15.2</td>
<td>10.9</td>
<td>42.4</td>
<td>38.3</td>
<td>18.0</td>
</tr>
<tr>
<td>21</td>
<td>8.8</td>
<td>21.0</td>
<td>21.2</td>
<td>22.2</td>
<td>72.4</td>
<td>3.1</td>
<td>4.7</td>
<td>2.6</td>
<td>50.6</td>
<td>8.7</td>
<td>4.9</td>
<td>9.1</td>
<td>7.6</td>
<td>24.1</td>
<td>22.5</td>
<td>9.9</td>
</tr>
<tr>
<td>51</td>
<td>9.4</td>
<td>35.4</td>
<td>19.6</td>
<td>28.1</td>
<td>42.4</td>
<td>2.8</td>
<td>3.9</td>
<td>5.3</td>
<td>67.9</td>
<td>8.5</td>
<td>5.6</td>
<td>8.1</td>
<td>6.3</td>
<td>26.9</td>
<td>24.4</td>
<td>10.8</td>
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<td>77</td>
<td>11.2</td>
<td>45.9</td>
<td>10.6</td>
<td>35.5</td>
<td>9.9</td>
<td>5.6</td>
<td>3.4</td>
<td>7.0</td>
<td>75.8</td>
<td>9.7</td>
<td>5.7</td>
<td>8.7</td>
<td>8.4</td>
<td>27.1</td>
<td>24.7</td>
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<tr>
<td>102</td>
<td>12.1</td>
<td>48.0</td>
<td>8.8</td>
<td>42.4</td>
<td>2.4</td>
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<td>83.0</td>
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<td>9.6</td>
<td>9.4</td>
<td>26.5</td>
<td>24.7</td>
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<td>127</td>
<td>8.3</td>
<td>50.3</td>
<td>4.3</td>
<td>44.9</td>
<td>1.9</td>
<td>6.8</td>
<td>2.3</td>
<td>6.0</td>
<td>84.7</td>
<td>10.6</td>
<td>5.4</td>
<td>8.6</td>
<td>8.7</td>
<td>26.3</td>
<td>24.8</td>
<td>13.9</td>
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<tr>
<td>147</td>
<td>9.2</td>
<td>61.3</td>
<td>5.9</td>
<td>52.9</td>
<td>1.8</td>
<td>8.2</td>
<td>3.2</td>
<td>8.4</td>
<td>38.9</td>
<td>12.2</td>
<td>6.8</td>
<td>9.9</td>
<td>10.7</td>
<td>32.3</td>
<td>29.4</td>
<td>16.3</td>
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<td>173</td>
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<td>9.2</td>
<td>3.0</td>
<td>7.8</td>
<td>39.2</td>
<td>14.0</td>
<td>7.0</td>
<td>11.5</td>
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<td>36.0</td>
<td>33.8</td>
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<td>87.8</td>
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<td>77.0</td>
<td>0.0</td>
<td>12.4</td>
<td>3.0</td>
<td>11.6</td>
<td>39.7</td>
<td>18.0</td>
<td>9.0</td>
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<td>16.5</td>
<td>47.2</td>
<td>44.5</td>
<td>25.2</td>
</tr>
</tbody>
</table>
Figure 3-11 Glutamine utilisation, glutamate and ammonia production during batch fermentation Culture 1.
Figure 3-12 Outline of metabolic pathways involved in energy and amino acid metabolism in transformed cells. 1: glutaminase, 2: mitochondrial glutamate pyruvate transaminase and glutamate oxaloacetate transaminase, 3: cytosolic glutamate pyruvate transaminase, 4: cytosolic amidotransferses, 5: the double arrows indicate two alternative routes for serine and glycine biosynthesis. Redrawn from Ljunggren and Häggström (1992).
Chapter 3.3 Time Course Study of IFN-γ Glycosylation

The highest rate of glutamine utilisation was in the first 24 h despite the higher growth rates occurring during the 24-72 h period of the growth curve Figure 3-7d). Since this rate is higher compared to glutamate and ammonia production it might suggest that there may be either an intracellular increase of these compounds or an initially high level of glutamine conversion to biosynthetic precursors. It has been suggested that glutamate can accumulate in the mitochondria (La Noue and Schoolwerth, 1979). Indeed, the diffusion of ammonium ions (NH₄⁺), generated from glutamine and glutamate deamination, out of the cell is very slow; although if converted to ammonia the diffusion rate is much higher (Martinelle and Häggström, 1993). However, Ljunggren and Häggström (1992) have suggested that the glutaminase reaction will be quickest when the glutamine concentration is high because of the poor affinity of a glutaminase isoenzyme. It is also noteworthy that there is a slight increase in the rate of glutamate and ammonia production following the complete depletion of glutamine. One possibility is that an absence of glutamine may prevent the synthesis of nonessential amino acids in preference for energy metabolism and the resultant increase in NH₄⁺. However, this would not explain the increased rate of release of glutamate.

Another fate of glutamate is as an amino group donor in serine biosynthesis. During complete glycolysis 3-P-glyceraldehyde is converted to pyruvate and then usually to lactate which is released. The lactate level is often monitored with respect to glucose concentration in the culture to determine the extent of complete glycolysis which would theoretically give a lactate-to-glucose ratio.
of 2. However, pyruvate may enter the tricarboxylic cycle in mitochondria. Transamination of 3-P-glyceraldehyde to serine will decrease this ratio and may result in an increase in extracellular serine. Indeed, in Figure 3-13 an increase in serine is observed. After an initial lag period the increase is linear throughout the time course. The large increase does suggest some lack of biosynthetic control of this nonessential amino acid. Serine can be converted to glycine but no increase in glycine was observed. Serine and glycine both generate NADH while glycine can be used in the formation of NADH and $\text{NH}_4^+$. NADH may add to the reduction of pyruvate to lactate in the cytosol.

![Figure 3-13 Changes in the concentration of serine and glycine during batch fermentation Culture 1.](image-url)
Glucose and glutamine are used competitively in the production of aspartate. During the fermentation culture there was an initial decrease in the level of aspartate to ~ 75% of its original level and fluctuated during the rest of the culture (Figure 3-14). This steady level of aspartate suggest little utilisation of this amino acid which is observed for many cell lines (Thomas, 1986). In contrast, the level of asparagine, a non-essential amino acid, fell continuously during the period of growth of the culture (Figure 3-14). This is unusual since most non-essential amino acids are overproduced during the culture of transformed cell lines. It is likely that asparagine may be essential for the CHO 320 cell line. Aspartate can be converted to asparagine in mitochondria by a transamination reaction with glutamine as the amino group donor. It is unlikely that glutamine would be used for this reaction with an excess of asparagine present in the culture medium.
The utilisation and production of the three branched amino acids isoleucine, leucine and valine are illustrated in Figure 3-15. There was a rapid utilisation of isoleucine and leucine during the first 24 h although by 48 h all three amino acids were at concentrations ~ 40% of their original value. The three amino acids share common enzymes in initial degradation steps before their final conversion to acetyl-CoA (isoleucine and leucine) and succinyl-CoA (isoleucine and valine). During the rest of the growth period there was no further increase in utilisation of any of the three amino acids. Indeed, after ~ 144 h there was an increase in the quantity of each amino acid back to and

Figure 3-14 Changes in the concentration of asparagine and aspartate during batch fermentation Culture 1.
exceeding their original starting concentrations. This may have been related to the decrease in cell viability and increase in cell lysis.

![Graph showing changes in concentration of isoleucine, leucine, and valine](image)

**Figure 3-15** Changes in the concentration of isoleucine, leucine, and valine during batch fermentation Culture 1.

### 3.3.3 IFN-γ analysis

#### 3.3.3.1 IFN-γ purification and N-glycosylation site occupancy

Recombinant IFN-γ was immunopurified from daily samples and separated by SDS-PAGE and micellar electrokinetic capillary electrophoresis.
Chapter 3.3 Time Course Study of IFN-γ Glycosylation

(MECE). The three site-occupancy variants: doubly glycosylated, 2N, (occupied at the Asn_{25} and Asn_{97} sites), singly glycosylated, 1N, (occupied at the Asn_{25} site only) and non-glycosylated species, 0N, or glycoforms, demonstrated by Curling et. al (1990) are visible in Figure 3-16. There is the characteristic heterogeneity visible within each glycoform and an increase in the degree of staining with time of culture (which is due to the increased concentration of IFN-γ). The proportions of each glycoform were determined by light densitometry and the peaks integrated. The changes in the proportions of each glycoform through culture are illustrated in Figure 3-17.

Figure 3-16 SDS-PAGE separation of IFN-γ from batch fermentation Culture 1, silver stained to identify the site-occupancy variants: 2N, 1N and 0N. Lanes 1-8 are the daily time course samples in decreasing time points, lane 9 is blank, the markers (lane M) are 14.4, 21.5, 31.0, 45.0, 66.2 and 97.4 kDa.
Figure 3-17 Changes in the relative proportions of IFN-γ glycoforms during Culture 1 determined by SDS-PAGE and densitometry.

During the culture the initial proportions of the three site-occupancy variants are ~ 55% 2N, 35% 1N and 10% 0N. The 2N form was seen to decrease during culture with a concomitant increase in the 2N form. The change in the relative proportions of these two glycoforms over the time course was ~ 10%. In contrast the 0N remained relatively constant, not varying by more than 5% over the course of the fermentation culture. Curling et. al. (1990) demonstrated a similar decline in the proportion of 2N but an increase in the 0N form rather than the 1N form. One difference between the cultures was that the presented data was collected from fermentation cultures under conditions of constant pH and dO₂. The work of Curling et. al. (1990) was completed in spinner vessels with no regulatory control of these two
parameters. Environmental control during fermentation culture may have influenced the glycosylation pattern in a positive manner. Indeed, Castro *et al.* (1995) demonstrated a positive influence of lipids on the glycosylation of IFN-γ expressed by this cell line.

MECE of purified IFN-γ obtained from daily sampling of Culture 1 also revealed three glycoforms. Three typical electropherograms are illustrated in Figure 3-18. The doubly glycosylated variants elute first (10-12 min), shortly followed by the singly glycosylated (12-13 min) and finally the non-glycosylated species (15-16 min). There is clearly additional heterogeneity evident within each site-occupancy variable (glycoform). These glycoforms have been characterised in more detail by higher resolution separations (James *et. al.*, 1994). This heterogeneity was suggested as being most likely due to the variable sialylation of the molecule. MECE chromatograms showed both consistency in elution times and similarities in the pattern of individual glycoform groups (Figure 3-18).
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Figure 3-18 The separation of recombinant IFN-γ by MECE of three Culture 1 samples. The glycoforms were separated using 200 mM SDS/borate with a 50 μm by 50 mm uncoated capillary.

Integration of the peaks and the summation to 100% allowed the quantification of changes in the proportions of the glycoform variants during culture (Figure 3-19). Recombinant IFN-γ is more than 90% glycosylated with progressively decreasing proportions of the 2N glycoform. During the culture the proportion of 2N glycoforms decreased gradually with a concurrent rise in the proportion of the 1N glycoform, both by about 10%. The proportion of 0N glycoforms remained constant during the culture lifetime. Curling et. al. (1990) also reported a decrease in the 2N glycoform by using SDS-PAGE although the decrease was greater.
Chapter 3.3 Time Course Study of IFN-γ Glycosylation

The reduction in glycosylation may have occurred extracellularly. It is possible that non-viable cells release intracellular proteases and glycosidases due to the loss of cell membrane integrity. Gramer and Goochee (1993) reported the presence of at least four glycosidases in cell culture medium. However, no significant protease activity was found to be present in this cell culture medium (PM Hayter, personal communication).

Figure 3-19 Changes in the relative proportions of IFN-γ glycoforms during Culture 1 determined by MECE.

The ‘deglycosylation’ of IFN-γ may have occurred at an intracellular level. The MECE data suggests that the Asn97 site is not being glycosylated at the expense of an increased glycosylation at the Asn25 site. This resulted in an equal increase in the amount of 1N species and decrease in the 2N glycoform.
There was only a negligible change in the 0N species. The impaired ability to add a second glycan moiety may be due to the influence of available glycosylation precursors and/or the health of the cell. Both these factors are dependent on the nutrient availability in the culture medium. Glucose is a known glycosylation precursor as well as a significant energy provider. Hayter et al. (1992) demonstrated that an increase in glucose concentration during a glucose-limited chemostat culture has a positive influence on the proportion of the 2N glycoform. The depletion of glucose in d is both non-linear and reaches zero after approximately 100 h. The decrease in the 2N glycoform is gradual and nearly linear.

3.3.3.2 Trypsin digestion and rp-HPLC separation of tryptic peptides

Purification of IFN-γ samples from both Cultures 1 and 2 by cation exchange and immunoaffinity chromatography yielded 0.4 to 0.6 mg of purified IFN-γ per litre of supernatant. The IFN-γ was 98% pure, as determined by silver-stained SDS-PAGE gels (data not shown). Trypsin cleavage of purified IFN-γ created at least 20 peptides (and glycopeptides) including individual peptides containing the two intact N-glycosylation sites. Peptide mapping by MALDI-MS, combined with N-terminal sequencing, was used in the laboratory to confirm the identity of these peptides (data not shown). Rp-HPLC separation of IFN-γ tryptic fragments allowed the isolation of the glycosylated peptides (glycopeptides) T4 and T19 (Figure 3-20). The T19 (Asn97) peptide possesses a variably occupied glycosylation site, since
both glycosylated and non-glycosylated T19 (Asn97) peptides were identified at significant levels. In contrast, the T4 (Asn25) peptide was almost completely occupied with little non-glycosylated peptide being detected.

![Retention time vs. absorbance graph](Image)

**Figure 3-20** Reverse-phase HPLC separation of tryptic glycopeptides from recombinant IFN-γ. Glycosylated peptides T4 and T19 are indicated as T4(G) and T19(G) and the corresponding non-glycosylated peptides as T4 and T19 respectively.

### 3.3.3 Site-specific oligosaccharide changes

Mass spectra of tryptic glycopeptides from recombinant IFN-γ, derived from early (24-72 h) and late culture (116-164 h) for all batch cultures revealed a heterogeneous array of N-linked oligosaccharides the proportions of
which changed during culture (Figure 3-21). Although the glycosylated T4 peptide was less effectively ionised than its T19 counterpart, this did not preclude the accurate mass assignment of individual oligosaccharides and their quantification based on relative signal intensities in desialylated spectra (Table 3-6 and Table 3-7). N-glycan mapping at each glycosylation site was deduced by comparing mass values of desialylated spectra before and after sequential exoglycosidase arrays (Edge et al., 1992; Sutton et al., 1994) and has been successfully applied to the analysis of IFN-γ from different host cell lines (James et al., 1995). The linkage and anomeric configuration of the determined N-glycan structures were consistent with the recognised glycosylation pathway in animal cells.

The predominant oligosaccharide at both glycosylation sites in all batch cultures studied was a complex biantennary structure, Gal$_2$GlcNAc$_2$Man$_3$GlcNAc$_2$, which was fucosylated when attached to Asn$_{25}$ but not when attached Asn$_{97}$ (Table 3-6 and Table 3-7). A large proportion of these N-glycans contained terminal NeuAc on one or both arms. Lower percentages of complex tri- and tetra-antennary structures were also identified. Significant levels of high-mannose type N-glycans were identified towards the end of culture, and in particular Man$_5$GlcNAc$_2$ at the Asn$_{97}$ glycosylation site. Other high-mannose structures (Man$_{6-9}$GlcNAc$_2$) and truncated hybrid glycoforms with a terminal mannose on one arm were also present at lower percentages. The percentage of glycoforms with structures that could not be determined was less than 5%.
Figure 3-21 MALDI-MS spectra of desialylated IFN-γ tryptic fragments containing the Asn25 and Asn97 glycosylation sites taken from batch Culture 1 at 45 and 147 h. N-glycan structures corresponding to individual peaks in the spectra were determined by exoglycosidase array sequencing.

The relative percentage of the N-glycans changed during the course of all 3 batch cultures (Figure 3-22). Most notably, the percentage of Man5GlcNAc2 and GlcNAc2Man3GlcNAc2 at the Asn97 glycosylation site increased by 2.7% and 2.1% during batch Culture 1 and by 9.8% and 4.2% during batch Culture 2 respectively. The percentage of the predominant species, Gal2GlcNAc2Man3GlcNAc2, over the same time periods decreased by 22.0% during batch Culture 1 and by 14.9% during batch Culture 2. There was a similar increase in Man5GlcNAc2 with a corresponding decrease in the
proportion of $\text{Gal}_2\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2\text{Fuc}_1$ observed for the $\text{Asn}_{25}$ glycosylation sites for both cultures. The second batch culture was unusual in possessing a particularly long lag-phase and produced higher levels of $\text{Man}_3\text{GlcNAc}_2$ at both glycosylation sites compared to the other batch cultures.

In summary, recombinant CHO cells secreted an increasing percentage of recombinant human IFN-γ with truncated or high-mannose type glycosylation late in the batch cultures. Robinson et al. (1994) found an increasing proportion of high mannose-terminated glycans at the H-chain, N-linked site of a recombinant antibody expressed by a NSO cell line during fed-batch culture. This phenomenon was reduced by maintaining a higher residual glucose concentration and a switch to a glutamine-free medium. However, Gawlitzek et al. (1995a) found no changes in the expression of complex-type N-glycans from a BHK-21 cell lines during short-term nutrient limitations. These type of changes in the glycosylation of recombinant protein are cell-line specific and should be monitored during the production of any new recombinant protein intended for therapeutic use.
Table 3-6 Percentages of N-glycan structures associated with the Asn_{25} glycosylation site during batch Cultures 1 and 2.

<table>
<thead>
<tr>
<th>Glycan</th>
<th>Glycan Peptide</th>
<th>Batch Culture 1 (% glycans)</th>
<th>Batch Culture 2 (% glycans)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>45 h</td>
<td>69 h</td>
<td>93 h</td>
</tr>
<tr>
<td>Biantennary</td>
<td>4022.1</td>
<td>73.3</td>
<td>73.5</td>
</tr>
<tr>
<td>Mannose</td>
<td>3291.4</td>
<td>0.5</td>
<td>0.0</td>
</tr>
<tr>
<td>man-5</td>
<td>3469.4</td>
<td>0.8</td>
<td>0.0</td>
</tr>
<tr>
<td>Truncated</td>
<td>2455.7</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>2601.8</td>
<td>1.8</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>2805.0</td>
<td>0.5</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>2967.2</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>3129.3</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>3494.6</td>
<td>1.8</td>
<td>0.0</td>
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<td></td>
<td>3348.5</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>3697.8</td>
<td>0.8</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>3860.0</td>
<td>14.0</td>
<td>2.2</td>
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<tr>
<td></td>
<td>4225.3</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Triantennary</td>
<td>4387.4</td>
<td>2.8</td>
<td>21.7</td>
</tr>
<tr>
<td>Tetrantennary</td>
<td>4752.8</td>
<td>3.7</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Monosaccharide structures are schematically represented as: ▲, galactose (mass = 162.1); ■, N-acetylgalcosamine (203.2 Da); ●, mannose (162.1 Da); and ★, fucose (146.1 Da).

Chapter 3.3 Time Course Study of IFN-γ Glycosylation
Table 3-7 Percentages of N-glycan structures associated with the Asn97 glycosylation site during batch Cultures 1 and 2.

<table>
<thead>
<tr>
<th>Glycan</th>
<th>Glycan</th>
<th>Peptide</th>
<th>Batch Culture 1 (% glycans)</th>
<th>Batch Culture 2 (% glycans)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>45 h</td>
<td>69 h</td>
</tr>
<tr>
<td>Biantennary</td>
<td>3146.2</td>
<td></td>
<td>76.5</td>
<td>70.5</td>
</tr>
<tr>
<td></td>
<td>3292.3</td>
<td></td>
<td>3.0</td>
<td>2.8</td>
</tr>
<tr>
<td>Mannose</td>
<td>2415.5</td>
<td></td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>man-4</td>
<td></td>
<td>0.0</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>man-5</td>
<td></td>
<td>3.3</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>man-6</td>
<td></td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>man-7</td>
<td></td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>man-8</td>
<td></td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Hybrid</td>
<td>2943.0</td>
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</tr>
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<td></td>
<td>3105.1</td>
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<td>1.9</td>
<td>1.7</td>
</tr>
<tr>
<td>Truncated</td>
<td>2608.7</td>
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<td>0.8</td>
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</tr>
<tr>
<td></td>
<td>2780.8</td>
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<td>1.2</td>
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</tr>
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<td>2821.9</td>
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<td>0.9</td>
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<tr>
<td></td>
<td>3025.1</td>
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<tr>
<td></td>
<td>3349.4</td>
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<td></td>
<td>3511.5</td>
<td></td>
<td>10.0</td>
<td>9.6</td>
</tr>
<tr>
<td>Triantennary</td>
<td>3657.7</td>
<td></td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td>Tetrantennary</td>
<td>3876.8</td>
<td></td>
<td>0.4</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Chapter 3.3 Time Course Study of IFN-γ Glycosylation
**Figure 3-22** Glycosylation changes during batch Cultures 1 and 2. The percentages of IFN-γ glycan structures from different time points are grouped as complex biantennary (♦), complex triantennary and tetra-antennary (▲) or hybrid, oligomannose and truncated (●).
3.4 Monitoring the Proteolysis and Sialylation of Recombinant Human Interferon-γ During the Batch Culture of CHO Cells

A fermentation culture was completed with identical operating procedures to those used previously with one exception: there was no carry over of spent medium from inoculum vessels into the fermenter. The cells demonstrated typical growth characteristics (Hayter et al., 1991) and reached a maximum viable cell density of $0.54 \times 10^6$ cells ml$^{-1}$ and a total cell density of $0.7 \times 10^6$ cells·ml$^{-1}$ after 95 h of culture (Figure 3-23). Cell viability dropped sharply after peak cell densities had been reached (Figure 3-23). Recombinant IFN-γ accumulated during the growth phase to concentrations of $11 \times 10^3$ IU ml$^{-1}$ (Figure 3-23). The specific growth rate of the CHO cells under these conditions was $0.028$ h$^{-1}$ ($\mu$) (Table 3-8). Maximum specific IFN-γ production rates ($q_{\text{IFN}}$) also were associated with this period of growth and the culture produced approximately $300$ IU $(10^6$ cells)$^{-1}$ h$^{-1}$. 

Figure 3-23 Fermentation profiles of CHO cells during batch suspension culture; (a) viable cell density and viability, (b) total cell density and IFN-γ production; (c) glucose consumption, lactate and ammonia production curves; (d) cell cycle distribution.
Table 3-8 Growth, productivity and metabolic quotients.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\mu$ (h$^{-1}$)</td>
<td>0.028</td>
</tr>
<tr>
<td>$q_{\text{IFN}}$ (IU·10$^6$·cells$^{-1}$·h$^{-1}$)</td>
<td>303</td>
</tr>
<tr>
<td>$q_{\text{Glucose}}$ (mM·10$^6$·cells$^{-1}$·h$^{-1}$)</td>
<td>0.269</td>
</tr>
<tr>
<td>$q_{\text{Lactate}}$ (mM·10$^6$·cells$^{-1}$·h$^{-1}$)</td>
<td>0.387</td>
</tr>
<tr>
<td>$q_{\text{Ammonia}}$ (mM·10$^6$·cells$^{-1}$·h$^{-1}$)</td>
<td>0.049</td>
</tr>
</tbody>
</table>

Glucose was utilised rapidly and was depleted after 100 h (Figure 3-23). The maximum specific glucose utilisation rate was 0.269 mM (10$^6$ cells)$^{-1}$ h$^{-1}$ ($q_{\text{Glucose}}$). Lactate and ammonia both accumulated during the culture (Figure 3-23c). Lactate showed a rapid initial increase reaching a final concentration of 18 mM; accordingly, the specific lactate production rate was 0.387 mM (10$^6$ cells)$^{-1}$ h$^{-1}$ ($q_{\text{Lactate}}$). Ammonia levels increased slowly and reached 2 mM; the rate of specific ammonia production was 0.049 mM (10$^6$ cells)$^{-1}$ h$^{-1}$ ($q_{\text{Ammonia}}$).

Cell cycle distributions in the CHO cell population are shown in Figure 3-23d. When the late exponential period had been reached an increase in the percentage of cells in the G1 phase occurred. G1 phase cells formed about 60% of the total cells after 100 h of growth. The proportion of cells in S phase decreased after an initial increase, changes that were the reverse of the
proportion of cells in G1. The quantities of cells in G2/M phase increased during the growth phase reaching 17%.

3.4.1 IFN-γ peptide mapping by reversed-phase HPLC and mass spectrometry

The elution profile of Asp-N peptides by rp-HPLC is shown in Figure 3-24. All the collected peaks were subjected to MALDI-MS and masses matched to predicted peptide fragments (Table 3-9). Because of its speed and sensitivity, MALDI-MS has been used with very little cost to the applications of peptide mapping and identification of modifications such as glycosylation (Mann and Talbo, 1996). It was evident that cleavage events were occurring at Glu residues in addition to Asp residues. This is to be expected with the digestion conditions employed: low protein:protease ratio, extended digestion time and denatured substrate. Protein sequence analysis predicts 20 peptides or amino acids ranging in size (Figure 3-25). The majority of the IFN-γ polypeptide was mapped in this manner (92%), with only small peptide fragments (< 500 Da) not amenable to analysis, due to interference by matrix ions. Those peptide fragments that could not be assigned on mass criteria alone were subjected to N-terminal sequencing to confirm their identity. The C-terminal peptide fraction eluted early (peak 1) and its identity was confirmed by N-terminal sequencing. Using endoprotease Asp-N, it was not possible to assign an N-terminal amino acid, however, previous studies have shown
unambiguously that the N-terminal amino acid of IFN-γ produced by CHO cells is pyroglutamic acid (James et al., 1996a).

Figure 3-24 Elution profile of endoprotease Asp-N digested purified IFN-γ samples separated by rp-HPLC. Peak numbers indicate the individually collected peaks analysed by MALDI-MS. The numbered peaks are identified in Table 3-9.
Table 3-9 Predicted and experimental masses of endoprotease Asp-N peptides of IFN-γ with N-terminal sequencing data. G = glycosylated; N = N-terminal sequenced; = possible Na⁺ adduct.

<table>
<thead>
<tr>
<th>Peak number</th>
<th>Measured Mass (Da)</th>
<th>Correlated Mass (Da)</th>
<th>Residues</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1658</td>
<td>4430.2</td>
<td>119-143</td>
<td>ELSPAAKTGKRRRSQ</td>
</tr>
<tr>
<td>2</td>
<td>1196</td>
<td>1193.4</td>
<td>102-111</td>
<td>DLNVQRKAIH</td>
</tr>
<tr>
<td>3</td>
<td>1777</td>
<td>1756.1</td>
<td>76-89</td>
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<td>93-101 (G)</td>
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<td>1431.5</td>
<td>90-101</td>
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<td>112-118</td>
<td>ELIQVMA (N)</td>
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<td>2668.2</td>
<td>41-61</td>
<td>DRKIMQSQIVSFYFKLFKNFK</td>
</tr>
</tbody>
</table>
3.4.2 Changes in the C-terminus

Mass spectra of collected C-terminal peptide fragments are shown collectively from various stages of the culture in Figure 3-26. A polypeptide truncation was revealed of at least 10 amino acids beginning at Gln133, corresponding to a maximum molecular weight of 1658 Da. This truncation was apparent in all samples; no full length IFN-γ molecules (143 amino acids) were found at any stage of the fermentation. In early culture there was only one major IFN-γ polypeptide species of 133 amino acids. This was also visible in the late and death phase samples. There was evidence of further truncation later in culture by the increase in peptide heterogeneity. These new peptide species were present at lower intensities during early culture and at
greater intensities at late and death stages of culture. The death phase sample showed the greatest heterogeneity due to the addition of a species of molecular mass 71 Da to several of the peptide fragments. A peak of molecular mass 1065 Da in the late and death phase samples was not conclusively identified.

![Mass spectra of IFN-γ time course samples. Early = 76 and 100 h, late = 122 and 146 h and death = 169 and 193 h. + = addition of Ala_{117}.](image)

**Figure 3-26** Mass spectra of IFN-γ time course samples. Early = 76 and 100 h, late = 122 and 146 h and death = 169 and 193 h. + = addition of Ala_{117}.

The major cleavage event occurs at Gln_{133}, the enzyme responsible for this cut is not known. James *et al.* (1996a) demonstrated by ESI-MS that CHO cell derived IFN-γ polypeptides from late culture terminated between Gly_{127} and Gln_{133}. Since this truncation occurs at all stages of the fermentation and at a point of relatively low cell death the event may be intracellular in origin. Protease levels in the medium during early culture are likely to be low due to
Chapter 3.4 Monitoring the Proteolysis and Sialylation of Recombinant Human Interferon-γ During the Batch Culture of CHO Cells

the relatively high culture viability. Curling et al. (1990) have demonstrated that proteolysis of IFN-γ occurs prior to secretion from CHO cells. The truncations seen in later culture may have occurred sequentially by the loss of individual amino acids, as indicated in Figure 3-26. Gawlitzek et al. (1995a) found that BHK-21 cells expressed N-terminal truncated variants of a IL-2 grown in the absence of glucose. Glucose depletion in late culture might also influence C-terminal truncations in the CHO 320 cell line.

It is also likely that the late and death phase modifications were caused by the action of cellular carboxypeptidases or serine proteases released into the culture supernatant. The C-terminus of IFN-γ is rich in basic amino acids which are good substrates for these enzymes. These types of enzymes are thought to be released by dead cells (Teige et al., 1994). In addition, the sequence Lys-Arg-Lys-Arg is the cleavage site for the endoprotease furin. The additional mass difference of 71 Da to several of the peptides in the late culture sample may be due to the presence of the residue Ala_{117}. The enzyme responsible for this event is not known although the endoprotease chymotrypsin acts weakly on the C-terminal of methionine. The species of mass 1065 present in late and death stages of culture could be a sodium-potassium adjunct of a species of mass 1005 Da that was seen only in the death phase sample.

This increase in product heterogeneity may have important implications for quality control and biological activity. There is an industrial practice of adding
protease inhibitors to serum free medium to prevent product degradation due to the action of released cellular proteases. This practice is unlikely to prevent the earliest truncation event at Gln133. However, these events are particularly cell line and product specific. The biological activity and receptor binding of IFN-γ are both linked to its C-terminal region (Farrar and Schreiber, 1993, Lundell et al., 1991). Death phase samples were found to bind to the IFN-γ receptor at a 20-fold lower rate than commercial grade IFN-γ from *E. coli* (data not shown). Recombinant IFN-γ from early culture is likely to be the most bioactive; the region Lys-Arg-Ser is of particular importance to the bioactivity of IFN-γ. Work is required to determine the mechanisms behind these proteolytic events and their influence on product activity. The number of cleavage sites in IFN-γ makes it an ideal model system for these studies.

### 3.4.3 Changes in IFN-γ sialylation

An elution profile of the separation of N-glycans from the IFN-γ polypeptide is illustrated in Figure 3-27. The IFN-γ N-glycans, which are non-absorbing at 210 nm, were collected during the more hydrophilic gradient section between 15 and 45 minutes. The IFN-γ polypeptides eluted as a single peak after approximately 57 min. The collected N-glycans were freeze-dried and labelled with 2-aminobenzamide (2-AB) (Oxford GlycoSystems Ltd.) as per manufacturer’s instructions. The labelled N-glycans were separated by charge separation HPLC and a typical trace is illustrated in Figure 3-28. The neutral species elute first and were quickly followed by a peak identified as
Chapter 3.4 Monitoring the Proteolysis and Sialylation of Recombinant Human Interferon-γ During the Batch Culture of CHO Cells

free 2-AB label (personal communication, Oxford Glycosystems Ltd.). The peaks eluted in order of increasing charge: neutral, mono, di, tri and tetra-sialylated N-glycans. Each peak was distinct and reproducible. There was noticeable heterogeneity in the neutral, di and tri-sialylated peaks but little in either the mono or tetra-sialylated peaks. It is potentially possible to resolve the heterogeneity apparent in the neutral peak by additional HPLC separation but this did not prove successful.

Figure 3-27 Separation of released N-glycans and IFN-γ polypeptides by rp-HPLC.

![Graph showing separation of glycans and polypeptides](image-url)
Chapter 3.4 Monitoring the Proteolysis and Sialylation of Recombinant Human
Interferon-- during the Batch Culture of CHO Cells

Figure 3-28 Elution profile of neutral and sialylated N-glycans separated by
charge profile HPLC.

After integration of the area under each peak sialylation variants ('sialoforms')
were weighted for their sialic acid content. This is possible because 2-AB
binds stoichiometrically to released N-glycans in a 1:1 ratio. The resulting
proportions provide a better appreciation of the influence of each sialoforms to
the sialylation of the molecule. No sialoforms accounted for more than 50%
with the neutral and disialylated species in greatest quantity (Figure 3-29).
During the time course there was a decrease in the majority of sialylated N-
glycans and an increase in the proportion of neutral N-glycans. The neutral N-
glycans changed the most during the batch culture (an increase of ~10%) and
the monosialylated N-glycans the least (a decrease of ~4%). The increase in
tetrasialylated species could have been an anomaly. The alteration in total
glycan sialylation is illustrated in Figure 3-30. The majority of N-glycans are sialylated (over 70%) and they decreased from early to late culture by ~10%.

*Figure 3-29* Changes in the proportions of individual sialoforms (weighted for sialic acid content) during batch suspension culture.
Figure 3-30 Changes in the proportions of neutral and sialylated N-glycans (weighted for sialic acid content) during batch suspension culture.

Hooker et. al. (1995) demonstrated that a large degree of N-glycans from the CHO 320 cell line were sialylated. However, methods employed in that work were not able to quantitate the amount of sialylation. This method is a simple and quantitative method of determining the degree and variety of sialylation. With additional site-occupancy information it is possible to determine a molar value for sialic acid content (Section 3.7). Morrell et. al. (1971) found that the sialic acid content determines the survival of an extensive range of glycoproteins in the circulation. More recently, Szudlindski et. al. (1993) reported that sialylation of recombinant human thyrotropin influenced in vivo activity as well as plasma half-life. Further work is required to confirm these changes in sialylation and to develop methods to determine the mechanisms
that produce these variations. Recombinant IFN-γ proves itself to be a useful tool in the study of sialylation. The range of sialylation and the influence of site-occupancy variation make the system a sensitive tool to investigate the influence of production system.
3.5 Monitoring the Performance of an ELISA for Recombinant Human IFN-γ

The utilisation of a non-commercial ELISA necessitated the constant renewal and validation of biological materials. These materials included the polyclonal antibody raised in rabbit against recombinant human IFN-γ (Brian Cover, University of Kent, U.K.), the monoclonal antibody (Celltech Biologies Ltd.) and the recombinant human IFN-γ purified from cell culture medium which was used as a standard. The purified IFN-γ was calibrated against a recognised human IFN-γ standard (NIBSC). When the batch of anti-IFN-γ polyclonal antibodies, designated R2PA, was low in quantity a series of immunogenic injections of IFN-γ were made with two rabbits in parallel. The increase in antibody titre was monitored by the same ELISA except that the monoclonal antibody was used to coat the plate and the undiluted polyclonal sera used as the second antibody (Figure 3-31).

Each sample of sera produced acceptable responses in the modified ELISA. Both sets of sera showed a concentration dependence and high absorbance values. Since the fourth booster injection produced no improvement in titre the sera was harvested and purified using Protein A Sepharose. Dilution testing of both R3PA and R4PA produced similar dilution curves to R2PA (Figure 3-32). The initial dilution chosen was based on the mid-point of the linear part of the curve, in Figure 3-32 this corresponds to ~1 in 50 dilution.
Chapter 3.5 Monitoring the Performance of an ELISA for Recombinant Human IFN-γ

The effectiveness of the dilution was confirmed by determination of IFN-γ culture samples of known titre.

![Graph showing absorbance (490 nm) against dilution](image)

**Figure 3-31** Test of activity of anti-IFN-γ polyclonal antibodies present in untreated rabbit sera. Purified R2PA was used as a comparison. Each boost is equivalent to a further presentation of recombinant IFN-γ after the initial injection.
Chapter 3.5 Monitoring the Performance of an ELISA for Recombinant Human IFN-γ

Figure 3-32 Dilution test of purified R2PA against 50 IU ml\(^{-1}\) of recombinant IFN-γ in culture supernatant.

Periodically, the stocks of purified recombinant IFN-γ were checked against an international human IFN-γ standard (NIBSC). In addition, newly purified IFN-γ had to be diluted to 200 IU ml\(^{-1}\) for use in the ELISA. This required the testing of several dilutions of newly purified material and comparison with standard IFN-γ until the correct dilution was made. In Figure 3-33 typical results of both these experimental procedures are illustrated. The comparison of the original standard and the NIBSC standard is very close and clearly acceptable. Dilution of newly purified IFN-γ was estimated from the results of a protein determination of the material and the specific activity of IFN-γ (2.4 x 10\(^7\) IU mg\(^{-1}\)). A typical dilution was usually in the range of 1000-2,000-fold.
Since the dilution used was not sufficient to give a correct standard curve further dilutions were made until the correct dilution to match the NIBSC standard curve was found.

Figure 3-33 Standard curves of a working IFN-γ standard, a commercial human IFN-γ standard (NIBSC) and a preparation of newly purified recombinant IFN-γ.

After preparation of suitable stocks of polyclonal antibody and IFN-γ standard a typical standard curve was normally achieved (Figure 3-34). The curve is sigmoidal with a proportionally large linear section which is used for the determination of unknown sample concentrations. However, when R3PA and R4PA were tested, R4PA produced a respectable curve, but that of R3PA’s was particularly shallow (Figure 3-35). It was decided to continue with R4PA and several ELISAs were performed of typical batch culture samples.
However, the ELISA consistently gave high background values and low positive values (Figure 3-36). In comparison R2PA gave good positive values and relatively lower blanks. It was considered that one possible source of the problem was the purification procedure for the polyclonal antibodies. Protein A is not specific for IFN-γ antibodies and the elution step is made at low pH which might be detrimental to the integrity of the antibodies. Purified IFN-γ was covalently bound to a HiTrap column according to the manufacturer’s instructions (Pierce and Warriner, Chester, U.K.) and a sample of R4PA serum was purified. Purified specific anti-IFN-γ polyclonal antibodies were dilution tested against IFN-γ from culture supernatant (Figure 3-37).

**Figure 3-34** Typical standard curve of purified recombinant IFN-γ using R2PA anti-IFN-γ polyclonal antibody.
Chapter 3.5 Monitoring the Performance of an ELISA for Recombinant Human IFN-γ

**Figure 3-35** Standard curves of purified recombinant IFN-γ using R3PA and R4PA anti-IFN-γ polyclonal antibodies.

**Figure 3-36** Contribution of blank and sample absorbance values to the total absorbance determined by ELISA of R4PA. R2PA is shown for comparison.
Chapter 3.5 Monitoring the Performance of an ELISA for Recombinant Human IFN-γ

HiTrap-purified anti-IFN-γ polyclonal antibodies were diluted at those dilutions giving high positive and low blank values (0.10-0.20 dilutions) and used in further ELISAs. However, the resolution remained very poor - different time course samples gave very similar values. It was decided to try a commercial anti-IFN-γ polyclonal antibody preparation. A coating polyclonal raised in goat (R&D Systems, Abingdon, U.K.) was obtained on recommendation by L. Monaco (Biotechnology Unit, Milan, Italy) who were using the same cell line. L. Monaco suggested a working dilution of 5 μg ml⁻¹, which was at least 10-fold higher than that recommended by the manufacturers. A dilution test was used to verify the appropriate dilution (Figure 3-38). The concentration of polyclonal antibody at mid-exponential
Chapter 3.5 Monitoring the Performance of an ELISA for Recombinant Human IFN-γ

corresponded to $\sim 4.5 \, \mu g \, ml^{-1}$ seeming to confirm the given recommendation. However, when the commercial polyclonal was used to test culture samples, absorbance values obtained were consistently low and calculated concentrations were 5-to-10-fold higher than expected. Even at twice the suggested concentration there was little improvement in the standard curves produced (Figure 3-39).

![Figure 3-38 Dilution test of a commercial anti-IFN-γ polyclonal antibody (R&D Systems) with 50 IU ml$^{-1}$ recombinant IFN-γ.](image)

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Chapter 3.5 Monitoring the Performance of an ELISA for Recombinant Human IFN-γ

Figure 3-39 Test of a commercial anti-IFN-γ polyclonal antibody (R&D Systems) at two dilutions with a range of standard IFN-γ concentrations.

Byron et al. (1992) used a combination of two commercial anti-IFN-γ antibodies (Genzyme, Cambridge, U.K.) to detect human IFN-γ in blood at similar concentrations to those in cell culture supernatant. The suggested concentrations of the two anti-IFN-γ antibodies were confirmed by dilution tests. The recommended dilution of anti-IFN-γ polyclonal antibodies to coat the plates was 1 in 3000. This dilution (equivalent to $3.33 \times 10^{-4}$) gave high absorbance values and low background values required for a sensitive ELISA (Figure 3-40). The same occurred for the anti-IFN-γ monoclonal antibody preparation whose recommended concentration was 1 μg ml$^{-1}$ (Figure 3-41). These polyclonal antibodies were tested according to the protocol described using human IFN-γ (NIBSC) as standard. The resulting standard curve is...
illustrated in Figure 3-42. The sigmoidal curves produced demonstrated good correlation with standard dilutions (R=0.99). The incubations were then made at room temperature as opposed to 4°C and for half the suggested incubation times. There was no loss in resolution or sensitivity. Since the cost of human IFN-γ standard (NIBSC) was minor in comparison to the cost of the commercial anti-IFN-γ antibodies it was used as the standard in all subsequent determinations.

Figure 3-40 Dilution test of anti-IFN-γ polyclonal antibodies (Genzyme) using culture supernatant at 50 IU ml⁻¹ IFN-γ.
Chapter 3.5 Monitoring the Performance of an ELISA for Recombinant Human IFN-γ

Figure 3-41 Dilution test of anti-IFN-γ monoclonal antibodies (Genzyme) using culture supernatant at 50 IU ml\(^{-1}\) IFN-γ.

Figure 3-42 Typical standard curve obtained with a modified version of an ELISA developed by Byron et. al. (1992).
3.6 Investigation of CHO Cell Growth and IFN-γ Production in Microcarrier Systems

To investigate the influence of culture system on the growth and IFN-γ production of CHO 320 cells microcarrier culture techniques were employed. Nilsson et al. (1988) and Smiley et al. (1989) had both demonstrated the feasibility of CHO cell production of IFN-γ on microcarriers. Three microcarriers were tested for their suitability for CHO 320 cell batch culture: Cytodex 3 (Pharmacia Biotech): a microporous gelatin-coated dextran microcarrier, Cytopore 2 (Pharmacia Biotech) a macroporous cellulose microcarrier and Cytoline 1 (Pharmacia Biotech), a macroporous polyethylene/silica microcarrier. All the microcarriers varied in diameter, relative and charge density and effective surface areas (Section 2.2). Cell yield is directly related to the available surface area and hence the concentration of microcarriers (Nilsson, 1989). Thus in order to provide a better comparison between the first two microcarriers the ratio of cell density to microcarrier surface area was kept equal by adjusting the microcarrier concentrations accordingly.

The Cytodex culture was set-up at a microcarrier density of 5 g l⁻¹ and the Cytopore culture at 2 g l⁻¹ microcarrier, both with a cell inoculation density of 2 x 10⁵ cells ml⁻¹. Growth and detachment of cells from the microcarrier was observed by fluorescein diacetate staining (Figure 3-43). The growth characteristics on each microcarrier type were similar to those observed in
Chapter 3.6 Investigation of CHO Cell Growth and IFN-γ Production in Microcarrier Systems

suspension culture (Figure 3-44a). The Cytopore culture had a typical lag phase and a lower maximum total cell density \((0.6 \times 10^6 \text{ cells ml}^{-1})\) compared to the Cytodex culture. The Cytodex culture demonstrated no lag phase and a typical value for maximum total cell density approaching \(1.0 \times 10^6 \text{ cells ml}^{-1}\). The difference in the extent of growth and presence of a lag phase might have been due to the irregular surface morphology of the Cytopore microcarriers making attachment or cell spreading slower. Alternatively, differences due to the matrix material such as surface charge can influence both attachment and spreading. However, further experiments would be required to determine a definite influence of microcarrier type.

In addition, it was observed that it was very difficult to remove all the cells from Cytopore microcarriers by the method suggested by the manufacturer. The inclusion of 1% (v/v) Triton X-100 to the dissociation and staining solution improved the release of nuclei from the microcarriers. However, some were still observed to have remained within the microcarriers despite extended passaging of the microcarriers through a syringe needle. The growth rates of cells on both microcarriers were comparable to batch suspension culture values: the Cytodex culture exhibited a specific growth rate of 0.019 h\(^{-1}\) compared to a value of 0.024 h\(^{-1}\) for the Cytopore culture (Table 3-10).
Chapter 3.6 Investigation of CHO Cell Growth and IFN-\(\gamma\) Production in Microcarrier Systems

**Figure 3-43** A Cytodex 3 microcarrier stained with fluorescein diacetate and observed by fluorescence microscopy. Cells are stained bright green, shadows on surface may represent previous attachment of cells.

**Figure 3-44** Growth and production of CHO cells on two different microcarriers. (a) total cell density and (b) IFN-\(\gamma\) concentration.
Chapter 3.6 Investigation of CHO Cell Growth and IFN-γ Production in Microcarrier Systems

Table 3-10 Growth, productivity and metabolic quotients.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cytodex 3</th>
<th>Cytopore 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>μ (h⁻¹)</td>
<td>0.019</td>
<td>0.024</td>
</tr>
<tr>
<td>qIFN [IU (10⁶ cells)⁻¹ h⁻¹]</td>
<td>136</td>
<td>315</td>
</tr>
<tr>
<td>qGlucose [mM (10⁶ cells)⁻¹ h⁻¹]</td>
<td>0.246</td>
<td>0.426</td>
</tr>
<tr>
<td>qLactate [mM (10⁶ cells)⁻¹ h⁻¹]</td>
<td>0.413</td>
<td>0.608</td>
</tr>
<tr>
<td>qAmmonia [mM (10⁶ cells)⁻¹ h⁻¹]</td>
<td>0.025</td>
<td>0.034</td>
</tr>
</tbody>
</table>

The production of IFN-γ was also similar to batch suspension kinetics (Figure 3-44b). Peak IFN-γ titres were associated with the period of maximum cell density and remained at the same level during the death phase of culture. The Cytopore culture exhibited a maximum IFN-γ concentration of 12 x 10³ IU ml⁻¹ and the Cytodex culture 10 x 10³ IU ml⁻¹. The mean specific IFN-γ production rate (qIFN) during the growth phase was 136 IU (10⁶ cells)⁻¹ h⁻¹ during the Cytodex culture and 315 IU (10⁶ cells)⁻¹ h⁻¹ for the Cytopore culture (Table 3-10). The analysis of metabolites also revealed a very similar series of profiles when compared to previous batch suspension culture results (Figure 3-45). Correspondingly, the metabolic rates were also comparable to batch suspension culture data (Section 3.3). However, the metabolite rates of the Cytopore culture were higher than those of the Cytodex culture because the total cell densities in the Cytopore culture were lower. Both cultures demonstrated the complete utilisation of glucose when peak cell densities were reached after about 80 h. Lactate utilisation occurred concurrently reaching a maximum of ~16 mM in both cultures at the end of the culture time.
Ammonia concentrations increased gradually during the two cultures reaching a maximum of 2 mM.

![Figure 3-45 Metabolite changes during the batch culture of CHO cells on (a) Cytodex 3 and (b) Cytopore 2 microcarriers.](image)

Every cell culture has a cellular requirement for cell-produced chemicals to avoid long lag phases. Since each microcarrier provides a different microenvironment every microcarrier culture demands a different inoculation density for optimum growth. Since cells do not readily migrate from one bead to another a minimum threshold number of cells per microcarrier is required for inoculation. For example, Hu et al. (1985) demonstrated that the critical number of cells per dextran bead was 6 for human foreskin fibroblasts. Therefore it is common to determine the inoculum concentration of cells per
Chapter 3.6 Investigation of CHO Cell Growth and IFN-γ Production in Microcarrier Systems

microcarrier for successful cultivation. Cytodex 3 was chosen for future study because of its good optical properties and the ease and reliability of cell removal.

Four cultures using Cytodex 3 were set up at different microcarrier densities but with an equal cell inoculation density of $2 \times 10^5$ cells ml$^{-1}$. This corresponded to an inoculum number of cells per bead of 50, 25, 10 and 1 cell per bead for microcarrier densities of 1, 2, 5 and 10 g l$^{-1}$ respectively. In both the 1 g l$^{-1}$ and 2 g l$^{-1}$ cultures the microcarriers grew to confluence (Figure 3-46a). At a microcarrier concentration of 5 g l$^{-1}$, although there was more surface area available for growth the extent of growth was less than for the lower microcarrier density cultures. It was also observed that after one day’s growth some of the microcarriers had none or very few cells attached to them and many remained in this state until the end of the time course. The 10 g l$^{-1}$ culture displayed a poor growth curve.

The maximum number of cells per bead, $x_{\text{max}}(D_m)$, where $x_{\text{max}}$ is the maximum cell density at confluence and $D_m$ refers to microcarriers of mean diameter, can give an indication of the capacity of each microcarrier for cell growth (Table 3-11). This was calculated from the maximum total cell density at confluence, microcarrier density and the number of microcarriers per g dry weight: $4 \times 10^6$. This demonstrated that the multiplication ratio of cells from the inoculation ratio of cells per microcarrier increased by 4.5, 4.3 and 3.5-fold for the 1, 2 and 5 g l$^{-1}$ cultures respectively. These values are typical for batch culture but
could possibly be improved by using microcarriers of selected diameter and an improved medium (Hu and Wang, 1986). The 10 g l\(^{-1}\) culture proved too high a density of microcarriers to achieve normal growth and was not analysed further. This suggests that although the minimum inoculation density is between 1 and 10 cells per microcarrier a better cell yield is obtained with at least 25 cells per microcarrier.

Recombinant IFN-\(\gamma\) production for the three densities of microcarrier culture was very similar (Figure 3-46b). However, maximum IFN-\(\gamma\) concentrations were higher than those normally encountered for batch suspension cultures by about 2-fold. Most animal cells are most productive when attached to a surface especially if they are of an epithelial morphology such as ovary cells. At the point of maximum cell density the IFN-\(\gamma\) concentration was the same as that achieved during batch suspension culture. However, the IFN-\(\gamma\) level continued to rise which was rarely seen during suspension culture. In addition, these levels were not seen during the initial Cytodex 3 experiment (Figure 3-44). The specific IFN-\(\gamma\) production rates for the three cultures during the exponential growth phase were similar to those obtained during suspension culture (Table 3-11). The 1 g l\(^{-1}\) culture demonstrated a higher rate due to the greater IFN-\(\gamma\) concentrations achieved during the initial 48 h (Table 3-11).
Figure 3-46 Growth and IFN-γ production of CHO cells on Cytodex 3 at four microcarrier densities. (a) Attached total cell density, (b) IFN-γ concentration, (c) total unattached cell density and (d) unattached viable cell density.
Table 3-11 Growth rate, IFN-γ productivity and confluent cell density of Cytodex 3 batch cultures at three microcarrier densities

<table>
<thead>
<tr>
<th>Parameter</th>
<th>1 g l⁻¹</th>
<th>2 g l⁻¹</th>
<th>5 g l⁻¹</th>
</tr>
</thead>
<tbody>
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<td>μ (h⁻¹)</td>
<td>0.029</td>
<td>0.023</td>
<td>0.029</td>
</tr>
<tr>
<td>q_{IFN} [IU (10⁶ cells)⁻¹ h⁻¹]</td>
<td>458</td>
<td>295</td>
<td>316</td>
</tr>
<tr>
<td>x_{max}(D_m) (cells bead⁻¹)*</td>
<td>224</td>
<td>108</td>
<td>35</td>
</tr>
</tbody>
</table>

*the maximum number of confluent cells per bead of mean diameter

Two parameters of microcarrier culture which are not normally monitored are the unattached total and viable cell densities (Figure 3-46c and d). The first allowed an extra indication of the attachment and detachment of cells from the microcarriers. According to the values of total attached cell density an average of 66% of the cell inoculum attached themselves to the microcarriers after 5 h (Figure 3-46c). However, the density of unattached cells after this period corresponded to only 4% of the original cell inoculum concentration.

Therefore a proportion of the cells inoculated were probably lysed during the initial attachment phase. A proportion of a cell inoculum is usually lost due to stress on transfer to a new environment. The presence of circulating microcarriers will result in the additional stresses due to collisions between microcarriers, unattached cells and newly attached cells.

The collision phenomena will influence the number of unattached cells by cells being knocked off the microcarriers by collisions, or, by a some physiological trigger. It was unlikely to be coincidental that the density of unattached cells
was higher in the cultures with a lower microcarrier density (Figure 3-46c). If microcarrier-to-microcarrier collisions are solely responsible for cell detachment we would have expected the 2 g l\(^{-1}\) to have a higher unattached cell density than the 1 g l\(^{-1}\) culture (both have similar growth curves) since these collisions will be more frequent. However, if the microcarrier-to-microcarrier or cell-to-microcarrier collisions are severe enough to cause cell lysis then we would expect the lower density culture to have the highest density of unattached cells. Indeed, there is a greater density of unattached viable cells in the lower microcarrier density cultures (Figure 3-46d). However, the data did represent a typical growth curve despite a long lag phase which suggested that these cells were growing independently of the microcarrier population. Perhaps the lower density microcarrier cultures provided a better environment e.g. less cell-microcarrier collisions for unattached cell growth than higher microcarrier density cultures. This result suggests that the total unattached cell density curve represents a delayed normal suspension curve with superimposed microcarrier detachment as cells lose viability.

The attractions of microcarriers to pharmaceutical production are ease of scale-up, the separation of product from the production system and the ability to use existing batch-type bioreactor systems. In addition, to produce high quantities of pharmaceuticals, such as antibodies, at a reduced medium cost microcarriers provide the ideal support for perfusion systems. One microcarrier used in such systems is Cytoline 1 (Pharmacia Biotech): a high density, highly porous polyethylene/silica microcarrier optimised for the culture of CHO cells in
Chapter 3.6 Investigation of CHO Cell Growth and IFN-γ Production in Microcarrier Systems

fluidised bed reactors. To determine its suitability it was used as a growth substrate in batch suspension culture of CHO 320 cells in a 500 ml spinner flask at an inoculation density of $2.3 \times 10^5$ cells ml$^{-1}$ at a microcarrier density of $2$ g l$^{-1}$. This microcarrier is non-transparent so acridine orange staining was used to monitor cell growth in the microcarriers although resolution was very poor (Section 2.4.3). However, it could be determined that in early culture each microcarrier had several pores occupied with cells which had formed dense pockets of cells during the latter part of culture. Very few cells were visible on the outer surface of the microcarriers. Cell growth was similar in pattern to that of batch suspension kinetics although the maximum attached cell density was lower, only reaching $4.0 \times 10^5$ cells (ml microcarrier)$^{-1}$ (Figure 3-47a). The growth rate, $0.016$ h$^{-1}$, was comparable if lower than other microcarriers suggesting that the initial growth conditions may be inhibitory (Table 3-10). However, it must be remembered that this calculation is based on the number of cells attached to 1 ml of microcarriers and not on the cells present in 1 ml of culture medium.

The IFN-γ concentration achieved was also lower than levels normally seen with suspension cultures, reaching a maximum of $9 \times 10^3$ IU ml$^{-1}$. The $q_{\text{IFN}}$ was $315$ IU $(10^6$ cells$)^{-1}$ h$^{-1}$ which was most similar to the other macroporous microcarrier, the Cytopore 2. The increase in the total density of unattached cells showed a more shallow increase than that seen with the Cytodex microcarriers (Figure 3-47b). This was partly due to the much smaller densities achieved on this particular microcarrier type. There was very little
non-microcarrier growth which was seen to decrease continually after the attachment phase of 5 h.

Figure 3-47 (a) Growth of CHO cells on Cytoline 1 and IFN-γ production; (b) attachment and detachment kinetics during culture.
Table 3-12 Growth rate and IFN-γ productivity of a Cytoline 1 batch culture.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cytoline 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>μ (h⁻¹)</td>
<td>0.016</td>
</tr>
<tr>
<td>qIFN [IU (10⁶ cells)⁻¹ h⁻¹]</td>
<td>313</td>
</tr>
</tbody>
</table>
change from red to yellow, indicating a drop in pH, within several hours of medium exchange.

The CHO cells grew exponentially reaching a maximum of $2.2 \times 10^6$ cells (ml microcarrier)$^{-1}$ for the Spinner 1 culture (Figure 3-48). Spinner 2 failed to grow normally and the cells were seen to detach from the beads in sheets or clumps. Spinner 1 was continued until the medium requirements of the culture became to great to maintain a glucose level above 2 mM. Due to the premature ending of the experiment IFN-γ analysis was not performed. The maximum microcarrier-bound cell concentration of a CHO cell line on Cytodex 3 is theoretically $4.6 \times 10^6$ cells (ml microcarrier)$^{-1}$ at a microcarrier concentration of 5 g l$^{-1}$ (Gawlitzek et al., 1995b). These authors also used medium exchange, replacing up to 90% of the total reactor volume to achieve increased production of interleukin-2 compared to a conventional suspended cell culture.
The results from the microcarrier work demonstrate the suitability of most of the microcarriers tested to the batch culture of CHO 320 cells. Cell growth comparable to suspension cultures has been demonstrated and there is some indication of increased product levels. However, to achieve a detailed understanding of the influence of microcarrier culture conditions on the glycosylation of IFN-γ greater product titres are required. One method which can achieve higher production levels is fed-batch culture. However, this method requires the use of fermentation conditions to maintain efficient production.
control of both nutrient and environmental conditions. Fluidised bed perfusion culture provides such a system as well as providing a more stable environment in comparison to fed-batch culture. It was for this reason that further microcarrier work was directed to this method.
3.7 Utilisation of a Fluidised Bed Bioreactor System for the Cultivation of CHO cells

The stability of cell populations and consistency of product quality in long term microcarrier perfusion cultures has been reported (Helgeson, 1996). However, most of the work with these systems has been in the fields of chemical and civil engineering. The influence on the animal cell growth and recombinant protein expression of these perfused systems compared to more conventional batch/fed-batch bioreactors has only recently been examined (Griffiths and Racher, 1994 and Gawlitzek, 1995b). The novel fluidised bed bioreactor system (Cytopilot-Mini, Pharmacia Biotech, described in Section 2.2) was investigated for its potential to improve the growth and production of CHO cells expressing IFN-γ in comparison to more conventional suspension bioreactors.

3.7.1 Initial studies

The description of a modular integrated fluidised bed bioreactor was first made by Reiter et al. (1991). These authors described a 2 l reactor system which had an internal recirculation loop, efficient oxygenation and the option of operating as a stirred tank-, packed bed- or fluidized bed reactor. The system is basically a reactor vessel with an extending cylinder and a liquid distributor plate (Figure 3-49). The liquid distribution plate is located in the lower chamber which houses the heating jacket, sampling and discharge
Chapter 3.7 Utilisation of a Fluidised Bed Bioreactor System for the Cultivation of CHO cells

facility, the magnetic stirrer and the probe nozzles for pH and dO₂. The upper chamber contains the draft tube, headplate with connectors and the retention sieve which prevents microcarriers entering the lower chamber. The magnetic stirrer can rotate in both directions which allows the operation of microcarrier cultures as either a fluidised or packed bed. Using typical conditions for mammalian cell growth Reiter et al. (1991) were able to culture CHO-K1 cells to a density of 1.2 x 10⁸ cells (ml microcarrier)⁻¹.
Figure 3-49 Schematic of the principle components and flow within the bioreactor during fluidised bed mode. 1: magnetic stirrer, 2: stainless steel double jacketed housing, 3: inlet and outlet for heating circuit, 4: probe nozzle, 5: porous gas/liquid distribution plate, 6: glass cylinder, 7: downflow section of draft tube, 8: retention sieve, 9: headplate with tube connectors. Redrawn from a Pharmacia Biotech data file (18-1060-74).
Chapter 3.7 Utilisation of a Fluidised Bed Bioreactor System for the Cultivation of CHO cells

The ability of CHO 320 cells to attach successfully and grow on Cytoline 1 (Pharmacia Biotech) microcarriers has been demonstrated in Section 3.6. The high sedimentation rate of these microcarriers allows the use of a high circulation rate required to supply sufficient oxygen to support high cell densities. The Cytopilot (Pharmacia Biotech) and the microcarriers were prepared according to manufacturer’s instructions. A microcarrier bed volume of 200 ml was chosen because fluidisation was enhanced considerably above 100 ml. An inoculation density of $2.0 \times 10^6$ cells (ml microcarrier)$^{-1}$ was used. Initially, the bioreactor was run as a packed bed at 150 rpm for 3 h which encouraged attachment of cells to the microcarriers. The reduction in cloudiness of the culture medium provided a useful indicator of cell attachment during this period. The direction of the stirrer was changed to fluidise the microcarrier bed and the agitation rate increased to 450 rpm. Initial cultures grew well but the microcarrier bed suffered a loss of fluidisation after 5-10 days. The growth of cells was not sufficient to account for an increase in microcarrier density and hence a reduction of fluidisation. Raising the agitation rate and/or momentarily switching to packed bed mode alleviated the problem only temporarily.

The Cytoline 1 microcarriers are made of polyethylene (weighted with silica) which is susceptible to temperatures above 121°C (information sheet, Pharmacia Biotech). The preparation of microcarriers was changed to reduce their exposure to high temperature. Instead of autoclaving the microcarriers to degas them the microcarriers were boiled in distilled water for 10 minutes.
Chapter 3.7 Utilisation of a Fluidised Bed Bioreactor System for the Cultivation of CHO cells

When the microcarriers had been treated with 0.1 M NaOH and then washed they were placed in the bioreactor. The system was then autoclaved at 118°C (rather than 121°C) for 40 minutes. These protocol changes resulted in a stable microcarrier bed height during culture. After about 150 h of perfusion culture the pH was observed to drop suggesting an increase in the lactate concentration. Since there was no spare port for alkali addition the central port used to connect a tube to the retention sieve was sacrificed. This tube had been used to flush the retention sieve with air to remove microcarriers. It was found that by reversing briefly the stirrer rotation the passage of medium, now flowing out of the retention sieve, was sufficient to remove most of any clogging microcarriers. The addition of 0.1 M NaOH to control pH was found to be insufficient during later culture (> 250 h) and was changed to a 0.5 M NaOH feed. The addition of an increased alkali concentration was deemed safe for two reasons. Firstly, the addition of alkali occurred at the top of the bioreactor away from the cells and was seen to be physically dispersed by the retention sieve across the medium surface. Secondly, the alkali was observed (by the change in medium colour) to be drawn down the draft tube where it would have been readily mixed in the stirrer assembly.

During culture there was a considerable degree of foam formation. This was reduced by the use of a pure oxygen supply for aeration and the addition of 0.1% w/v Pluronic F-68 and 10 ppm Antifoam C. In addition to these additives the medium was also supplemented with penicillin (100 U ml⁻¹) and streptomycin (100 g ml⁻¹). These antibiotics were added because of the
increased risk of contamination due to the increased length of culture time and the intensive sampling and medium addition regime. To determine that the additives used were not deleterious to growth or IFN-γ production 100 ml batch suspension cultures were made in duplicate with and without the aforementioned additives. Four cultures were inoculated from the same seed stock at $1.7 \times 10^5$ cells ml$^{-1}$ and samples taken daily for cell counts and IFN-γ analysis. In Figure 3-50a the growth curves of both the control and test cultures are shown.

The cells in the test medium demonstrated a higher maximum cell density than the test cultures reaching $0.78 \times 10^6$ cells ml$^{-1}$ compared to $0.65 \times 10^6$ cells ml$^{-1}$. Both cultures had the same specific growth rate ($\mu$) of $0.016 \text{ h}^{-1}$ (Table 3-13). In addition there seemed to be a delay in the onset of the death phase. It was observed that during culture no foam was present in the test shake flasks but was present in the control vessels. The gas-liquid interface in the control cultures was therefore sufficient to produce sufficient air entrainment to cause bubble and foam formation. Murhammer and Goochee (1990) found that Pluronic F-68 had shear-protective properties in a surface-aerated bioreactor. Goldblum et al. (1990) concluded that Pluronic F-68 adsorbs to cell membranes to form protective layers on cells. Bavarian et al. (1991) observed that cells could be trapped in the foam layer and this resulted in a cell-enriched upper surface. Therefore it is possible that Pluronic-F-68 and/or the antifoam resulted in an increased cell density due to the protective function of Pluronic-F-68 or the absence of foam caused by the antifoam.
The production of IFN-γ during batch culture did not seem to be as affected as cell growth (Figure 3-50b). Both cultures demonstrated typical profiles of IFN-γ production - the maximum concentration occurring at the point of maximum cell density. The control cultures reached a maximum IFN-γ concentration of \( \sim 16.0 \times 10^3 \text{ IU ml}^{-1} \), the test cultures attained a peak value of \( \sim 14.5 \times 10^3 \text{ IU ml}^{-1} \). These differences were reflected in the specific IFN-γ production rates (\( q_{\text{IFN}} \)) which were lower for the test culture by \( \sim 8\% \) (Table 3-13). It is possible that one or more of the medium additives may be slightly toxic to the cells and cause an increased metabolic burden which could result in a decrease in the metabolic energy available for protein production. The suggested upper limit of Antifoam C concentration for use in culture is 50 ppm (Sigma catalogue) while concentrations of Pluronic F-68 are quoted as high as 0.5% w/v. However, there was no difference in cell viability between the control and test cultures (data not shown). In conclusion, it was decided that the levels of these additives were not sufficiently detrimental to warrant their removal from the medium.
Figure 3-50 Growth (a) and IFN-γ production (b) of CHO 320 cells during batch culture in the presence of 0.1% w/v Pluronic F-68, 10 ppm Antifoam C, penicillin (100 U ml⁻¹) and streptomycin (100 g ml⁻¹).

Table 3-13 Specific growth rate and IFN-γ productivity of a batch culture in the presence of 0.1% w/v Pluronic F-68, 10 ppm Antifoam C, penicillin (100 U ml⁻¹) and streptomycin (100 g ml⁻¹).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Cytopilot medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>μ (h⁻¹)</td>
<td>0.016</td>
<td>0.016</td>
</tr>
<tr>
<td>q_{IFN} [IU (10⁶ cells)⁻¹ h⁻¹]</td>
<td>334</td>
<td>309</td>
</tr>
</tbody>
</table>
3.7.2 Perfusion culture

The development of the perfusion enabled CHO cells to be grown over a period of at least 500 h. During this period the cells were observed to attach to the microcarriers and colonise the microcarrier pores. In Figure 3-51 a Cytoline 1 microcarrier, observed by SEM, is displayed which demonstrates the macroporous nature of the substrate. In Figure 3-52 it is possible to see the CHO cells occupying one of the microcarrier’s pores. The cell density on the microcarriers, monitored by nuclei counts, was observed to increase approximately linearly after ~ 100 h (Figure 3-53a). The total attached cell density reached a maximum of about 3.8 x 10^7 cells (ml microcarrier)^{-1} and appeared to plateau at that cell density. The value of $\mu$, 0.010 h^{-1} (Table 3-14), was lower than the values obtained during most batch suspension cultures with this cell line made by the author.

The glucose consumption rate was calculated daily and was used as a guide to determine the required perfusion rate (Figure 3-53c). The glucose concentration was maintained as close as possible to 2 mM during the culture. The glucose consumption rate was observed to decrease after ~ 410 h (data not shown). This suggested that the microcarrier bed was nearly fully occupied or that this particular cell line had reached a maximum population density for this mode of culture. The perfusion rate reached the theoretical maximum rate of 0.20 l h^{-1} or 25-fold bed matrix bed volumes per day (data file, Pharmacia Biotech). This is probably based on the typical glucose consumption rates of
animal cells, the maximum achievable cell concentration on the microcarriers and glucose concentrations in typical cell culture media.

Figure 3-51 A low magnification photograph of a Cytoline 1 microcarrier after 142 h of perfusion culture viewed by SEM.

Figure 3-52 A high magnification photograph of a Cytoline 1 microcarrier after 142 h of perfusion culture viewed by SEM.
Figure 3-53 Fermentation profiles of CHO cells grown in a 2 l perfused fluidised bed reactor; (a) cell growth and perfusion rate, (b) IFN-γ production and viability of unattached cells; (c) glucose consumption, lactate and ammonia production curves; (d) cell cycle distribution.
Figure 3-53 Fermentation profiles of CHO cells grown in a 2 l perfused fluidised bed reactor; (a) cell growth and perfusion rate, (b) IFN-γ production and viability of unattached cells; (c) glucose consumption, lactate and ammonia production curves; (d) cell cycle distribution.
Table 3-14 Growth rate and IFN-γ productivity during fluidised bed perfusion culture.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\mu$ (h⁻¹)</td>
<td>0.010</td>
</tr>
<tr>
<td>$q_{IFN} , \text{[IU (10}^6 \text{cells)}^{-1} \text{h}^{-1}]$</td>
<td>397</td>
</tr>
</tbody>
</table>

The production of IFN-γ was monitored by ELISA and was observed to rise steadily during the perfusion culture (Figure 3-53b). The maximum IFN-γ concentration was $\sim 21 \times 10^3$ IU ml⁻¹, which is higher than that achieved during batch suspension culture. This increase in IFN-γ concentration is expected because of the much higher cell densities present in the reactor. The cell density of a typical batch suspension culture is $0.9 \times 10^6$ cells ml⁻¹, thus we have an approximately 4-fold increase if the culture systems are compared on the basis of reactor volume (the 200 ml microcarrier bed occupied 10% of the total reactor volume). The perfusion system has the ability to be operated with a 500 ml microcarrier bed volume and so a culture has the potential for a 10-fold increase in cell density over batch suspension.

The average value of $q_{IFN}$ of 397 IU $(10^6 \text{cells})^{-1} \, \text{h}^{-1}$ was higher than that achieved during most batch suspension cultures (Section 3.3 and 3.4). It has already been stated and demonstrated that the productivity of animal cells is often higher on an attached surface (Section 3.6). A perfused system has the advantage of constant nutrient levels and low toxic metabolite levels. However, this is often difficult to achieve in a microcarrier suspension culture.
Chapter 3.7 Utilisation of a Fluidised Bed Bioreactor System for the Cultivation of CHO cells

system; most of these culture systems use microporous microcarriers in various fed-batch culture modes. In the environment of a fluidised macroporous microcarrier bed system the cell population has the additional advantages of a low shear environment and increased metabolic communication of the cells inside the pores.

The determination of cell viability poses a particular problem in such a system. A sample of microcarriers double stained with a fluorescein diacetate and ethidium bromide solution observed by confocal microscopy is shown Figure 3-54. The viable cells stain green, the non-viable cells stain red. This procedure is dependent on fast preparation and staining of the microcarriers so that the cells do not lose viability. This staining technique has been used to corroborate evidence that the viability of unattached cells is an accurate indicator of cell viability within microcarriers (G. Blüml, Applied Institute of Microbiology, Austria, personal communication). With the appropriate computer software Blüml determined the proportions of viable and non-viable cells within each microcarrier pore and matched these values with those of unattached cells. Blüml concluded that cells are lost from the microcarriers regardless of their viability. The viability of unattached cells was monitored during the perfusion culture and is illustrated in Figure 3-53b. During the majority of the culture lifetime cell viability remained above 70%. However, after 120 h the cell viability was observed to drop as low as 57% before increasing again after 210 h.
Chapter 3.7 Utilisation of a Fluidised Bed Bioreactor System for the Cultivation of CHO cells

Figure 3-54 Cells inside a pore of a Cytopore 1 microcarrier taken after 340 h of culture stained with fluorescein diacetate (green) and ethidium bromide (red) and observed by confocal microscopy. The pore is approximately 150 μm in diameter and does not extend beyond the area of staining.

The drop in unattached cell viability (150-250 h) may have been due to the decrease in glucose concentration from its initial value of 11 mM to less than 2 mM (Figure 3-53b and c). After an adaptation period to this lower glucose level the cell viability recovered. Glucose concentration has been shown to influence this cell line: Hayter et al. (1993) found an increased level of IFN-γ glycosylation following an increase in glucose concentration from 2 to 4 mM during a glucose-limited chemostat culture of this CHO cell line. The initial decrease in glucose concentration was matched by a characteristic sharp increase in lactate concentration (Figure 3-53c). When glucose reached 2 mM the lactate concentration reached an initial peak concentration of 14 mM after 140 h, a typical value for suspension cultures at the end of batch growth. The lactate concentration oscillated during the following 300 h before reached an apparently more steady level of ~ 8.5 mM. The ammonia concentration
increased steadily during the first 250 h reaching a maximum of 1.6 mM. It then fluctuated before reaching a final value of 1.2 mM.

During the perfused fermentation the cell cycle of cells attached to microcarriers was monitored. The cell cycle proportions of newly inoculated cells in suspension culture are typically of the proportions: 55% G₁, 35% S and 10% G₂/M (Section 3.3 and 3.5). After 140 h of perfusion culture the cell cycle proportions of attached cells had become 58% G₁, 30% S and 12% G₂/M (Figure 3-53d). After 190 h the proportions of cells in G₁ and S phases decreased at similar rates while the proportion of cells in G₂/M phase increased. These observations suggest that the perfusion culture caused the cells to arrest in the G₂/M phase. Bloemkolk et al. (1992) found that lower temperatures caused cells to arrest in the G₁ phase of the cell cycle during the batch suspension culture of S4B6 hybridoma cells expressing an anti-interleukin-2 antibody. Since monoclonal antibody synthesis is assumed to take place in the G₁ phase, a prolonged G₁ phase was expected to enhance the specific antibody production, however no increase was observed by the authors.

The assumption that cells removed from microcarriers do so with no prejudice to their viability has been stated (G. Blüml, personal communication). To determine whether cell cycle analysis could add weight to this hypothesis the unattached cells were analysed during the perfusion culture (Figure 3-55). The proportion of cells in the G₁ phase increased quickly the first 190 h of
cultivation and then decrease steadily over the following 300 h. Conversely, the proportion of S phase cells decreased during that first period and then increased gradually during the final 300 h. Cells in the $G_2/M$ phase increased in relative quantity throughout the culture lifetime.

![Figure 3-55 Changes in the proportion of unattached cells in different phases of the cell cycle during perfusion culture.](image)

When we compare samples taken from the same time points we can determine any relationship between the two cell populations (Figure 3-56). This comparison reveals a poor correlation between the two populations. The linear regression demonstrates an $R$ value of only 0.72 despite the equation of the line being similar to the line of equivalence. If the populations had been identical with regard to the proportions in each phase of the cell cycle we would have expected the relative proportions to have fallen on the equivalence
line. Only G_1 phase cells were similar in proportion when taken from the two cell populations. Cells taken from the microcarriers had a lower proportion of cells in the G_2/M phase and a higher proportion of cells in the S phase than cells from the unattached population.

![Graph](image)

**Figure 3-56** Scatter plot of cells in each cell cycle phase from both attached and unattached populations during perfusion culture. Both the linear regression line (−) and equivalence line are shown (−−). Sample times in h are given next to the respective data points.

However, this result does not invalidate the usefulness of monitoring the viability of unattached cells. If the cell viability is not linked to cell cycle then it may still be a good indicator of the health of the microcarrier population.
The significance of the cell cycle data is that it suggests that cells in the G$_2$/M and possibly the G$_1$ phase detach more easily than cells in the S phase. It is known that cells round-up when they prepare for cell division during the G$_2$/M phase. This change from an attached morphology, where the cells are usually flatter with a greater proportion of the cell in contact with the surface, results in an increased tendency to be detached from a surface. Therefore newly formed cells in the G$_1$ phase would also share this tendency before they begin to spread out across the microcarrier surface.

3.7.3 Analysis of amino acid metabolism

The determination of amino acid concentrations during perfusion culture were analysed as described in Section 3.3 but glycine and threonine were not determined because of poor resolution by the second HPLC system (Millipore) employed. The concentrations of amino acids during the colonisation of the microcarrier bed is given in Table 3-15. Samples were taken every 1-2 days and diluted 1 in 25. Every amino acid with the exception of glutamate and serine demonstrated a sharp decrease during the first 101 h. After 190 h many of the amino acids reached a reasonable equilibrium which was maintained during the rest of the culture. The utilisation of amino acids is also given in Table 3-15. This gives an indication of likely ‘steady state’ levels during more prolonged cultures assuming that the perfusion rate is maintained at the same level and there is no increase in cell density.
Glutamine was especially well utilised, reaching a level of 3.5 mg l\(^{-1}\) after 101 h (Figure 3-57). After 190 h it had recovered and was maintained at a mean level of 59 mg l\(^{-1}\) (S.D. 16) for the rest of the culture time. It was also the most heavily utilised amino acid during the culture (79%), only asparagine was utilised to nearly the same extent (59%). Glutamate, the glutamine by-product, accumulated during most of culture but was observed to return to feed concentration levels after ~ 329 h. Therefore there was no net production on utilisation of glutamate for the rest of the culture period.
Table 3-15 Changes in the extracellular levels of amino acids during batch fermentation culture.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Asp</th>
<th>Glu</th>
<th>Asn</th>
<th>Ser</th>
<th>Gln</th>
<th>His</th>
<th>Arg</th>
<th>Tyr</th>
<th>Met</th>
<th>Phe</th>
<th>Val</th>
<th>Iso</th>
<th>Leu</th>
<th>Lys</th>
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<tr>
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<td>51.7</td>
<td>33.1</td>
<td>307.3</td>
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<td>15.3</td>
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<td>3.9</td>
<td>220.7</td>
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<td>8.6</td>
<td>14.3</td>
<td>5.6</td>
<td>37.7</td>
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<td>411</td>
<td>9.9</td>
<td>17.0</td>
<td>13.6</td>
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<td>179.2</td>
<td>10.5</td>
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<td>8.1</td>
<td>38.3</td>
<td>32.2</td>
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<tr>
<td>483</td>
<td>13.8</td>
<td>21.5</td>
<td>21.0</td>
<td>24.5</td>
<td>66.0</td>
<td>5.6</td>
<td>170.5</td>
<td>9.8</td>
<td>11.2</td>
<td>15.9</td>
<td>8.1</td>
<td>39.3</td>
<td>31.8</td>
<td>9.9</td>
</tr>
</tbody>
</table>

Utilisation (%) 51 0 59 26 79 12 8 21 27 48 37 46 51 35

Difference between time = 0 h and 483 h.

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Figure 3-57 Glutamine utilisation and glutamate production during perfusion culture.

The concentration of serine present in the perfusate remained relatively stable during the first half of the culture period (Figure 3-58). After ~ 192 h it decreased in concentration until the end of the culture where it demonstrated a utilisation of 26% compared to feed levels. The utilisation of serine was in sharp comparison to batch culture where a steady production of serine was observed after some initial utilisation (Section 3.3). The decrease in the level of serine could be due to a requirement for glycine synthesis. Unfortunately, without being able to determine the glycine level this can only be suggested.
Both asparagine and aspartate demonstrated more batch-type characteristics (Section 3.3) - both were well utilised during perfusion culture (Figure 3-59). Both amino acids demonstrated a rapid decrease in concentration during the initial 101 h. By ~ 190 h both amino acids reached a more steady level of utilisation of 51% and 59%, for asparagine and aspartate respectively. The branched amino acids also demonstrated similar characteristics during the culture. After an initial decrease isoleucine, leucine and valine remained at reasonably constant concentrations throughout the culture. Compared to concentrations in the feed medium isoleucine and leucine were more heavily utilised at the end of culture compared to valine (Figure 3-60). The remaining four amino acids, showing more than 20% utilisation by the end of culture, were lysine, methionine, phenylalanine and tyrosine. These 4 amino acids also
demonstrated similar characteristics to the branched amino acids (Figure 3-61).

In summary, all the amino acids appear to be essential for the cell line during the colonisation period of perfusion culture with the exception of glutamate.

**Figure 3-59** Changes in the concentration of asparagine and aspartate during perfusion culture.
Figure 3-60 Changes in the concentration of isoleucine, leucine and valine during perfusion culture.
Figure 3-61 Changes in the concentration of lysine, methionine, phenylalanine and tyrosine during perfusion culture.

3.7.4 IFN-γ analysis

3.7.4.1 IFN-γ purification and N-glycosylation site occupancy

Samples of perfusate were collected at 6 time points during perfusion culture and IFN-γ was immunopurified from 1 l sample volumes (Table 3-16). There was some variation in the quantity of IFN-γ recovered but the amount purified was sufficient for subsequent analyses. The determination of IFN-γ macroheterogeneity by MECE revealed a consistent production of the three glycoforms during perfusion culture (Figure 3-62). The approximate
proportions were: 80% 2N, 17% 1N and 3% 0N. This compares to typical batch culture values from the exponential growth phase of 75% 2N, 20% 1N and 5% 0N (Section 3.5). The increase in overall glycosylation cannot be due exclusively to the glucose concentration which is no higher during perfusion culture than that present during the exponential growth phase of batch culture. Curling et al. (1990) and Hayter et al. (1991) found that a high initial glucose concentration (22 mM) did not affect macroheterogeneity but transient additions during glucose-limited chemostat culture did increase the proportion of 2N glycoforms (Hayter et al., 1992)

Table 3-16 Recovery of IFN-γ from perfusate.

<table>
<thead>
<tr>
<th>Sampling time (h)</th>
<th>Purified protein (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>142</td>
<td>1.28</td>
</tr>
<tr>
<td>213</td>
<td>0.87</td>
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<tr>
<td>262</td>
<td>0.68</td>
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<tr>
<td>329</td>
<td>1.20</td>
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<tr>
<td>411</td>
<td>1.32</td>
</tr>
<tr>
<td>483</td>
<td>1.81</td>
</tr>
</tbody>
</table>
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Figure 3-62 Changes in the relative proportions of IFN-γ glycoforms during perfusion culture determined by MECE.

3.7.4.2 IFN-γ digestion with PNGaseF and 2-AB labelling of released glycans

Recombinant IFN-γ from perfusate samples was treated by PNGaseF (Oxford Glycosystems) and the released glycans labelled with 2-AB (Oxford Glycosystems) according to manufacturer’s instructions (Section 2.3). Separation of the 2-AB-labelled glycans revealed a typical elution pattern of the neutral and sialylated variants. The elution patterns of each sample showed a similar distribution of charged and uncharged species (Figure 3-63). After integration of the peaks and weighting of the species according to their
sialic acid content it was possible to determine the changes in relative proportions of sialylated and neutral species (Figure 3-64). This procedure demonstrated that the disialylated sialoforms were the dominant species followed by the trisialylated with the tetrasialylated and monosialylated species in similar proportions. The neutral species did not account for more than 10% of the population of the monitored glycans. During the perfusion culture the neutral and monosialylated species decreased slightly with a greater decrease in the relative proportion of disialylated species. In comparison, the trisialylated species increased by the greatest proportion while the tetrasialylated species also increased. In summary, there was an increase in the relative proportions of the highly sialylated species with a suggested decrease in the less sialylated forms.
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Figure 3-63 Separation of sialylated and non-sialylated species of IFN-γ during perfusion culture by rp-HPLC. Samples were collected at 142 h and 483 h.
Figure 3-64 Changes in the relative proportions of neutral and sialylated species of recombinant IFN-γ determined from 2-AB labeling of released glycans and weighted for sialic acid content.

When the sialylated species were summed it was observed that at least 80% of the glycans were sialylated (Figure 3-65). The sialylated species were observed to increase in proportion to neutral species during perfusion culture. The overall change in the proportion of sialylated and neutral species was ~6%. In contrast to samples taken from batch culture there was a much greater proportion of sialylated species under conditions of perfusion culture (Section 3.5). This increase in sialylated species cannot be due to an increase in the addition of glycans because this was not observed by MECE analysis of glycoform quantities. Therefore this increase in glycosylation is due to a specific increase in sialylation. It is possible to combine these two
sets of data to determine the molar sialylation of IFN-γ (Figure 3-66). Gawlitzek et al. (1995b) demonstrated an increase in sialylation of interleukin-2 variant glycoprotein expressed by BHK cells in microcarrier culture when compared to non-microcarrier culture in suspension culture.

Figure 3-65 Changes in the proportion of neutral and sialylated species during perfusion culture.
Chapter 3.7 Utilisation of a Fluidised Bed Bioreactor System for the Cultivation of CHO cells

3.7.4.3 Capillary isoelectrofocusing of IFN-γ

An alternative method for monitoring the sialylation of a recombinant protein is capillary isoelectrofocusing (CIEF). Recombinant IFN-γ purified from perfusion culture was analysed by capillary isoelectrofocusing according to manufacturer’s instructions (Beckman). Purified IFN-γ samples were analysed by CIEF, a chromatogram of typical markers is illustrated in Figure 3-67. From the elution times a calibration plot can be created to determine the isoelectric point of subsequent IFN-γ samples. The addition of pI markers did not affect the elution time of IFN-γ. Eleven major peaks were repeatedly

Figure 3-66 Change in the molar sialylation of recombinant IFN-γ during perfusion culture determined by MECE and HPLC separation of 2-AB labeled released glycans.
eluted during an elution time of 50 minutes (Figure 3-68). These species according to their isoelectric points which are most likely to be influenced by sialic acids. The cleavage of sialic acids by neuraminidase removes all IFN-γ heterogeneity when separated by conventional gel isoelectric focusing (Figure 3-69). This procedure revealed at least 8 pI variants below pI 5.9 (Lane 2, Figure 3-69). After removal of the sialic acids most of the heterogeneity was lost and the pI increased to ~ pI 9-10. Galway et al. (1990) found that CHO mutant cell lines lacking the ability to sialylate expressed recombinant human follicle-stimulating hormone variants with a pI of between 1-2 units more than wild type cells. The much larger shift in the pI of IFN-γ after desialylation is due in part to its large content of basic amino acids (Section 1.8.2). The presence of sialic acids results in a decrease in the net pI and a corresponding increase in the elution time.
Chapter 3.7 Utilisation of a Fluidised Bed Bioreactor System for the Cultivation of CHO cells

**Figure 3-67** Electropherogram of four marker proteins separated by capillary isoelectrofocusing with calibration line created from the peak elution times of each marker.
Figure 3-68 Electropherogram of immunopurified recombinant IFN-γ from a fluidized bed perfusion bioreactor separated by capillary isoelectrofocusing (pI 3-10) at 13.5 kV.
Chapter 3.7 Utilisation of a Fluidised Bed Bioreactor System for the Cultivation of CHO cells

Figure 3-69 Gel isoelectrofocusing of immunopurified recombinant IFN-γ. M: isoelectric point markers; -: recombinant IFN-γ; +: recombinant IFN-γ digested with neuraminidase. Reproduced from Granados Reyes (1996).

Each peak (Figure 3-68) was integrated and the distribution of IFN-γ species is presented in Figure 3-70. The majority of IFN-γ species elute with an estimated pI of between pI 3-5, with the majority between pI 4-5. There were smaller proportions of species above and below this pI range. The greatest variation in the proportion of individual species was at the extremes of the pI range. There was an observed shift in the distribution of pI variants to the lower end of the pI range during the course of the perfusion culture. In Figure 3-71 the mean adjusted pI for the IFN-γ molecule demonstrates more clearly the variation in pI during the perfusion culture. This shows a clear decrease in
the pI of the molecule after 200 h of culture. This correlates with the increase in molar sialylation determined by MECE and 2-AB labelling. This decrease is probably due to an increase in the proportion of sialylated IFN-γ species and not to any increase in site occupancy. MECE demonstrated the stable distribution of the three site-occupancy variants (Figure 3-67), 2-AB labelling of released glycans quantitatively determined the distribution of sialylation variants and their influence on the molar sialylation of IFN-γ. CIEF demonstrates the influence of sialylation heterogeneity on pI of the molecule and the possibility for fast determination of variations in sialylation.
Figure 3-70 Changes in the distribution of IFN-γ molecules of differing pl during perfusion culture. Samples were taken at the indicated times.
Figure 3-71 The mean pI of immunopurified recombinant IFN-γ during perfusion culture.

3.7.4.4 Site-specific oligosaccharide changes

Tryptic peptides were prepared as described in Section 3.3. Mass spectra, illustrated in Figure 3-72, revealed a heterogeneous population of N-linked oligosaccharides identical to those observed during batch culture (Section 3.3). Structures were confirmed by exoglycosidase array sequencing and were consistent with those determined by Hooker et al. (1995). The predominant structures were biantennary with some triantennary structures also evident (Figure 3-73). The majority of structures were sialylated and the proportions of each glycopeptide appeared to remained consistent as a function of culture time. However, this a qualitatively judgement since negatively
sialylated glycopeptides reduce the ionisation efficiency, making the sialylated spectra non-quantitative (James et al., 1995).

The predominant desialylated oligosaccharide at both glycosylation sites in all batch cultures studied was a complex biantennary structure, \( \text{Gal}_2\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2 \), (Figure 3-73). Poor resolution of the Asn_25 only allowed quantitative data of the Asn_97 site (Table 3-13). Spectra from this site did not demonstrate sufficient sensitivity to identify all the N-glycans determined in Section 3.3. However, it did reveal those structures which are present in the greatest quantity (including all components which contributed to more than 5% of the total population demonstrated in Section 3.3). Other structures at the Asn_97 site included three truncated glycans and a single non-fucosylated triantennary glycan. The proportion of biantennary structures was similar to data from batch Culture 2 (Section 3.3.2.3 and Table 3-17). The proportions of truncated and triantennary N-glycans were higher than those observed in batch suspension culture. This is probably due to the fewer species resolved. The sensitivity was not sufficient to determine increases in tetrantennary structures or other minor truncated structures.

During the perfusion culture the proportion of triantennary structures was observed to increase in proportion while the biantennary species decreased (Table 3-17 and Figure 3-74). The increase in triantennary structures supports the data obtained from 2-AB labelling of released N-glycans (Section 3.3.2.3). The decrease in mono- and disialylated structures observed by that technique.
were observed to decrease during perfusion culture. These structures are represented by the truncated and biantennary structures determined by mass spectrometry. However, the truncated data can include monosialylated as well as neutral variants and the biantennary data can include mono-, disialylated and neutral variants. The increased production of more highly branched structures is likely to be a result of the culture conditions employed. The synthesis of these types of structures, as well as increased sialylation, may be a consequence of a better physiological environment: constant nutrient supply and low waste metabolite levels or an altered physical state: attached growth and higher cell densities. Or indeed a combination of both.
Figure 3-72 MALDI-MS spectra of sialylated IFN-γ tryptic fragments containing the Asn\textsubscript{25} and Asn\textsubscript{97} glycosylation sites taken from perfusion culture at 142 and 483 h. N-glycan structures corresponding to individual peaks in the spectra were determined by exoglycosidase array. Monosaccharide structures are schematically represented as: ♦, sialic acid (mass = 291.3); ▲, galactose (162.1); ■, N-acetylgalactosamine (203.2 Da); ●, mannose (162.1 Da); and ★, fucose (146.1 Da).
Figure 3-73 MALDI-MS spectra of the desialylated Asn$_{97}$ IFN-γ tryptic fragment taken from perfusion culture at 213 and 411 h. N-glycan structures corresponding to individual peaks in the spectra were determined by exoglycosidase array. Monosaccharide structures are schematically represented as: ▲, galactose (162.1); ■, N-acetylg glucosamine (203.2 Da) and ●, mannose (162.1 Da).
Table 3-17 Percentages of N-glycan structures associated with the Asn97 glycosylation site during perfusion culture.

<table>
<thead>
<tr>
<th>Glycan category</th>
<th>Glycan structure</th>
<th>Peptide mass</th>
<th>Sampling Point (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>142</td>
</tr>
<tr>
<td>Biantennary</td>
<td>△○○○○○-N</td>
<td>3146.2</td>
<td>43.4</td>
</tr>
<tr>
<td>Truncated</td>
<td>△○○-N</td>
<td>2618.7</td>
<td>12.3</td>
</tr>
<tr>
<td></td>
<td>△○○○-N</td>
<td>2780.8</td>
<td>15.1</td>
</tr>
<tr>
<td></td>
<td>△○○○○-N</td>
<td>2983.3</td>
<td>13.3</td>
</tr>
<tr>
<td>Triantennary</td>
<td>△○○○-N</td>
<td>3511.5</td>
<td>15.9</td>
</tr>
</tbody>
</table>

Monosaccharide structures are schematically represented as: △, galactose; ○, N-acetylglucosamine; and ●, mannose.
Figure 3-74 Glycosylation changes during perfusion culture. The percentages of IFN-γ glycan structures from different time points are grouped as complex biantennary (♦), complex triantennary (▲) or truncated (●).

In combination with 2-AB labelling MALDI-TOF and exoglycosidase sequencing can provide a detailed picture of the subtleties of glycosylation heterogeneity of a recombinant protein during culture. However, linkage information is not provided by these methods. CHO cells lack the Gal α2,6-sialyltransferase and are only able to synthesise the NeuAca2,3Galβ1,4GlcNAc-R sequence. This type of structural difference may be important for metabolic clearance rates (Fiete et al. 1991). The alteration of these type of N-terminal oligosaccharide sequences has been demonstrated in CHO cells (Lee et al. 1989). It has also been possible to
Chapter 3.7 Utilisation of a Fluidised Bed Bioreactor System for the Cultivation of CHO cells

dually alter the terminal sialylation of recombinant proteins for intended therapeutic use. The importance of terminal oligosaccharide sequences to in vivo activity has encouraged the rapid advancement of analytical techniques to the identification of these moieties. During the development of a new recombinant cell line the decision of which particular clone to take through to production may no longer be dominated by its productivity. The sensitivity of these methods enables the analysis of very small quantities of a recombinant product. However, many of these techniques are time-consuming and often several are required to achieve a full characterisation of the product. One solution is process intensification to achieve a rapid throughput of samples (Harmon et al., 1996). After scale-up the physiological state of the cells must not be ignored because of its influence on product synthesis. To this end more sophisticated control techniques must be developed to maintain improvements in product optimisation (Konstantinov, 1996). Only by a combination of detailed and rapid analysis with cell-based process control can a recombinant product with consistent glycosylation characteristics be obtained.
Chapter 3.8 Final Conclusions

3.8 Final Conclusions

In the investigation of the influence of culture environment on the growth of a CHO cell line and the expression of IFN-γ several overall conclusions may be drawn:

- Capillary electrophoresis was demonstrated to be a fast and quantitative method of monitoring the variable site occupancy of a recombinant protein. Recombinant IFN-γ expressed in CHO 320 cells, analysed by MECE, displayed the following distribution of glycoforms during batch culture: 40-55% 2N, 35-45% 1N and 5-15% 0N.

- The oxygen mass transfer coefficient of a 15 l suspension bioreactor was determined for differing sparger systems and media volumes. An equation for determining the correct agitation speed at different operating volumes was determined to maintain a constant power to volume ratio during culture:

\[
N = 3 \sqrt{\frac{0.019 W L^{-1} \times \text{working volume}}{0.018}} \text{ rps.}
\]

- The batch suspension culture of CHO 320 cells was achieved for the first time at the 15 l scale. The culture was monitored for parameters of growth, viability, nutrient utilisation, metabolite build-up and product synthesis. During exponential growth all of the amino acids were utilised with the exceptions of glutamate and serine. The site occupancy of IFN-γ was observed to shift from the 2N to the 1N site occupancy variant by ~ 10%
Chapter 3.8 Final Conclusions

during the culture when analysed by SDS-PAGE or MECE. Mass spectrometry and exoglycosidase sequencing enabled the characterisation of a spectrum of glycan moieties present at individual glycosylation sites. The primary glycosylation structure was a complex biantennary which was fucosylated at the Asn\textsubscript{25} site. During the culture the proportion of these type of complex structures decreased and truncated and high mannose structures increased.

- Peptide mapping by mass spectrometry revealed that native IFN-\(\gamma\) was subject to a C-terminal truncation of ten amino acids and the sequential loss of further amino acids during batch suspension culture. Fluorescent labelling of released N-glycans revealed a highly sialylated population. At least 70\% of the population was sialylated with the disialylated type in the majority. There appeared to be a decrease in the proportion of sialylated N-glycans with culture time.

- The CHO 320 cell line was successfully cultivated on three types of microcarriers supplied by Pharmacia Biotech. Fed-batch culture using Cytodex 3 microcarriers demonstrated the potential to achieve high cell densities, > 2 \times 10^6 cells ml\(^{-1}\), in small suspension cultures.

- A perfused fluidised bed reactor was used for the cultivation of CHO cells to a high cell density, > 30 \times 10^6 cells (ml microcarrier\(^{-1}\), and a more concentrated product stream, > 20 \times 10^3 IU ml\(^{-1}\). The cells were observed to detach in a cell cycle dependent manner, \(G_2/M\rangle\rangle G_1\rangle S\), and utilisation of most of the amino acids in the culture medium was observed. There was an increased glycosylation site occupancy compared to batch suspension
Chapter 3.8 Final Conclusions

culture: 80% 2N, 17% 1N and 3% 0N, whose proportions remained constant during the culture. The proportion of sialylated N-glycans was also higher: at least 80% were sialylated which increased slightly during the culture time. This was largely due to an increased synthesis of more highly branched sialoforms. This shift was reflected in an increase of 0.25 sialic acids per IFN-γ molecule after 200 h of perfusion culture. Capillary isoelectrofocusing determined at least eleven pI variants which were attributed to variable sialylation states. During the perfusion culture the pI decreased by 0.3 units after 200 h of culture. Mass spectrometry demonstrated the same predominant biantennary structure present in batch culture. There was an increase in triantennary structures and a decrease in biantennary structures.

The aim of the project was to investigate the influence of time and bioreactor modality on the growth and product synthesis of the CHO 320 cell line has been met.
Chapter 3.9 Final Discussion

3.9 Final Discussion

It is now well recognised that the carbohydrate moiety of a recombinant glycoprotein may affect the immunogenicity, half-life, bioactivity and stability of a potential therapeutic product. Indeed, regulatory authorities such as the US Food and Drug Administration are requiring increasingly sophisticated carbohydrate analyses as part of the ‘well-characterised product’ or process validation. Multiple bioprocess factors are known to affect recombinant protein heterogeneity, and control of recombinant protein glycosylation may possibly be achieved at a number of levels: choice of host cell, genetic engineering of glycan processing or control of bioprocess parameters such as culture environment, method of cell culture and culture time etc. Since about half of all proteins are glycosylated the ability to monitor and modify carbohydrate moieties is of growing importance in the biotechnology industry.

The use of an industrial cell line producing a commercial therapeutic drug provided a solid foundation on which to investigate the potential of rapid and detailed product monitoring and the implications of product heterogeneity. Micellar electrokinetic capillary electrophoresis was applied to the separation of IFN-\(\gamma\) and for the first time demonstrated on-line quantification of changes in the proportions of IFN-\(\gamma\) glycoforms. The results showed a decrease in the glycosylation at the Asn97 site to increase the proportion of the 1N form. The significance of this decreased glycosylation state is open to interpretation.
Chapter 3.9 Final Discussion

However, since IFN-γ purified from peripheral blood lymphocytes is predominantly of the 2N variety it is likely that the increasing reduction in this form would produce a less human-like response for human applications.

The development of bioreactor systems for mammalian culture has not been of prime importance in the design of production processes. Once it was realised that suitable modifications to microbial fermentation systems were sufficient for batch growth of mammalian cells few risks were taken by industry. Despite some exceptions: Celltech Biologies use of airlift bioreactors and British Biotech's use of the Verax fluidised bed reactor there has been little major variation on the trusted agitated suspension bioreactor. Indeed, during this project when major pressure was put on the suspension bioreactor in the form of a decreasing working volume it still achieved typical cell cultures for the CHO 320 cell line. This was accomplished by the development of an engineered equation, specific to the bioreactor and culture medium, which could maintain a constant energy input to the culture.

Mass spectrometry is a versatile tool which has probably not been better applied than to the field of glycoproteins. MALDI-MS has become one of the two most successful forms of this analytical technique (the other being electrospray ionisation mass spectrometry). It's has been used in this work to monitor the site-specific changes in glycosylation to a degree of detail not seen before. Only has erythropoietin received the same level of detailed study. The discovery that the glycosylation changes at site content level, as well as at the
site occupancy level already determined, confirms the level of detail that may be required to satisfy regulatory authorities in the near future. The confirmation by MS of the C-terminal truncation of recombinant IFN-\(\gamma\) during batch culture adds an additional level of product heterogeneity. More importantly with regard to human IFN-\(\gamma\) is that an intact C-terminal is necessary for receptor binding. More work needs to be focused on the influence of released proteases and glycosidases in culture medium. The increased tendency towards serum free media results in the removal of protective agents present in sera specific. Alternative non-protein protective agents should be developed to meet this trend. Another approach would be to extend cell viability by the inhibition of apoptosis using cells genetically engineered to resist apoptosis. Work has already been targeted to this area with some success.

Most human glycoproteins which have been targeted for therapeutic use are of the complex variety. This has tended to steer cellular production to several cell lines which have demonstrated complex glycosylation. The growing realisation that sialylation is the most critical carbohydrate addition may well focus this choice even further. The realisation that the presence of terminal sialic acids is the most important positive factor in circulatory half-life as well as being critical to the activity of many glycoproteins are powerful reasons for its importance. This work has demonstrated a informative and quantitative technique to the elucidation of the types of sialylation present on recovered N-glycans. This demonstrated a decrease in sialylation during batch culture.
which was not surprising given the observed increase in truncated and high mannose structures. In combination with MECE it was possible to determine the molar sialylation of recombinant IFN-γ during perfusion culture. This is the first time a therapeutic glycoprotein has been characterised in this way. This demonstrated an increase in the tri- and tetrantennary forms of the molecule. It has been observed with erythropoietin that the more highly branched forms demonstrated an increased plasma half-life. Ideally, all glycoprotein variations should be described in tandem with \textit{in vivo} and certainly the work detailed here would have benefited from these type of studies.

The influence of perfusion culture on the microheterogeneity but not the macroheterogeneity is interesting. During batch culture the decreases in the glycosylation site occupancy were accompanied by decreases in complex glycosylation and sialylation. The likelihood that an overall decrease in the physiological health of the cell resulted in a decrease in glycosylation capacity is not unlikely. However, the switch from mono- and biantennary forms to tri- and tetrantennary with no increase in overall glycosylation during perfusion culture is more difficult to interpret. One suggestion is that is perhaps ‘easier’ to add sugars rather than to create carbohydrate branches. This would explain why branches are only created in the environment of constant nutrients, reduced shear and low metabolic wastes.
Chapter 3.9 Final Discussion

A second possibility is that the greatly increased cellular communication due to the dense cell clumps may result in a physiological trigger to form branched sugar moieties. The phenomenon also suggests that there may be a limit to the addition process of sugar chains to the polypeptide. It might have been expected to observe an increase in the 2N form at the continued expense of the 1N form. Perhaps there is a limited rate to which the transfer from dolichol phosphate can be achieved in comparison to the rate at which polypeptides are synthesised. It is of course possible that the ‘ideal’ balance of glycoforms is in the ratio observed (80% 2N, 15% 1N and 5% 0N). It is known that in vivo there is usually a variety of glycosylation variants of a particular glycoprotein and that this heterogeneity is maintained.

The potential to influence this heterogeneity to a balance that is more human-like is obviously desirable. It may even be possible with sufficient in vivo information to select for less humanlike varieties which may in fact be of better therapeutic potential. Although product characterisation would ideally be performed at an early stage of drug development this is usually achieved in small cultures which do no resemble the final production system. The possibility of selecting a bioreactor for its influence on product quality is an intriguing one. Because perfusion has the potential for a continuous and high product titre it has been considered mainly as a system for products which are required in high dose formulations e.g. antibodies. The increase in highly branched forms which are sialylated as well as the maintenance of the site occupancy range may be an attractive persuader in the use of these systems for other low dosage products. There is still some education required for the
industry to take up a system which has received its fair share of criticism. However, novel systems like that demonstrated successfully here for the first time in the U.K. demonstrates the potential of these ‘alternative’ bioreactor systems.
Chapter 3.10 Future Work

3.10 Future Work

The possibilities for future work could follow two lines of research:

1. Further work with the CHO 320 cell line:
   a) A quantitation of the sialylation present at the two individual glycosylation sites during culture.
   b) Further investigation of the influence of reactor modality on growth and product synthesis.
      i) Cultivation on the same microcarriers but in the 15 l suspension bioreactor. This would determine whether influences on product synthesis were due to the cell line's attached state or the physicochemical environment.
      ii) A more detailed study using fluidised bed technology might include:
          a) For example, when a stable cell population is obtained the perfused medium could be switched to protein free (or even serum containing).
          b) Variations in pH, temperature and dO₂ concentration.
Chapter 3.10 Future Work

2. Application to other cell lines:
   
a) The analytical techniques demonstrated here are suitable for application to many other cell lines expressing a recombinant protein e.g. the CHO 43 cell line (a high IFN-γ expression clone).

b) The use of fluidised bed technology for mammalian cells has not reached widespread use despite its potential to achieve highly concentrated product streams for extended culture periods. Until the commercial and economic potential of these type of systems is demonstrated they will remain an unpopular choice of production system for large scale pharmaceutical production. However, research which demonstrates detailed and perhaps unforeseen advantages of these systems may help to catalyse the introduction of these systems.
4. References


Chapter 4 References


Chapter 4 References


Chapter 4 References


Chapter 4 References


Chapter 4 References


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