OPTIMISATION OF CHO CELL GROWTH AND RECOMBINANT INTERFERON-γ PRODUCTION

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by

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To my parents

ABSTRACT

The optimisation of recombinant protein production by animal cell cultures is important for the economic feasibility of these processes. Simultaneously with product yield, product authenticity is a crucial aspect to consider as it may *per se* affect the therapeutic value of such proteins. More defined culture media are being developed, particularly to ensure batch product consistency. A Chinese Hamster Ovary cell line (CHO 320) producing human interferon-γ (IFN-γ), a glycosylated protein, was chosen to investigate the effects of the culture environment on (1) cell growth, (2) product yield and (3) product authenticity.

A statistical approach was used to identify important culture components for cell growth and IFN-γ production. When the concentration of the resulting positive variables was initially increased in culture, improvements of approximately 40% in both of these parameters were achieved; the glycosylation of IFN-γ was not affected. The former analysis also indicated that different stimuli were required for growth and production. Fed-batch feeding of glucose and glutamine, components depleted early from culture, did not prolong cell growth or IFN-γ production but the initial glycosylation pattern of IFN-γ was a function of glutamine concentration.

Bovine serum albumin (BSA) was shown to have important role(s) in culture and cell growth was not possible in its absence. Pluronic F68, alone or in combination with a lipid mixture or linoleic acid, was able to restore cell growth in low BSA (1 mg/ml) cultures. However, IFN-γ production was significantly reduced and the extent of IFN-γ glycosylation also changed. These effects were related to: (1) BSA concentration, (2) BSA type, and ultimately, (3) lipid composition of the culture.

The results reported in this thesis exhibit the necessity to consider the effects of the culture environment not only on cell growth and product yield but also on product authenticity throughout any optimisation process.

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NOMENCLATURE

AIDS	Acquired Immune Deficiency Syndrome
Ala	alanine
Arg	arginine
Asn	asparagine
Asp	aspartate
α-kG	α- ketoglutarate
BHK	Baby Hamster Kidney cells
BSA	bovine serum albumin
C127	Mouse Mammary cells
CSF	cerebrospinal fluid
CD-4	human T-4 differentiation antigen
CHO	Chinese Hamster Ovary cells
CMC	carboxymethycellulose
CMP	cytidine monophosphate
Cys	cystine
Da	dalton
DHFR	dihydrofolate reductase
DMEM	Dulbecko's Modified Essential Medium
DMSO	dimethyl sulfoxide
Dol-P-P	dolychol-P-P
EBM	ephithelial basement membranes
EDTA	[ethylenedinitrilo] tetra-acetic, disodium salt
EGF	epidermal growth factor
ELISA	enzyme linked immunosorbant assay
EPO	erythropoietin
ER	endoplasmic reticulum
FAF-BSA	fatty acid-free bovine serum albumin
FCS	foetal calf serum
FGF	fibroblast growth factor
GDP	guanosine diphosphate
Glc	glucose
GlcNAc	N-acetylglucosamine

Gln	glutamine
Glu	glutamate
Gly	glycine
h	hour
HDL	high density lipoprotein
HPLC	high performance liquid chromatography
IFN	interferon
IFN-γ - *x*N	interferon-γ glycosylated at *x* sites
IU	International Units
Hdp	hydroxyproline
His	histidine
HIV	human immunodeficiency virus
IGF	insulin-like growth factor
IgG	immunoglobulin G
IgM	immunoglobulin M
Iso	isoleucine
IL	interleukin
l	litre
Leu	leucine
Lip mix	lipid mixture
LL-O	lipid-linked oligosaccharide
Lys	lysine
m	minute
Mab	monoclonal antibody
man	mannose
MDCK	Madin-Darby Canine Kidney cells
MEM	Minimum Essential Medium
MES	2-[N-morpholino] ethanesulfonic acid
Met	methionine
mPL-I	mouse placental lactogen-I
MTX	methotrexate
MW	molecular weight
μ	specific cell growth rate (h^{-1})
OPA	orthophthalaldehyde
P	phosphate

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PEG	polyethylene glycol
Phe	phenylalanine
Pro	proline
pSV2	mammalian expression plasmid vector
q_{amm}	specific production rate of ammonia *($\mu mol/10^6 cells/h$)*
q_{glc}	specific consumption rate of glucose *($\mu mol/10^6 cells/h$)*
q_{gln}	specific consumption rate of glutamine *($nmol/10^6 cells/h$)*
q_{IFN}	specific production rate of interferon-γ *($IU/10^6 cells/h$)*
q_{lac}	specific production rate of lactate *($\mu mol/10^6 cells/h$)*
RPMI	Roswell Park Memorial Institute medium
rscu-PA	recombinant single chain urokinase-type plasminogen activator
SDS	sodium dodecyl sulphate
SE	standard error
Ser	serine
SV40	Simian virus 40
TCA	tricarboxylic acid
Tris	2-amino-2-[hydroxymethyl]1,3-propandiol
Thr	threonine
TNF	tumour necrosis factor
t-PA	tissue-type plasminogen activator
Try	tryptophan
Tyr	tyrosine
UDP	uridine diphosphate
V	variance
Val	valine
$Y_{amm/gln}$	yield of ammonia on glutamine *(mol/mol)*
$Y_{lac/glu}$	yield of lactate on glucose *(mol/mol)*

1- INTRODUCTION

1.1- Animal cells: a potential resource

1.1.1- General introduction

The first reports on cultivation of animal cells date back to the beginning of the century. In 1907, Harrison suspended dissected nerve tissue from frog embryos in lymph fluid, allowed it to clot as a droplet on the underside of a microscope cover slip sealed with wax, and found that the embryonic nerve cells were able to grow for several weeks under these conditions (Harrison, 1907). The more recent area of cell culture technology started in the early 1950s with the work of Eagle, who analysed the nutritional requirements of animal cells in culture (Eagle, 1955a,b,c), and the number of available cell lines has increased significantly since this initial work was carried out.

The approach of using cells which naturally produced the product of interest has presented several limitations. The producer cells often could not be cultivated and the productivity of the cells was extremely low. The ability to fuse cells of different types emerged in the 1970s, making it possible to produce genetically stable hybridomas, capable of continuous secretion of specific monoclonal antibodies (Köler and Milstein, 1975). The development of the recombinant DNA technology also in the 1970s, with the expression of mammalian genes in bacteria, led to the production of mammalian proteins in bacterial cultures (Cohen *et al.*, 1973). This technology was then applied to mammalian cells and many types of cells were transformed to continuous cell lines capable of infinite growth capacity while others were genetically manipulated to produce selected products. The developments which occurred in recombinant DNA technology have made possible the production of many previously scarce human proteins in large quantities (Bebbington and Hentshell, 1985) and as a result several

recombinant mammalian cells capable of high specific production of selected compounds are now available.

The development of animal cell technology has been a continuous process and is the result of technological advances in different areas, such as bioreactor design, cultivation techniques, genetic engineering techniques, media design and protein concentration and purification techniques. Much effort is being dedicated to widen the knowledge of cellular physiology and protein processing, the mechanisms controlling gene expression and the activity of specific gene products. Development in the area of process control is also required.

1.1.2- Why use animal cells?

It was believed in the late 1970s that most of the biologicals naturally produced by animal cells would be produced in the future either by prokaryotic cells, e.g., bacteria, or by eukaryotic cells, e.g., yeast. In recent years it became clear that there are several advantages in using animal cells as hosts, instead of bacterial or yeast systems, despite the perceived economical advantages of the latter ones. Animal cells, unlike bacteria or yeast systems, have the ability to secrete proteins in a correctly folded functional state and with the correct post-translational modifications, such as glycosylation (Berman and Lasky, 1985; Bialy, 1987). These features may be essential for the biological function of a protein (Dube *et al.*, 1988; Delorme *et al.*, 1992). Glycosylation is one of the most significant post-translational modifications and can influence the biological activity, immunogenicity and life time of therapeutic proteins (Parekh *et al.*, 1989a). It should be considered though that cells may not be able to perform all the modifications necessary for a specific protein, and even proteins expressed in mammalian cells may display reduced activity because of incorrect processing (Yan *et al.*, 1989).

A detailed analysis of the biosynthetic capabilities of existent expression systems (bacteria, yeast, insect cells, animal cells) has been made by Bialy (1987). When a mammalian gene is introduced into an heterologous host, such as a bacterium, some aspects of the complex protein biosynthetic apparatus can be modified and they may have significant problems in modifying, over-expressing and folding foreign proteins. Bacteria are not able to perform post-translational modifications associated with mammalian cell proteins, like proteolytic cleavage, subunit association or addition reactions, especially glycosylations, phosphorylations, acylations and methylations. Inactive protein, altered or unexpected activity and increased difficulty in purification are some of the possible consequences. Yeasts constitute another alternative for the expression of some human proteins. They are able to perform post-translational modifications to secreted proteins (Smith *et al.*, 1985) although some of these may differ from those occurring in animal cells.

Another factor that needs consideration when comparing animal cell systems with bacterial systems is the usual absence of an effective secretion mechanism in the latter one. In bacterial systems, unless the product of the inserted gene is associated with a secretory component it will remain within the bacteria. The development of renaturation procedures for product recovery may be required, and this can limit the practicability of a bacterial production process, as it has been reported for the production of tissue-type plasminogen activator (t-PA) by *Escherichia coli* (Datar *et al.*, 1993). Proteins from animal cells are secreted to the medium, enabling product extraction to be made from culture supernatants, which is much more convenient than extraction from the cell lysate, although it exposes the product to the possible harsh environment of the culture. In addition, endotoxins can be part of the bacterial lysate, and have to be completely removed before use of the desired protein (Butler, 1987).

The major drawbacks of using animal cells are still the cost of fermentation and the lower productivities achieved. The first point is particularly stressed when serum is used in the

culture medium. A comparative economic analysis of the recovery process of t-PA from either Chinese Hamster Ovary (CHO) cells or *Escherichia coli* has shown that the ratio of fermentation to recovery materials cost for CHO-tPA is 3:1 while for t-PA from *Escherichia coli* the ratio is 1:7 (Datar *et al.*, 1993), highlighting the expensive mammalian cell culture fermentations and the complex downstream processing required for products obtained from bacteria cultures.

Nevertheless, animal cells seem to produce the most structurally accurate human proteins, when compared to bacterial and yeast systems. Ability of performing post-translational modifications, accurate polypeptide folding, efficient assembly of subunits and secretion of proteins are the most significant aspects which enhance the importance of the use of animal cells.

1.1.3- Products obtained from animal cells in culture

A wide range of biological products are produced by animal cells in culture. Vaccines, monoclonal antibodies, growth factors, hormones, plasminogen activators and lymphokines are among the large group (Mizrahi, 1988; Leist *et al.*, 1990; Sanders, 1990; Birch, 1991). Examples of recent animal cell products, some of which are already in therapeutic use, are presented in Table 1.1. Sanders (1990) presented an extensive list of genes currently expressed in specific animal cell types.

The cell lines which are cited in Table 1.1 are the ones that are most commonly used for protein production. Among these, the CHO cell line is the best characterised and most widely used. CHO cells were first isolated by Puck, who in 1957 established a fibroblastic cell line from the ovary of a Chinese hamster (Puck *et al.*, 1958). The CHO host/vector system that has been most used is a result of the co-amplification of the genes for dihydrofolate reductase

(DHFR) and the product of interest in DHFR$^-$ cell lines, which present very high efficiency of amplification and expression of recombinant genes (Kaufman and Sharp, 1982). A high degree of similarity between oligosaccharide structures derived from CHO cells and human glycoproteins has been reported for products such as interleukin-2 (Conradt *et al.*, 1989), interferon-β_1 (Kagawa *et al.*, 1988), erythropoietin (Takeuchi *et al.*, 1988) and tissue-plasminogen activator (Spellman *et al.*, 1989). This makes CHO cells a desirable host for recombinant therapeutic protein production.

Table 1.1- Examples of products obtained from animal cells.

Protein	**Cell line**	**Reference**
Tissue-plasminogen activator*	CHO*	Lubiniecki *et al.*, 1989; Spellman *et al.*,1989
	Mouse L	Browne *et al.*, 1985
Erythropoietin*	CHO*	Takeuchi *et al.*, 1988
Interleukin-2	CHO	Conradt *et al.*, 1989
	Mouse T	Yamada *et al.*, 1987
Human growth hormone*	C127*	Pavlakis and Hamer, 1983
	CHO	Friedman *et al.*, 1989
Factor VIII	CHO	Kaufman *et al.*, 1988
	BHK21	Pavirani *et al.*, 1987
Hepatitis B surface antigen	CHO	Michel *et al.*, 1985
Interferon-ß	C127	Zinn *et al.*, 1982
	CHO	McCormick *et al.*, 1984; Kagawa *et al.*, 1988
Interferon-γ	CHO	Scahill *et al.*, 1983; Hayter *et al.*, 1991
Antithrombin III	CHO	Zettlemeissi *et al.*, 1987
Monoclonal antibodies	Mouse hybridomas	PMA report, 1988
Recombinant antibodies	CHO, mouse myeloma	Rhodes, 1989
Factor IX	BHK	Busby *et al.*, 1985

* cited in Birch (1991) as already in therapeutic use. CHO= Chinese hamster ovary cells; BHK21= Baby hamster kidney cells; C127= mouse mammary cells.

Pharmacologically active proteins for application in diagnosis and therapy are of special interest and therapeutic products from animal cells are expected to make an increasing commercial impact in the next five years (Klausner, 1993). Approximately 150 recombinant proteins are at the present at some stage of clinical development (Table 1.2) and among them it is estimated that about 100 represent novel substances, with no precedents in medical therapy (Drews, 1993). It is anticipated that 30-40 products from this group will be successfully marketed over the next 5-6 years.

Table 1.2- Recombinant products in clinical development.

Protein	Number	Selected indications
Growth factors e.g. TNF, CSF, EPO, FGF, PDGF	27	Cancer, anemia, wound-healing, viral and bacterial infections, bone marrow transplantation
Hormones e.g. insulin, IGF, relaxin	13	Diabetes, growth disorders, osteoporosis
Interferons	11	Cancer, viral infections
Interleukins	19	Cancer
Fibrinolytics e.g. tPA	14	Cardiovascular diseases
Vaccines	28	Hepatitis B, AIDS, malaria, pertussis, typhus, influenza
Recombinant proteins	22	
Recombinant live vaccines	6	
Recombinant monoclonal antibodies	11	Cancer, infections, inflammation
Soluble receptors e.g. CD-4, IL-1-receptor	2	Inflammation, HIV-infection
Others e.g. Factor VIII, DNase	18	Enzyme deficiencies
Total	**143**	

Source: Pharmaprojects Data Base, February, 1993 (after Drews, 1993).

It is evident that the role of mammalian cell culture in the production of therapeutic proteins has significantly increased over the last years, and still other uses may be found in the future.

1.2- Cultivation systems for animal cells

The growth of animal cells, in contrast with microbial cells, is restricted to a narrow range of environmental conditions. These will determine the growth rate and maximum cell density that may be achieved, affecting the product yields obtained in the final culture medium. An appropriate cultivation system should provide cells with a suitable physical environment, but the choice requires an understanding of the cell growth characteristics and cell behaviour towards the surrounding environment. Together with the medium composition, environmental parameters such as dissolved oxygen, hydrodynamic forces, pH and temperature are among the most important factors to consider when designing a cultivation system for mammalian cells. Most of the effects on the growth and productivity of the cells are the result of complex interactions between those factors.

1.2.1- Kinetics of growth and product formation

The growth of animal cells in suspension culture follows a similar profile to that observed for most microorganisms. Distinct growth phases can be identified. During an initial stage, known as the lag phase, cells adapt to the fresh medium. After that, they begin an exponential growth phase, increasing in population with a typical doubling time of 18 to 24 hours. In a batch culture, the exponential growth is usually followed by a transition stage and subsequently cells reach a stationary phase, which is characterized by some kind of growth limitation, such as nutrient limitation, product inhibition or accumulation of toxic products.

Several parameters, such as medium composition and environmental parameters affect the cell

growth and productivity in culture. These are discussed in the following sections. The kinetics of cell growth, nutrient consumption and product formation in a batch culture are usually described mathematically as:

$$\frac{dx}{dt} = \mu \cdot x \qquad (1.1)$$

where,

x = cell concentration
μ = cell specific growth rate

$$\frac{ds}{dt} = -\frac{\mu x}{Y_S} - m_S \cdot x \qquad (1.2)$$

where,

s = nutrient concentration

Y_S = cell yield based on substrate

m_S = maintenance coefficient

$$\frac{dp}{dt} = q_p \cdot x \qquad (1.3)$$

where,

p = product concentration

q_p = specific productivity

The main objective in an animal cell culture is usually to obtain a biologically active product secreted by the cells. The production can either be growth associated, non growth associated or both. If the product is growth associated, the exponential growth phase of the culture should be extended. If the product is non growth associated, it is desirable to maintain the population in the stationary phase. When it is irrelevant if the cells are growing or not, it is desirable to achieve high-density cell growth and then maintain the cell density for as long as possible. This will therefore influence the choice of both cultivation method and medium design.

1.2.2- Cultivation techniques

Animal cells can generally be classified according to their anchorage dependence. This is the first factor to consider for the design of a culture system. While anchorage-dependent cells need a surface to attach in order to grow, anchorage-independent cells can grow fully submerged in suspension culture. Most of the continuous cell lines (e.g. CHO, BHK21, hybridomas, myelomas) can be grown in suspension while most 'normal' cells, such as fibroblasts and some continuous cell lines, e.g. mouse C127, are anchorage dependent; a third group can either grow in suspension or attached to a surface, e.g. CHO and HeLa cells, which includes transformed cells derived from anchorage-dependent cell types (Hu and Dodge, 1985; Birch, 1991).

The subject of animal cell bioreactor technology has been extensively examined (Hu and Dodge, 1985; Adamson and Schmidli, 1986; Prokop and Rosenberg, 1989; Leist *et al.*, 1990). Non-anchorage dependent cells can be grown in suspension, either floating free or immobilised in appropriate systems, e.g., microcarriers; anchorage dependent cells have to be attached to a surface. Suspension cultures are usually preferred. Homogeneity of the culture, reproducible and defined culture conditions, simplicity of scale-up, reliable on-line analysis and process control are listed as the most important features accomplished by using suspension cultures. At the laboratory scale, suspension cultures can be performed in shaker flasks and spinner flasks. Anchorage-dependent cells are usually cultured in T-flasks and roller bottles. For scaling-up of suspension cultures stirred or air-lift bioreactors are normally used (Rhodes and Birch, 1988; Lubiniecki *et al.*, 1989; Rhodes, 1989; Merchuk, 1990). More recent culture systems are based on the continuous passage of fresh medium through a bioreactor in which the cells are immobilised, known as perfusion cultures. These include hollow-fiber (Tharakan and Chau, 1986; Piret and Cooney, 1990) and porous microcarriers (Looby and Griffiths, 1990) bioreactors. The latter technology can be used in stirred tank bioreactors, and is applied

to both anchorage dependent and non-anchorage dependent cells (Fammiletti and Fredericks, 1988; Nilsson *et al.*, 1988; Smiley *et al.*, 1989; Schmidt *et al.*, 1992).

Stirred-tank reactors and air-lift reactors still remain the more widely used, due to the homogeneous environment they provide, their suitability to scale-up and process control and because substantial knowledge from traditional microbial fermentation literature is available.

Different operation modes can be utilised for the cultivation of animal cells, which are generally classified as batch, fed-batch, continuous or perfusion cultures. Examples of use of each technique for the production of several biologicals are presented in Table 1.3. Each mode has its own advantages and the use of systems other than a batch culture is an attempt to overcome some of the limitations of that one.

In a batch system no medium components are added and no waste products are removed, and growth becomes limited either by nutrient limitation or inhibitory products accumulation. In a fed-batch culture essential nutrients, or even a certain percentage of the whole growth medium, are continuously or intermittently fed into the culture, in order to extend cell growth and/or production phase. Metabolic products are not removed, which may then become the limiting growth factor. In order to efficiently design a fed-batch process, the requirements of the specific cell line must be known and such a system can be very useful when dealing with nutrients which are utilised according to their availability in the culture medium, such as glucose and glutamine (see also section 1.3.2.1). In continuous culture there is a constant feed and harvesting rate of the culture medium, and once a steady-state is attained, the culture can be maintained at a constant cell density and product yield for long periods. In a perfusion system the cells are physically retained in the bioreactor and there is a constant addition of fresh medium and removal of spent medium. Although higher cell density can be attained, these systems are more complex than the conventional stirred tank and air-lift bioreactors, require large supplies of medium and usually the culture is not homogeneous. Furthermore,

this technique becomes inefficient if producing recombinant products that are growth associated, as the immobilised cells inside the reactor are not in the growth phase.

Table 1.3- Examples of recombinant protein production by different cultivation techniques.

Operation method	Product	Reference
Batch	Interferons	Phillips *et al.*, 1985
	t-PA	Rhodes and Birch, 1988
	Recombinant antibodies	Rhodes, 1989
	Lymphokines	Shaughnessy and Kargi, 1990
	Interferon-γ	Hayter *et al.*, 1991a
Fed-batch	Monoclonal antibodies	Reuveny *et al.*, 1986; Luan *et al.*, 1987; Miller *et al.*, 1988; Rhodes and Birch, 1988
	*Interferon-γ	Smiley *et al.*, 1989
	Human IL-2	Lucki-Lang and Wagner, 1991
Continuous	Monoclonal antibodies	Ray *et al.*, 1989; Merten *et al.*, 1993
	Lymphokines	Shaughnessy and Kargi, 1990
	Interferon-γ	Hayter *et al.*, 1992
Perfusion	t-PA	Rhodes and Birch, 1988; Lin *et al.*, 1993
	rscu-PA	Avgerinos *et al.*, 1990
	Monoclonal antibodies	Büntemeyer *et al.*, 1991
	Human IL-2	Wagner *et al.*, 1989
	Factor VIII	Boedeker *et al.*, 1993

* indicates fed-batch in relation to the whole growth medium

Although the environment of the cells in a batch or fed-batch culture is continuously changing, these processes have several advantages in terms of large-scale production, namely flexibility, simplicity of plant and operation, clarity of batch definition for quality assurance and safety by reducing the quantity of product at risk (Rhodes and Birch, 1988).

1.2.3- Hydrodynamic environment of the cells

1.2.3.1- Aeration and agitation

Oxygen is the terminal electron receptor in oxidative phosphorylation, being essential for energy generation. Supplying sufficient oxygen to the culture to allow cell growth and product biosynthesis is one of the design criteria for a bioreactor. For this, it is important to know the optimum oxygen level that should be provided. Fleischaker and Sinskey (1981) have compiled specific rates of oxygen uptake of several cultured cells. Below the critical dissolved oxygen tension, consumption is limited by oxygen availability (Lin and Miller, 1992) and above the critical oxygen tension varies with the glucose consumption rate (Frame and Hu, 1985; Miller *et al.*, 1989a). Each cell type seems to have an optimum range of dissolved oxygen partial pressures for growth. CHO cells cultured under low oxygen concentrations (Kurano *et al.*, 1990a) had a specific growth rate half of its normal value, but cell growth and metabolism was found to be approximately constant between 50-100% air saturation. The studies conducted by Kilburn and Webb (1968) with mouse L cells have demonstrated the growth inhibitory effects of high oxygen levels and cultivation of cells out of the optimum range, either at lower or higher oxygen levels, limits their growth (Kilburn *et al.*, 1969). The production of oxygen-derived free radicals, which can damage cellular components such as lipids, DNA and proteins, may account for the toxic effect of oxygen (Lin and Miller, 1992). In fact, non-lethal levels of oxygen or H_2O_2 exposures may decrease the specific rate of production of t-PA by CHO cells by at least 50% (Lin *et al.*, 1993). They have also found that while CHO cell growth rate, cell titre and t-PA production rate were not affected by mild hypoxic conditions (50% reduction in specific oxygen consumption rate), the exposure of cells to anoxia led to a drastic decrease in cell titre and t-PA production rate.

Unlike other nutrients, oxygen has relatively low solubility in aqueous media, approximately

0.2 mmol/l at 37°C, and so it would be rapidly depleted from a high density cell culture, if not continuously replaced. Both agitation and aeration are required to maintain an adequate mixing of the culture and to provide enough oxygen to the cells. However, these are also the main sources of hydrodynamic shear stress in bioreactors and the mechanical-fluid forces generated may be of a magnitude that causes damage to the cells. Shear sensitivity has long been reported to vary between cell lines (Augenstein *et al.*, 1971) and culture parameters, such as agitation history of the cells, concentration of specific metabolites and cell growth phase (Petersen *et al.*, 1990; Ozturk and Palsson, 1991), with cells becoming more susceptible to shear in the stationary growth phase. A dependence of shear sensitivity on nutrient limitation, preventing maintenance of the structural integrity of the cell, has been suggested (Petersen *et al.*, 1990). When considering the scale-up of cell culture processes, this parameter may become critical.

In shaker-flask cultures, agitation is simply provided by placing them in shaker incubators. In larger scale cultures, agitation is usually provided by mechanical means, e.g. impellers, paddles, coiled stirrers, or in the case of air-lift bioreactors, the contents are pneumatically agitated by a stream of air (see Leist *et al.*, 1990). An important source of fluid shear in bioreactors is the agitation of the culture by such mechanical devices (Dodge and Hu, 1986; Peterson *et al.*, 1990). According to Bliem and Katinger (1988), the cell tolerance rates for agitation fall in the range of 100-200 revolutions per minute for industrial reactors (500-5000 l batch reactors). However, Kurano *et al.* (1990a) cultured CHO cells in small scale bioreactors at 400 rpm; adverse effects on specific cell growth rate were only noticed at 600 rpm. Kunas and Papoutsakis (1990) also reported that in a bubble vortex-free stirred tank bioreactor, the growth of hybridoma cells was not affected at 600 rpm agitation speed, concluding that cell damage in stirred and sparged bioreactors is mainly caused by the presence of bubbles.

As the agitation rate used is usually low to prevent damage of the cells, the low degree of fluid

mixing in the gas-liquid interface does not promote good oxygen transfer and as the ratio volume/surface increases head-space aeration becomes insufficient to supply the required oxygen. Several techniques have been developed to provide oxygen to cell cultures, e.g. use of surface aerators, air sparging (Aunins *et al.*, 1986; Hu *et al.*, 1986; Prokop and Rosenberg, 1989). Air sparging provides good oxygen transfer, but excessive foaming and cell damage due to the shear force caused by rising air bubbles and bubble break-up have been reported (Handa-Corrigan *et al.*, 1989; Gardner *et al.*, 1990; Kunas and Papoutsakis, 1990; Papoutsakis, 1991b; Zhang *et al.*, 1992b). Sparged systems are usually used for the culturing cell lines which are more robust, such as BHK 21, Namalva, some CHO lines and several hybridomas, whereas microcarrier-based cultures and other cell lines seem to be predominately affected by direct bubble sparging (Bliem and Katinger, 1988). A compromise between minimum physical damage to the cells and maximum oxygen transfer from the gas phase to liquid medium has to be established for each culture system.

1.2.3.2- Additives for cell protection from fluid-mechanical damage

Several additives have been reported as "shear protectants" from agitation and aeration in animal cell cultures. Among them are serum, bovine serum albumin, carbohydrate polymers and pluronic alcohols. A list with several examples where this protective effect was involved is shown in Table 1.4. Papoutsakis (1991a) has recently reviewed this area.

Serum and albumin have been shown to allow better cell growth in agitated and/or aerated cultures, and mostly the effects reported are dose-dependent. Although serum has an important nutritional role (see section 1.3.1) there are clear indications that this effect is not only due to faster cell growth caused by higher serum concentrations. The effect seems to be mainly related to modifications in the bubble-burst characteristics (physical effect) (Handa-Corrigan *et al.*, 1989; Michaels *et al.*, 1991; Smith and Greenfield, 1992), although evidences for a

biological mechanism, resulting from metabolic interactions rendering the cells more resistant to shear were also reported (Michaels *et al.*, 1991; Smith and Greenfield, 1992). Leist *et al.* (1990) postulated that a biological/biochemical effect, probably due to serum proteins, is the basis for the protection mechanism. Ramirez and Mutharasan (1991) partially attributed the protective effect of serum to its property of decreasing the plasma membrane fluidity through the transfer of cholesterol from lipoproteins to the plasma membrane. BSA seems to exert a physical protective effect in nature (Hülscher and Onken, 1988; Smith and Greenfield, 1992), by a mechanism similar to that proposed by Handa-Corrigan *et al.* (1989) for Pluronic F68, although a limited biological interaction has also been suggested (Smith and Greenfield, 1992).

Table 1.4- Protection of freely suspended animal cells from agitation and aeration damage.

Additive	Cell line	Reference
Serum	Hybridomas	Kunas and Papoutsakis, 1990; Handa-Corrigan *et al.*, 1989 Ramirez and Mutharasan, 1990; Ozturk and Palsson, 1991; Michaels *et al.*, 1991; Smith and Greenfield, 1992
	Human melanoma	Leist *et al.*, 1986
	BHK-21	Handa-Corrigan *et al.*, 1989
BSA	Hybridomas	Hülscher and Onken, 1988; Takazawa *et al.*, 1988; Smith and Greenfield, 1992
Pluronic F68	Mouse LS	Kilburn and Webb, 1968
	BHK-21	Handa-Corrigan *et al.*, 1989
	Hybridomas	Gardner *et al.*, 1990; Ramirez and Mutharasan, 1990; Michaels *et al.*, 1991; Smith and Greenfield, 1992; Zhang *et al.*, 1992a,b; Lakhotia *et al.*, 1993
	Insect cells	Maiorella *et al.*, 1988; Murhammer and Goochee, 1988
PEG	Hybridomas	Michaels *et al.*, 1991
CMC	Human melanoma	Leist *et al.*, 1986

BSA- bovine serum albumin; PEG- polyethylene glycol; CMC-carboxymethylcellulose.

The elimination of serum and even proteins from the culture media may significantly affect the susceptibility of animal cells to damage caused by adverse hydrodynamic conditions. The increasing tendency to develop these types of culture and the increasing need for large scale productions led to the search of additives to protect cells from shear damage in bioreactors. The nonionic surfactant Pluronic F68 has been the polymer most widely used as it protects cells against damage in mixed, agitated and/or aerated cultures. The mechanism through which Pluronics prevent cell damage is not clear yet and opinions diverge. These additives are known to lower the interfacial tension and/or stabilise the formed foams and gas-liquid interfaces. The protective effect of Pluronic F68 in fermenter cultures has been attributed to the surface active properties of the molecule, by changing the properties of the gas-liquid interfaces (bubbles) and stabilising surface foams (Handa-Corrigan *et al.*, 1989; Zhang *et al.*, 1992b). Murhammer and Goochee (1988) proposed that Pluronic F68 incorporates in the cell membrane and stabilises it, contributing to its protective effect in fermenter cultures. Ramirez and Mutharasan (1990) also suggested that the presence of a semi-stagnant film at the cell surface of hybridomas decreases the apparent fluidity of the cell membrane and leads to the formation of more shear resistant cells, providing evidence that Pluronic F68 alters the cell plasma membrane. This is supported by the work of Zhang *et al.* (1992a), which demonstrated that Pluronic F68 increases the strength of cell membranes. The latter effect was attributed to the adsorption of Pluronic to the cell membranes, and it was postulated to partially account for its protective effects in sparged, agitated bioreactors. In fact, a cumulative protection mechanism, based on the models mentioned above, has been proposed by Zhang *et al.* (1992b). Although Pluronic F68 is postulated to interact with the cell membrane, the work of Michaels *et al.* (1991) has indicated that these possible interactions do not affect the shear sensitivity of the cells, and that the effect is solely physical, i.e., resulting from hydrodynamic changes in the culture environment; the same was found for polyethylene glycol. It is possible that the effect depends on the cell line and culture conditions. Along with these reports, Pluronic F68 has also been shown to affect cell growth independently of shear damaging conditions, e.g., in

static cultures (Mizhari, 1975; Bentley *et al.*, 1989).

While much effort has been dedicated to the characterisation of the fluid-mechanical forces that cause damage to freely suspended cells, less work has been done on their actual effect on cell physiology. Recently, Lakhotia *et al.* (1993) have reported a reduction in the content of specific surface proteins of the cells, CD13 and CD33, caused by an increase in agitation rate from 80 to 400 rpm with a simultaneous detrimental effect on cell growth. However, even at mechanical forces of a magnitude that did not cause any apparent cell damage (270 rpm), a reduction in the content of the same proteins was still observed. Pluronic F68 was shown to have a protective effect on the CD33 content per cell of cultures subjected to hydrodynamic stress but not on the content of CD13. It was suggested that the protective effect of Pluronic F68 is surface protein specific.

1.2.4- Temperature and pH

Temperature and pH are important extracellular parameters in culture. Animal cells are routinely grown at a temperature of 37°C but they can grow over a fairly wide range of temperatures, which allows the isolation of temperature sensitive mutants. Temperatures ranging from 34 to 40°C have been shown to allow the growth of CHO cells (Gottesman, 1985), and an optimum of 37°C has been reported (Kurano *et al.*, 1990a). Different cell lines have different pH optimum values for growth and production. CHO cells have been reported to grow from pH 7.0 to pH 8.2 in static cultures, but outside that range cells would not grow well (Gottesman, 1985). CHO cell growth in agitated cultures was shown to be limited to a more narrow range, pH 7.0 to pH 7.9 (Kurano *et al.*, 1990a), which was suggested to be related to an increase in shear sensitivity under agitation. Optimum culture pH was reported to be between 7.6-7.8 (Gottesman, 1985; Kurano *et al.*, 1990a), but reported values for CHO cell cultures vary between 7.0 and 7.2 (Conradt *et al.*, 1989; Curling *et al.*, 1990).

1.3- Cell nutritional environment- the culture medium

The cell culture medium must provide the nutrients required by mammalian cells for the synthesis of cell biomass and products, and the additional function of maintaining suitable physiochemical conditions. This medium is far more complex and expensive than culture medium for microbial cells, based on an osmotically balanced mixture of vitamins, minerals and amino acids, often supplemented with animal serum and/or proteins. Quantitative studies on the effect of medium components on cell growth and product formation are essential for process development, as they vary widely with cell lines, culture systems and culture conditions. A good quantitative understanding of the nutrient requirements of the cell line to use is an important step for process optimisation.

1.3.1- Development of serum-free medium

Traditionally, supplementation of the culture medium with serum had been required to support long-term cultivation of animal cells. The present trend in industry is to move towards more well-defined and simpler culture medium and during the past decade there was a significant shift towards the development of serum-free media. The inclusion of serum is not only expensive but also introduces much variability to media formulations due to its own complexity and undefined nature. Thus, serum also represents a potential source of contamination and a potential carrier of growth and/or production inhibitors. Downstream processing in large scale operations is also impaired by the use of serum (Jäger, 1991). Although only a few of the components existent in serum are growth promoting, it has an important nutritional role in the culture media. Barnes (1987) reviewed the serum components considered to be potentially active on cultured animal cells. These include nutrients, such as vitamins, amino acids, lipoproteins and fatty acids, hormones and growth factors for the stimulation of growth and metabolism, binding proteins for lipids and small compounds, such

as albumin and transferrin, attachment factors and also enzymes. Furthermore, serum proteins also seem to play an important role in providing physical protection to the cells against shear damage (see section 1.2.3.2) and serum-free media that support cell growth in shake-flask cultures may not be efficient in large scale fermenters, where cells are more exposed to mechanical stresses.

The development of serum-free media has not always been straightforward, mainly due to difficulties in isolating the growth promoting factors which are present in the serum and because each cell type has specific requirements. When growing cells in serum-free medium, several of its functions have to be replaced by supplementation with components able to perform them. The developments in the understanding of cell nutritional requirements have made possible the growth of many cell types in the complete absence of serum (Barnes and Sato, 1980; Barnes, 1987). Serum-free medium for hybridomas has been often reported (Murakami *et al.*, 1982; McHugh *et al.*, 1983; Glassy *et al.*, 1988; Takazawa *et al.*, 1988, Minamoto *et al.*, 1991). Cell lines usually used for the expression of recombinant protein, such as CHO cells (Hamilton and Ham, 1977; Gasser *et al.*, 1985; Hayter *et al.*, 1989; Ganne and Mignot, 1991; Ogata *et al.*, 1993), have been grown in serum-free medium.

Although several advantages arise from culturing cells in serum-free media, its apparent cost if supplemented with hormones and growth factors may become higher than serum supplemented media. However, serum-free media with low protein supplementation usually becomes more cost effective and does allow more efficient large scale operation for the production of biological products.

1.3.2- Cell requirements in serum-free medium

1.3.2.1- Energy sources

Glucose and glutamine are typically the main energy and carbon sources for most of the culture media. Glucose is usually included in the medium at a concentration of 5 to 20 mM and glutamine at a concentration varying from 0.7 to 5 mM. Figure 1.1 shows the major metabolic pathways of animal cells. The operation of particular metabolic pathways is dependent on the cell line and on the culture conditions (Eigenbrodt *et al.*, 1985; Mckeehan, 1986). Glucose is metabolised to pyruvate and lactate through the glycolytic pathway to produce energy (Eagle *et al.*, 1958). Although glycolysis can supply cells with much of their energy requirements, its major role has been reported as the provision of anabolic substrates for biosynthesis (Hume *et al.*, 1978, Wice *et al.*, 1981); it is a major source of carbon, providing ribose for nucleic acid synthesis (Zielke *et al.*, 1976, 1978). Glutamine is usually the most abundant amino acid in cell culture media, being typically metabolised to glutamate and ammonia (McKeehan, 1986). Its metabolism leads to different levels of energy production, depending on whether it is completely oxidised to CO_2, or incompletely oxidised to either aspartate and lactate (Reitzer *et al.*, 1979). It is the major amino group donor for the synthesis of purines and pyrimidines, amino sugars, pyridine nucleotides and asparagine (McKeehan, 1986). It can additionally be involved in the synthesis of lipids (Zetterberg and Engstrom, 1981). Despite this, a high flux rate of glutamine carbons to pyruvate through glutaminolysis may not be essential for cell proliferation (McKeehan, 1986).

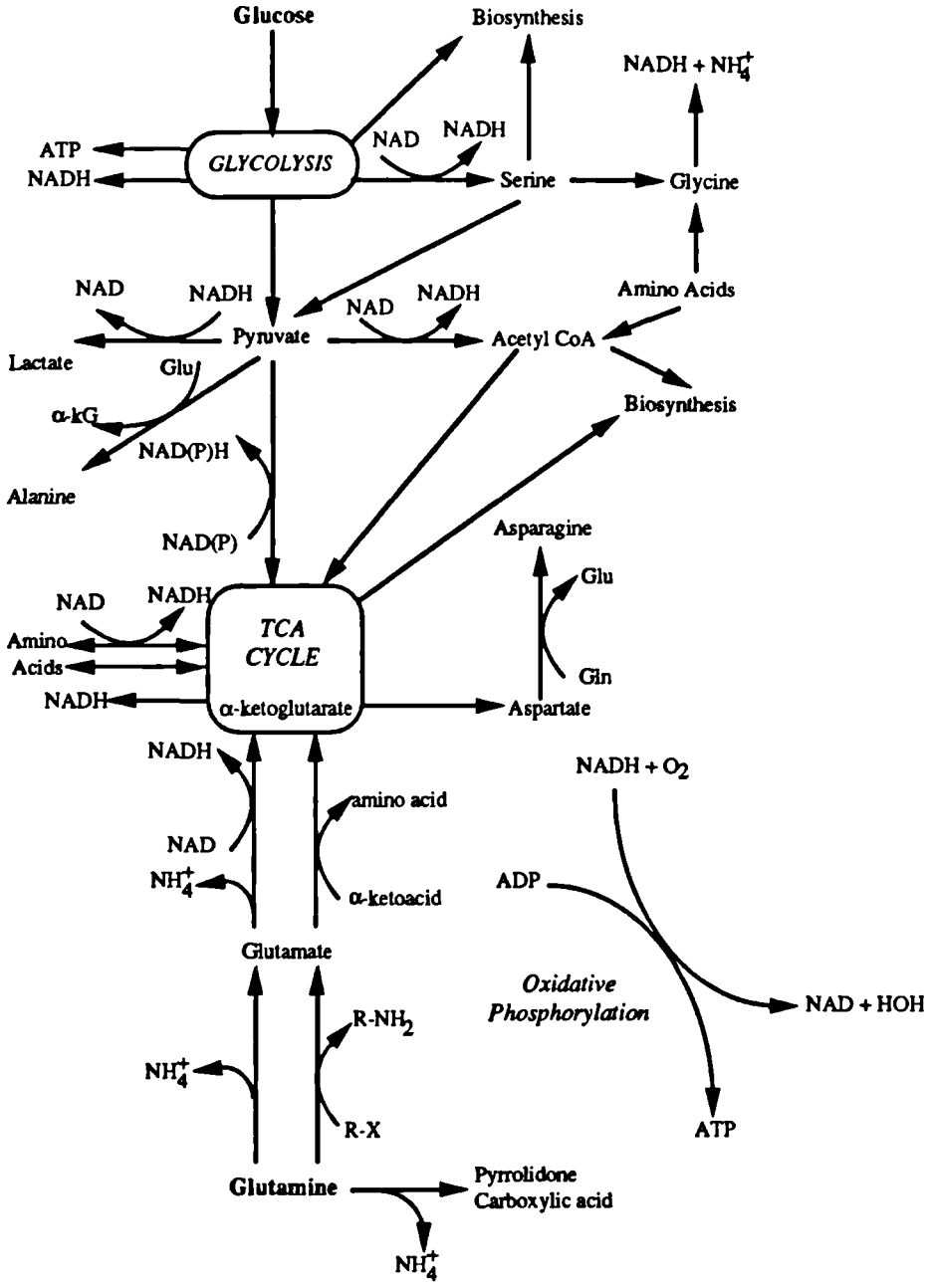


Figure 1.1- Diagram of the major metabolic pathways of animal cells (derived from Miller *et al.*, 1989a).

Glucose and glutamine metabolism are complementary to the production of other metabolites (Zielke *et al.*, 1978). The relative amounts of energy provided to the cells by glucose and glutamine vary according to the cell line. If both nutrients are present in the culture medium, glutamine oxidation can contribute up to 30 to 50% of the cell energy requirements (Zielke *et al.*, 1984). Even at high glucose concentrations, it has been stated that glycolysis can only supply 50% of the cell energy requirements, glutamine oxidation contributing 40% and pyruvate and fatty acid oxidation 10% (Eigenbrodt *et al.*, 1985). In the wild-type ovary cell line, in the presence of glucose, about 40% of the energy is supplied by glutamine (Donnelly and Scheffler, 1976). Despite this, cells can rely on the energy produced either by glycolysis, if glutamine is limited, or by glutaminolysis, if carbohydrates are limited, and it was suggested that flux through neither pathways is compulsory for cell growth (Eigenbrodt *et al.*, 1985).

The availability of glucose can reduce the rate of glutamine utilisation by mammalian cells (Zielke *et al.*, 1978, Reitzer *et al.*, 1979, Lazo, 1981) and a decrease in glucose concentration has been shown to increase glutamine oxidation rates (Zielke *et al.*, 1984). However, additions of either glucose and glutamine to culture medium has been shown to increase the rates of utilisation of both nutrients (Glacken *et al.*, 1986; Miller *et al.*, 1989a,b; Hayter *et al.*, 1991a), decreasing the nutrient utilisation efficiency. It is important to consider this type of relations when designing a culture medium, in terms of providing glutamine and glucose.

Cells are able to grow in media containing other types of carbohydrates, such as fructose or galactose, as first shown by Eagle (1958). The substitution of glucose by fructose, galactose or maltose has been achieved by others, who reported a decrease in the glycolytic rate under those conditions (Reitzer *et al.*, 1979; Imamura *et al.*, 1982, Nahapetian *et al.*, 1986). However, an increase in glutamine oxidation rate (Zielke *et al.*, 1984) also results from this metabolism. Mannose, fructose and galactose have been reported to support the growth of some recombinant CHO cell lines (Morgan and Faik, 1986).

1.3.2.2- Amino acids

Amino acids essentially act as precursors in the biosynthesis of proteins. They may also serve as an energy source, through oxidation to acetyl-CoA and tricarboxylic acid (TCA) cycle intermediates. Eagle, working with a mouse fibroblast and a human carcinoma cell line, reported the requirement of at least 13 amino acids for animal cell growth (Eagle, 1955a,b, 1959). These amino acids included arginine, cystine, glutamine, histidine, isoleucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine and valine and are most of the times considered essential for normal and established cell lines.

It was realised early that amino acid utilisation varies considerably with cell line. A high requirement of serine, glycine and proline was reported for normal human pituitary cells in the early 1960's (McCarthy, 1962). Culture medium must be designed in order to accommodate the needs of individual cell lines. As typical examples, the branched amino acids leucine, isoleucine and valine are consumed at high rates by some cell lines, namely Madin-Darby canine kidney (MDCK) cells (Butler and Thilly, 1982), human fibroblasts (Lambert and Pirt, 1975), hybridomas (Duval *et al.,* 1991) and CHO cells (Hayter *et al.*, 1991a). CHO cells are proline auxotrophs, requiring the presence of this amino acid in the culture medium (Kao and Puck, 1967), while it is produced in culture by myeloma cells (Ljunggren and Häggström, 1992).

There is a strong interrelation between the metabolic pathways shown in Figure 1.1. Amino acid quantification in culture supernatants is a straightforward means of determining the metabolic requirements and status of the cells. Specific amino acid utilisation depends on the availability of the major energy sources in culture, e.g., specific consumption of leucine, isoleucine, valine and serine was shown to be higher at lower glutamine concentrations (Miller *et al.*, 1989b). The production of amino acids, e.g., alanine, glycine and glutamate by some

cell lines has been reported (Butler and Thilly, 1982; Lanks, 1987; Miller *et al.*, 1989a,b, Duval *et al.*, 1991; Hayter *et al.*, 1991a). Alanine accumulation may reflect the transamination of glutamate to pyruvate and its production rate seem to be regulated by both glucose and glutamine consumption rates (Miller *et al.*, 1989a,b). Glycine and glutamate are a reflection of serine and glutamine metabolism, respectively.

Lately, more attention has been given to the concentration of amino acids in serum-free cultures and its importance for growth and production in animal cell cultures should be recognised for the development of any process. Due to the metabolic complexity between medium components, it is not possible to look at each component separately. Furthermore, no general rules can be made, as the amino acid metabolism varies between cell lines and even with the fermentation mode for the same cell line (Büntemeyer *et al.*, 1991).

1.3.2.3- Lipids

Incorporated lipids are important for animal cells as a source of energy and as structural components of membranes (King and Spector, 1981). The fatty acid composition of animal cells, both cellular storage lipids and membrane phospholipids, resembles the lipid composition of the culture medium and thus cell function is affected by changes in the medium lipid content (Doi *et al.*, 1978). The majority of cells can synthesise *de novo* cholesterol and most of the fatty acids they require from acetylcoenzyme-A. When the culture medium has an adequate supply of lipids *de novo* synthesis is inhibited and when cells are exposed to an excess of lipids, they accumulate excessive triglycerides and cholesterol esters in the form of cytoplasmic inclusions (Schmid, 1991). Linoleic and linolenic acid families can not be synthesised *de novo* by animal tissues; linoleic acid is essential for animals from a nutritional point of view (King and Spector, 1981) but whether the same is true for animal cells in culture is not clear yet.

Lipids are included in most serum-free and/or protein-free medium formulations (Barnes and Sato, 1980; Glassy *et al.*, 1988) and several lipids, e.g. cholesterol, oleic acid, linoleic acid and certain phospholipids, have been shown to stimulate the growth of several cell lines (Darfler and Insel, 1982; Kovar and Franek, 1986; Sato *et al.*, 1987; Minamoto *et al.*, 1991; Jayme, 1991). Serum and serum albumin are major sources of lipids in the culture medium and when using serum-free and protein-free media they can be supplemented to the medium in the form of isolated plasma lipoprotein fractions, free fatty acids complexed to serum albumin or fatty acid/phospholipid microemulsions (Schmid *et al.*, 1991). Pluronic F68 has also been used as an emulsifying agent (Jayme, 1991).

Quantitative data available on the subject is scarce (Schmid *et al.*, 1991) and although analysis of total fatty acids was shown to be an unreliable indicator of cell culture performance, individual quantitative analysis has allowed the identification of critical lipids for certain cell culture applications (Jayme, 1991). Most of the studies on the influence of lipids on culture performance focus on cell growth and production data are most of the times unavailable.

1.3.2.4- Vitamins and minerals

Addition of vitamins and trace elements is common practise in serum-free cultures. Several forms of water-soluble vitamins (vitamin B complex), such as riboflavin and thiamine, were found to be essential for the growth of some cell lines, e.g. mouse L and HeLa cells (Eagle, 1955c). Supplementation of vitamins to most media is mostly made based on those early nutritional studies and not much work has been done lately on the consumption of vitamins by cultured cells. Biotin, choline, folic acid, inositol, niacinamide, pantothenic acid, pyridoxine, riboflavine and thiamine are present in almost all basal media formulations. The lipid-soluble vitamins, A, D, E and K, are required by higher animal species, but they are not normally used in culture media. A more recent study on the consumption and stability of four vitamins,

ascorbic acid, nicotinamide, choline and thiamine (Kurano *et al.*, 1990c) in CHO cell culture has shown that while nicotinamide and choline were stable in culture medium, ascorbic acid and thiamine were not. Furthermore, ascorbic acid did not appear to be required for the growth of the CHO lines but growth limitation by choline at high cell densities was suggested.

At least 15 trace elements have been reported as essential or beneficial for the growth of several cell lines (Nielsen, 1981). Among these are copper, iron, manganese, selenium and zinc. Knowing the specific cell requirements for these nutrients is very important when using serum-free medium and specific supplementations have been developed according to the cell line. Some inorganic ions are essential for cell growth, as reported by Eagle (1955c): sodium, potassium, calcium, magnesium, chloride and phosphate and they are all present in basal media formulations.

1.3.2.5- Protein supplementation

The proteins insulin, transferrin and bovine serum albumin are usually included in most serum-free media formulations (Barnes and Sato, 1980; Glassy *et al.*, 1988), in order to replace some of serum functions. The function of the latter ones is mainly related to their binding capacities of several components, e.g., lipids (albumin) and trace elements (transferrin).

Insulin and transferrin are usually included in the medium at low concentration, ca. 5 µg/ml. Insulin is a peptide hormone known to control the metabolic activities of cells and affects cell proliferation. However, the reasons for its stimulatory effect are still unknown. Although extensively used in most serum free media formulations it has been shown that is not strictly required (Kovar and Franek, 1984) and complete deletion of insulin from serum-free medium has been reported (Schneider, 1989; Fike *et al.*, 1991). Transferrin is an iron-binding serum protein, and it acts via specific receptors on the cell surface (Aisen and Listowsky, 1980),

exerting a stimulatory effect on cell proliferation. Most cell lines under serum-free conditions require its presence to proliferate (Iscove and Melchers, 1978; Murakami *et al.*, 1982; Kovar and Franek, 1984). However, the concept that transferrin would never be replaced in the medium seems less likely as the replacement of transferrin by others sources of iron such as e.g., ferric citrate (Kovar and Franek, 1987; Scheneider, 1989) and iron gluconate (Minamoto *et al.*, 1991) has been reported.

Unlike insulin and transferrin, BSA is usually included in the medium at relatively high concentration, 1-5 mg/ml. BSA may serve as a carrier for metals, lipids and hormones (Barnes and Sato 1980; Iscove *et al.*, 1980) and it has been shown to strongly inhibit copper-stimulated peroxidation and generation of *free* hydroxyl radicals from systems containing copper and H_2O_2, preventing toxicity by oxidation of unsaturated fatty acids in the membranes (Darfler and Insel, 1983; Halliwell, 1988). It may also provide the cells with protection from shear damage (see section 1.2.3.2). It is most likely that its stimulating properties are due to the effects of unidentified molecules bound to it. Because BSA is derived from a somewhat raw material, bovine plasma and serum, even the purified fraction will reflect some of this natural variability. It is thus a component that may be a major source of variability in cell culture media .

There are several advantages in removing proteins from the culture media and efforts towards the development of protein-free medium are being made. In fact, the lower the level of exogenous proteins in the crude product the easier the downstream processing process. Also, these proteins may represent a possible source of contamination. Complete replacement of those proteins is not however a very easy task and protein-free medium is not necessarily more cost effective, specially if it is supplemented with hormones and lipid precursors. Each production system will always have to be analysed individually, as there is a clear dependence on the cell line and culture method. Successful protein-free media for the production of

monoclonal antibodies by hybridomas have been reported by Kovar and Franek (1987), Schneider (1989), Darfler (1990) and Fike *et al.* (1991). The former ones are quite complex and use hormone supplements (hydrocortisone, oestradiol, progesterone) while the detailed formulation for the latter one is only available for FDA purposes, but the authors do not refer to any hormone supplementation. While Darfler (1990) mostly reported cell growth in static cultures, the others included suspension cultures in their investigations, which is an important consideration for the scaling-up of the process. Production of recombinant interleukin-2 by suspension cultures of BHK 21 cells in a rather simple protein-free medium has also been reported (Lucki-Lange and Wagner, 1991). Clonal growth of the parental CHO-K1 in protein-free medium has been long achieved by Hamilton and Ham (1977), but suspension cultures would most likely behave differently. Furthermore, the level of contaminants existent in the chemicals at that time would probably be a problem in reproducing the same conditions. Substantial work is still required in order to routinely cultivate recombinant cells in totally protein-free medium.

1.3.3- Toxicity in animal cell culture

Ammonia and lactate are the usually reported potential toxic compounds accumulated in animal cell culture. As stated in section 1.3.2.1, ammonia mainly derives from the metabolism of glutamine by glutaminase activity while lactic acid is a result of the catabolism of glucose by anaerobic glycolysis.

The sensitivity of animal cells to ammonia and lactate varies considerably between cell lines. Inhibition by those compounds can become one of the limitations for cell growth. In a batch culture ammonia production levels usually reach 2 mM, which may be inhibitory to many cells (Butler and Spier, 1984). Glacken *et al.* (1986) have reported growth inhibition of MDCK cells above 4 mM ammonia concentration. Lower inhibition levels, 2-2.5 mM, have been

found for some hybridoma cell lines (Reuveny *et al.*, 1986; Hassel *et al.*, 1991) while L-mouse cells have been found to be inhibited by concentrations as low as 0.5 mM (Ryan and Cardin, 1966). On the other hand, the accumulation of lactic acid in culture may exceed the buffering capacity of the medium, leading to a decrease in the culture pH and to suboptimal conditions for cell growth (Reuveny *et al.*, 1986) but usually no inhibition is found up to 25 mM lactate. Concentrations as high as 40 mM did not any effect on the growth of several hybridoma cell lines (Hassel *et al.*, 1991).

The sensitivity of CHO cells to lactate and ammonium was investigated by Schlaeger and Schumpp (1989), who reported inhibition constants (50% growth inhibition) of 8-10 mM ammonia and ca. 75 mM lactate. The work of Hayter *et al.* (1991a) with CHO cells gave support to the previously reported results, showing that cell growth is not inhibited by ammonium concentration of 2 mM although inhibitory effects were seen at 4.5 mM, and cells seemed to be relatively insensitive to lactate as concentrations of 17.5 mM were reported to have little effect on cell growth. Kurano *et al.* (1990b) also found that CHO cell growth was not affected by lactate itself and an inhibition constant of about 7.5 mM was found for ammonia.

An interactive effect between ammonia and lactate inhibition has been suggested by Hassel *et al.* (1991). They found that at lower concentrations of lactate, the apparent toxicity of ammonia decreased. Reuveny *et al.* (1986) also found that the addition of lactate to culture medium stimulated the growth of some hybridomas. Ammonia inhibition was also reported to be pH dependent (Doyle and Butler, 1990), e.g., as the culture pH was reduced from 7.8 to 6.8 the initial concentration of ammonium chloride causing 50% growth inhibition shifted from 4 mM to 7.6 mM.

Some strategies have been proposed to overcome the problem of ammonia and lactate

accumulation in culture medium, when they become the bottleneck of the process. The use of slowly utilised carbohydrates results in less lactate production (Eagle *et al.*, 1958; Imamura *et al.*, 1982). The glucose concentration in the medium is known to affect the lactate production: the rate of glucose utilisation is higher at higher glucose concentrations (Zielke, 1978; Glacken *et al.*, 1986; Miller *et al.*, 1989a; Hayter *et al.*, 1991a), increasing the flux of glucose carbon through glycolysis to lactic acid. The same rational is applied to glutamine utilisation and consequent ammonia formation (Glacken *et al.*, 1986; Miller *et al.*, 1988; Hayter *et al.*, 1991a). Controlled feeding of glucose and glutamine into the culture medium, in order to keep their concentration within acceptable limits has been shown to be successful in reducing lactate and ammonia production (Glacken *et al.*, 1986; Ljunggren and Häggström, 1990). The accumulation of metabolic end products can be also limited by perfusion culture (Glacken *et al.*, 1986; Glacken, 1988), but this may not be economically viable. Kurano *et al.* (1990b) cultured CHO cells in glutamine-free and asparagine supplemented medium, reducing ammonia production by 40%. More recently, *in-situ* removal of ammonia through electrical means has been reported (Chang *et al.*, 1993). It has also been reported that potassium ion can be used as an ammonia detoxifying agent in murine myeloma cell cultures, by inhibition of inward transport of ammonium ions (Martinelle and Häggström, 1993).

1.4- Production of glycoproteins

A great proportion of proteins produced by animal cell systems are proteins which have oligosaccharide structures covalently attached to the polypeptide bone, which are known as glycoproteins. These structures may have a marked effect on properties which are critical to the protein therapeutic value and are known to be affected by the culture environment (Goochee and Monica, 1990; Goochee *et al.*, 1991). The importance of analysing the product in this respect throughout any production system is now well recognised. There are two

different types of glycosylation, N-glycosylation and O-glycosylation, but here we will only refer to the former.

1.4.1- Significance of glycosylation

Glycosylation is one of the several post-translational modifications of human proteins. It may have a major role on the biological properties of a protein, often dictating its therapeutic value. Biological properties, such as plasma clearance rate, antigenicity, immunogenicity, specific activity, solubility and resistance to proteolytic attack, may all be affected by the oligosaccharide structures of a glycoprotein (Goochee *et al.*, 1991). The importance of glycosylation for biological function and stability has however to be evaluated for each protein individually. While N-glycosylation is essential for full biological activity in vivo of EPO (Delorme *et al.*, 1992) and dramatically increases the functional anticoagulant activity of human Protein C (Grinnell *et al.*, 1991), it has also been reported that an increased glycosylation of recombinant antithrombin causes a decrease in the affinity for heparin (Björk *et al.*, 1992) and decreases the specific activity of t-PA (Einarsson *et al.*, 1985).

A review on the assembly of N-linked oligosaccharides has been written by Kornfeld and Kornfeld (1985). A diagram with the glycosylation pathway is shown in Figure 1.2. The oligosaccharides are covalently attached to the polypeptide chain through an asparagine residue, at the consensus sequence Asn-X-Ser/Thr. Each step in the glycosylation pathway is catalysed enzymatically. Initially, a $Glc_3Man_9GlcNAc_2$ structure is synthesised on a polyisoprenoid derivative, the dolichol-P-P ($Glc_3Man_9GlcNAc_2$-P-P-dol) and transferred *en bloc* to the asparagine residues of the nascent polypeptide chain in the endoplasmic reticulum (ER). Several "trimming" reactions are then catalysed by exoglycosidases in the ER to yield high mannose N-linked structures. These high mannose structures are variably modified in the Golgi compartments by several exoglycosidases and glycosyltransferase reactions, leading to

the formation of "complex type" oligosaccharide structures, terminated by sialic acids. Not all the potential sites for glycosylation are used in a protein, leading to forms that differ in the number of oligosaccharide chains attached. Variation in the terminal sugars and chain branching may also occur and ultimately affect protein behaviour *in vivo* (Maiorella *et al.*, 1993).

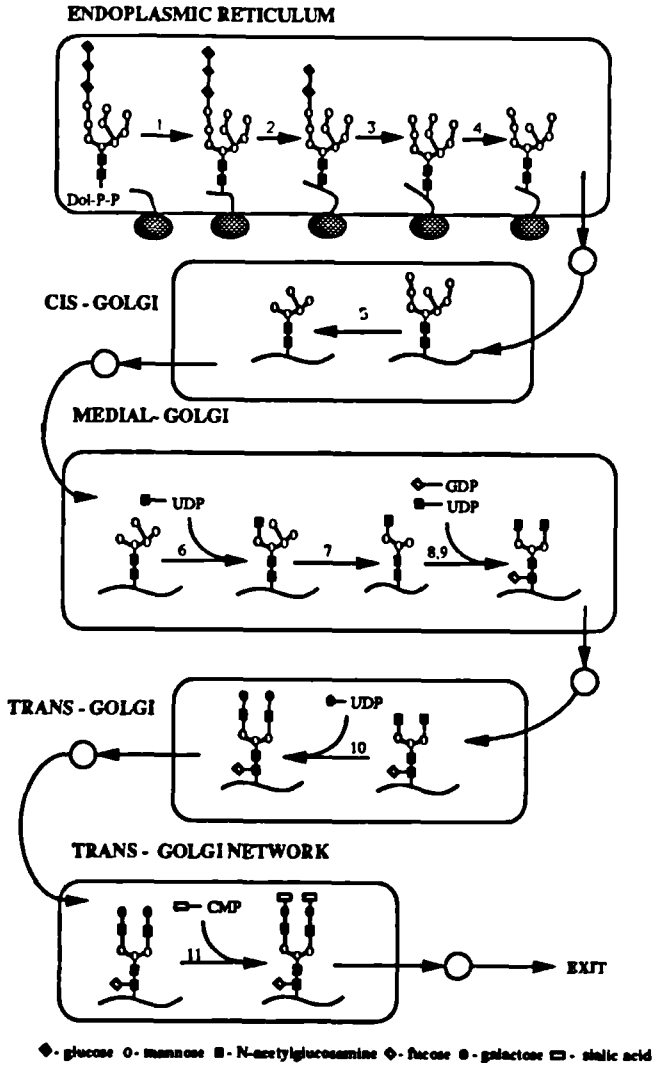


Figure 1.2- N-linked oligosaccharide processing in mammalian cells (derived from Goochee *et al.*, 1991). Enzymes: 1. oligosaccharyltransferase, 2. α-glucosidase I, 3. α-glucosidase II, 4. ER α(1,2) mannosidase, 5. Golgi α-mannosidase-I, 6. N-acetylglucosaminyltransferase I, 7. Golgi α-mannosidase-II, 8. N-acetylglucosaminyltransferase II, 9. α(1,6) fucosyltransferase, 10. β(1,4) galactosyltransferase, 11. α(2,3) sialyltransferase. Dol-P-P= dolichol-P-P; UDP= uridine diphosphate; GDP= guanosine diphosphate; CMP= cytidine monophosphate.

1.4.2- The host and its environment

How to control the variation of the oligosaccharide structures remains unclear, but several studies on the factors which may affect that variation have been carried out. It is now well recognised that the glycosylation profile of a given glycoprotein is host-dependent. Host-dependent oligosaccharide structures have been reported for IFN-ß (Utsumi *et al.*, 1989), t-PA (Parekh *et al.*, 1989b) and EPO (Goto *et al.*, 1988). The degree of similarity between oligosaccharide structures derived from CHO cells and human glycoproteins has been found to be quite high. This has been reported for several products, such as IFN-ß1 (Kagawa *et al.*, 1988), EPO (Takeuchi *et al.*, 1988) and t-PA (Spellman *et al.*, 1989). This fact seems to put CHO cells in a very favourable position when selecting a cell line for the synthesis of recombinant human proteins. It has to be pointed out that CHO cells have a limited capacity of γ- carboxylation and an inability for oligosaccharide sulphation (Goochee *et al.*, 1991).

Glycosylation patterns also vary with the extracellular environment of the cells and more attention now is being given to this subject. Some examples reported in the literature are shown in Table 1.5.

As the glycosylation profile of a protein may significantly affect its therapeutic value, glycosylation analysis throughout the development of production processes for such proteins becomes a major concern. The development of new strategies and instrumentation to allow a rapid definition of the glycosylation status is a major area of research (Parekh and Patel, 1992; James *et al.*, 1993).

Table 1.5- Factors affecting the glycosylation pattern of recombinant proteins.

	Cell line	Product	Reference
Age of culture	CHO	IFN-γ	Curling *et al.*, 1990
	Hybridomas	Mab	Robinson *et al.*, 1993
Culture method			
Ascites/serum-free/serum-supplemented	Hybridomas	IgG-1	Patel *et al.*, 1992
Ascites/serum-free AL/HF perfusion	Hybridomas	IgM	Maiorella *et al.*, 1993
pH	CHO	mPL-I	Borys *et al.*, 1993
Growth status	HepG2	Transferrin	Hahn and Goochee, 1992
	CHO	IFN-γ	Hayter *et al.*, 1993
Polypeptide folding	Cell-free	t-PA	Bulleid *et al.*, 1992
Medium components			
Serum content	murine carcinoma	EBM	Megaw and Johnson, 1979
Glucose	CHO	LL-O	Rearick *et al.*, 1981
	CHO	IFN-γ	Hayter *et al.*, 1992
Sodium butyrate		t-PA	Kopp *et al.*, 1993

AL= airlift; HF= Hollow fiber (1% serum).

1.4.3 - Interferon-γ

Human IFN-γ is a cytokine normally secreted by activated T-lymphocytes as part of an immune response to viral and mitogenic stimulation. The subject has been reviewed by Trinchieri and Perussia (1985). The molecule is composed of three major molecular weight variants with molecular masses of 17, 20 and 25 Kdaltons. Such heterogeneity is due to variability in the glycosylation state of the molecule, the 20 Kda form corresponding to glycosylation at Asn_{28} and the 25 Kda form to glycosylation both at Asn_{28} and Asn_{100} (Rinderknecht *et al.*, 1984). The development of recombinant DNA technology made possible to isolate the gene for IFN-γ and the first cloning was described by Gray *et al.* (1982) in *Escherichia coli* and monkey cells. This was then cloned and expressed in insect, yeast and

CHO cells (Hitzman *et al.*, 1983; Scahill *et al.*, 1983; Mutsaers *et al.*, 1986). The amino acid sequence predicted a mature polypeptide chain of 146 amino acids with a molecular mass of 17.1 Kda (Gray *et al.*, 1982). Figure 1.3 shows the sequence of the mature polypeptide. There are two major cleavage sites of the molecule, which seem to be at the C-terminal end of the 17.1 KDa mature polypeptide (Rinderknecht *et al.*, 1984; Pan *et al.*, 1987) and proteolysis seems to occur before secretion (Curling *et al.*, 1990). Also, the proteolytic cleavage increases significantly towards the end of a batch culture, as the percentage of non-viable cells increase, affecting especially the non-glycosylated IFN-γ form, which may be due to liberated intracellular proteases (Curling et *al.*, 1990).

MKTSYILAFQLCIVLGSLG ^{1}CYCQDPYVKEAENLKKYFNAGHSDVADNGTLFLGILKNWKEES
^{44}DRKIMQSQIVSFYFKLFKNFKDDQSIQKSVETIKEDMNVKFFNSNKKKRDDFEKLTNYSVTD
^{106}LNVQRKAIHELIQVMAELSPAAKTGK ↑ RKRSQMLFRGR ↑ RASQ

Figure 1.3- Human interferon-γ polypeptide sequence. The sequence in italics represents the initial peptide signal. **N-** glycosylation sites; ↑- cleavage sites.

The carbohydrate structure of CHO derived IFN-γ was reported to consist of biantennary N-linked oligosaccharides attached to either Asn_{28} or Asn_{100} (Mutsaers *et al.*, 1986) showing heterogeneity in the terminal sialic acids, like the human glycoprotein. The IFN-γ glycan structure is shown in Figure 1.4, as determined by Mutsaers and co-workers in 1986.

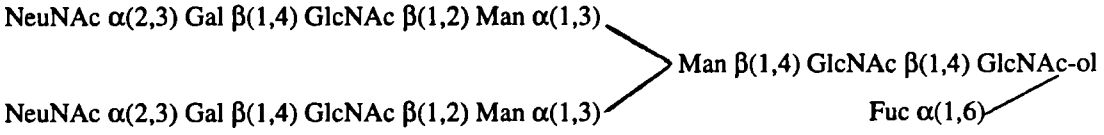


Figure 1.4- Biantennary N-glycans associated with IFN-γ (derived from Mutsaers *et al.*, 1986).

Curling *et al.* (1990) reported up to twelve molecular weight forms of IFN-γ secreted by CHO cells. This heterogeneity was due both to variability in glycosylation, leading to partially, fully

and non-glycosylated forms and to proteolytic processing of the glycosylated and non-glycosylated forms at two separate sites of the polypeptide. At least six variants of the fully glycosylated form were found, suggesting some variation in the oligosaccharide structure. More recent studies have shown that the glycan structures attached to IFN-γ are more complex than the ones presented earlier by Mutsaers *et al.* (1986) and there are also differences between the glycans attached to Asn_{28} and Asn_{100} (James *et al.*, 1993). In Figures 1.5a,b the oligosaccharide structures linked to Asn_{28} and Asn_{100} are represented. Not only biantennary but also more complex triantennary structures were found in the oligosaccharides at both Asn_{28} and Asn_{100}. Furthermore, truncated structures were found at both sites, ca. 16.5% for Asn_{28} and 10.5% for Asn_{100}. However, while ca. 87% of the structures linked to Asn_{28} were fucosylated, that fraction was only 3.5% for Asn_{100} structures. Thus, there is great heterogeneity between the oligosaccharide structures linked to the core polypeptide, which is designated as microheterogeneity.

Although core polypeptide processing of both natural and recombinant IFN-γ has been reported, whether this affects the biological properties of the product is a subject of debate. According to Hogrefe *et al.* (1989) the NH_2-terminal end is critical in maintaining the anti-viral activity but there are different opinions regarding the role of the carboxy-terminus for full biological activity *in vivo* (Seeling *et al.*, 1988; Sakaguchi *et al.*, 1988). A recent study (Ealick *et al.*, 1991) has shown that IFN-γ exists as a dimer, which was reported to be required intact for biological activity, and may include the COOH-terminal of one subunit and the NH_2-terminal of the other. The significance of glycosylation for the biological properties and pharmacokinetics of IFN-γ has not been firmly established. Recombinant IFN-γ obtained from bacteria cultures, which is not glycosylated, did not show any apparent change in activity (Perez *et al.*, 1990) but it has not been reported whether IFN-γ glycosylation affects other properties *in vivo*, such as the half-life of the product or the loss of a specific receptor-mediated activity (Bialy, 1987).

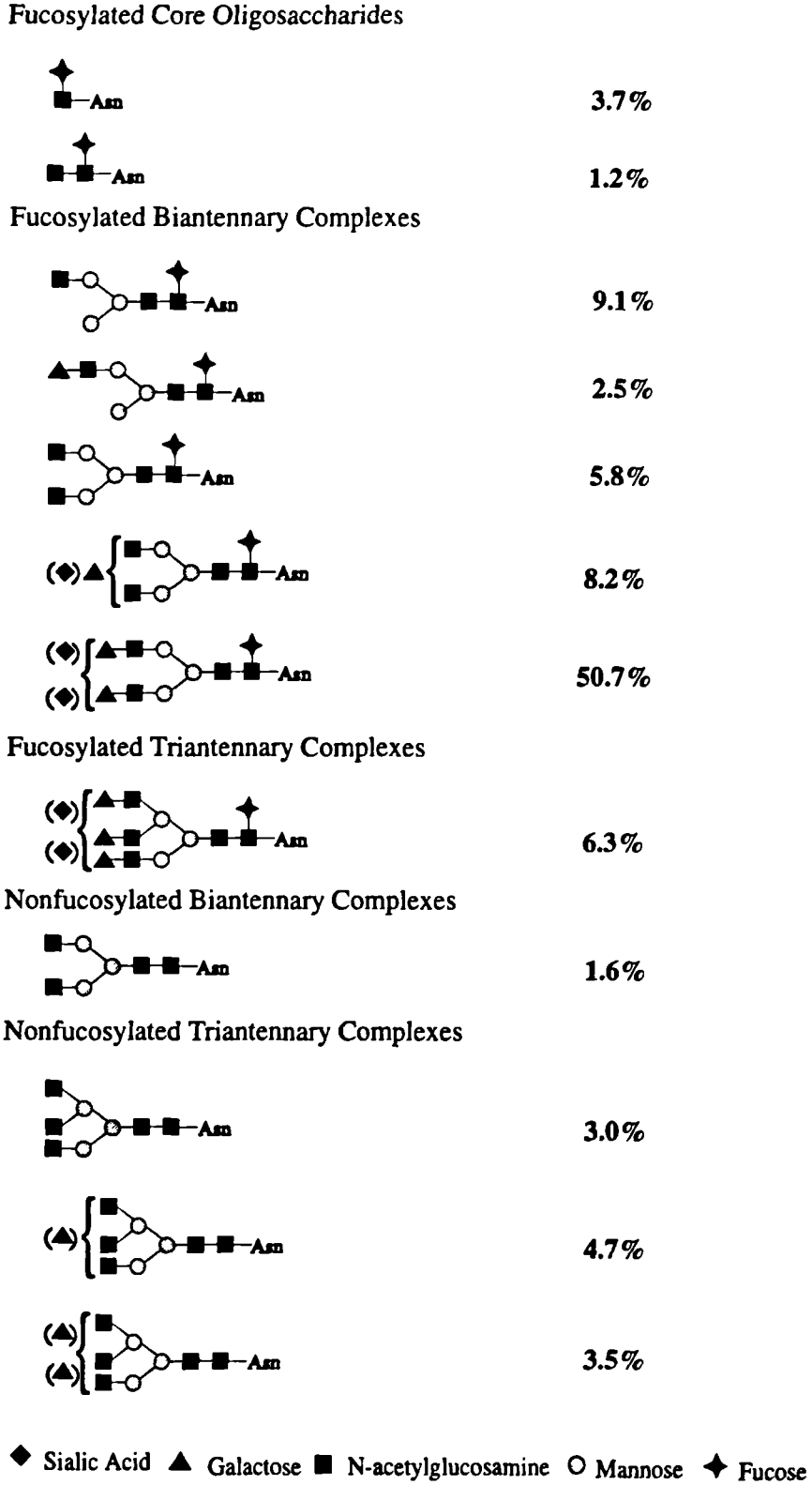
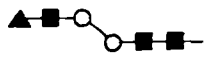


Figure 1.5- N-glycans associated with IFN-γ (derived from James *et al.*, 1993). a) Asn28 linked oligosaccharide structures.

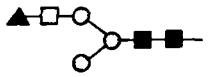
Nonfucosylated Core Oligosaccharides

2.6%

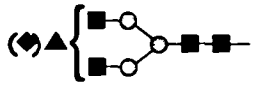
Nonfucosylated Biantennary Complexes



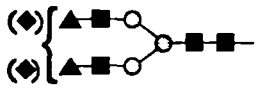
3.6%



4.3%

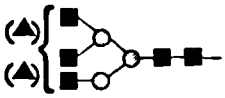


4.1%

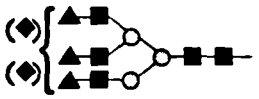


67.5%

Nonfucosylated Triantennary Complexes

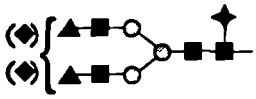


1.4%



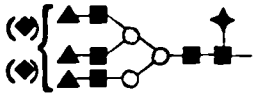
13.1%

Fucosylated Biantennary Complexes



3.1%

Fucosylated Triantennary Complexes



0.4%

◆ Sialic Acid ▲ Galactose ■ N-acetylglucosamine ○ Mannose ✦ Fucose

Residues in parentheses may be present or absent.

Figure 1.5- Contd. b) Asn_{100} linked oligosaccharide structures.

Product heterogeneity in relation to glycosylation is not necessarily a restraint to the product licensing process. However, in order to achieve batch to batch product consistency it is important to characterise and understand the reasons underlying such heterogeneity.

1.5- Scope of the work

The relatively low cell densities and product titres usually achieved with animal cell culture systems still strongly encourage studies on the cell culture environment. The optimisation of cell recombinant protein production by animal cell cultures is important for the economic feasibility of these processes. The design of the culture environment is clearly an aspect to look at very carefully throughout the development of any production system and its relevance can not be underestimated.

In early studies, experiments were developed in terms of nutrient requirements for cell growth (Eagle, 1955a,b, 1959) and many following studies still used this as the only criterion for developing cell culture and assigning levels of importance to specific components in the culture (Mather and Sato, 1979; Iscove *et al.*, 1980; Barnes and Sato, 1980; McHugh *et al.*, 1983; Gasser *et al.*, 1985; Kovar and Franek, 1985; Kovar, 1986). Recent reports focus both on cell growth and protein production (Luan *et al.*, 1987; Takazawa *et al.*, 1988; Duval *et al.*, 1991; Fike *et al.*, 1991; Lucki-Lange and Wagner, 1991; Minamoto *et al.*, 1991; Schmid *et al.*, 1991, 1992; Maiorella, 1992; Gaertner and Dhurjati, 1993; Lin *et al.*, 1993). Approaches that include the production component during the investigations will be of greater value. Optimisation schemes based on cell growth are not always the best rationale to take, as cell growth and production may not be enhanced by the same stimuli (Schmid *et al.*, 1991; Gaertner and Dhurjati, 1993). This may have prominent implications on the design of the culture and reinforces the importance of optimising the culture environment aiming to improve

the desired biological response.

Although an increase in product yield is most desirable, product authenticity is also a crucial aspect to consider as it is known to be affected by the culture environment and may *per se* affect the product therapeutic value (section 1.4). When producing proteins for therapeutic applications, the quality of the product throughout the investigations should be analysed and becomes an element that needs evaluation. Given the complexity of the process and the information required to evaluate such systems, the final choice for an appropriate culture system may not be straightforward and can not be based only on parameters such as product titre, fermentation cost and ease of downstream processing. The quality and consistency of the product from batch to batch will also have to be considered. The definition of the objectives of an optimisation scheme at an early stage of the investigation is thus of primary importance.

A Chinese hamster ovary (CHO) cell line producing human interferon-γ (IFN-γ) under serum-free culture, was used in this investigation. Manipulation of the culture design in order to increase the production of IFN-γ by CHO cells while investigating the effect of different culture environments on product authenticity were set as the main objectives. At the same time, the studies considered the trend of developing more defined cell culture media to allow better process reproducibility.

The importance of serum-free medium components on CHO cell growth and IFN-γ production with an experimental statistical design was first investigated (Chapter 3). Follow-up studies were based on that screening and include (1) the use of fermenter fed-batch cultures of glucose and glutamine with a view to extend CHO cell growth and IFN-γ production phases (Chapter 4) and (2) an investigation of the possibility of reducing the bovine serum albumin content of the culture, while exploring its major effects (Chapter 5). The glycosylation pattern of IFN-γ was simultaneously analysed.

2- MATERIALS AND METHODS

2.1- Cell culture system

2.1.1- Cell line

The cell line used throughout this investigation was a Chinese hamster ovary cell (CHO 320), kindly provided by Wellcome Biotech (Beckenham, Kent, UK). It was derived from a dihydrofolate reductase deficient ($DHFR^-$) mutant of CHO-K1, which has been co-transfected with the DHFR and the human IFN-γ genes. The IFN-γ gene was inserted using a plasmid vector derived from pSV2-dhfr, which is under the control of the SV40 early promoter. The IFN-γ gene was ampliflied by methrotrexate selection (McCormick *et al.*, 1984).

2.1.2- Culture medium

All the procedures involved in the final preparation of the culture medium and any other medium supplementation were carried out in a laminar flow hood model Gelaire BSB 4A (Flow Laboratories, High Wycombe, Buckinghamshire, UK), under sterile conditions. The sterile disposable cell culture material used was obtained from Fisons (Loughborough, Leicestershire, UK), Falcon (Becton-Dickinson, Oxford, UK) or Greinier (Dursley, Gloucestershire, UK), unless otherwise indicated. All the sterile filter units were obtained from Gelman Sciences (Ann Arbor, Michigan, USA). The media supplements were obtained from Sigma (Poole, Dorset, UK), unless otherwise indicated.

2.1.2.1- Serum-free medium

The culture medium was a serum-free medium based on Roswell Park Memorial Institute

(RPMI) 1640 (Imperial Laboratories, Andover, Hants, UK) and supplemented with proteins, bovine serum albumin (BSA), bovine insulin and human transferrin, glutamine, trace elements and methotrexate (MTX) developed by the Animal Cell Culture group at the University of Kent (Hayter *et al.*, 1989). The concentrations of the low molecular weight nutrients were based on those described by Hamilton and Ham (1977). The final concentrations of each component in the culture medium are presented in Table 2.1.

Table 2.1- Cell culture medium supplements and final concentrations in RPMI 1640.

Component	**Concentration**
Proteins (x20)	
Bovine serum albumin (Pentex Fraction V)*	5 g/l
Human transferrin	5 mg/l
Bovine insulin	5 mg/l
L-glutamine (x100)	2 mM
Sodium pyruvate (x100)	1 mM
L-alanine (x100)	0.1 mM
Putrescine (x100)	1 μM
Trace Elements (x100)	
$FeSO_4$	3 μM
$ZnSO_4$	3 μM
Na_2SeO_3	10 nM
$CuSO_4$	10 nM
Methotrexate (x1000)	0.1 μM

*Miles Biochemicals, Slough, Bucks, UK. Each group of supplements is added to the culture medium as a concentrated solution, as indicated in brackets.

The RPMI 1640 base medium was prepared using ultra pure water from a Milli-Q Plus system (Millipore, Watford, Hertfordshire, UK) and filtered through a 0.22 μm Micro Capsule filter unit and stored at 4°C. Unless otherwise indicated, all the supplements added to the culture

medium were prepared using the same Milli-Q water. The protein working stock (x20) was filtered using a Gelman Micro Capsule filter and stored at -20°C in 50 ml aliquots. The glutamine working stock (x100) was filtered using an Acrocap filter and stored at -20°C in 10 ml aliquots. The trace elements working stock (x100), prepared in combination with sodium pyruvate, L-alanine and putrescine (all x100), was filtered through an Acrocap filter and stored at 4°C in 10 ml aliquots. A methotrexate concentrated stock (x100000) was prepared in RPMI 1640, filtered through an Acrodisc and stored at -20°C in 100 µl aliquots; the working stock (x1000) was prepared by diluting asceptically the concentrated stock 100 times in RPMI 1640 and storing 1ml aliquots at -20°C. The final serum-free medium was prepared by warming up each working stock in a 37°C water bath and adding the appropriate volume to the required amount of RPMI, which was also pre-warmed at 37°C before being used. Frozen working stocks were only used at the time of thawing, and any excess was discarded.

2.1.2.2- Specific media supplementation

In each of the investigations carried out in the present study (Chapter 3, 4 and 5), different media formulations were used by adding specific medium supplements. For all the supplementation studies, the basal serum-free medium was first prepared as above, divided into appropriate portions, after which the specific nutrients were added. Thus, within each set of experiments the basal medium was the same, to minimise variability between experiments and attribute the effects to each specific supplementation rather than variability in the medium preparation.

A. Statistical studies on the components of the serum-free medium

In Chapter 3, the screening of several of the culture components with a statistical design of experiments is described. Increased concentrations of those components in the culture medium

was necessary and they were varied between two concentrations, i.e., a low and a high level. The concentration present in the serum-free medium was designated as the low level; the high level was taken as twice that concentration, except for arginine, glutamine and BSA, which were increased only by 50%. Each individual nutrient or group of nutrients was prepared as concentrated stock solutions, which were added to the basal medium in order to obtain the required high levels (Table 2.2). The actual concentration of each nutrient in the serum-free medium (low level), was taken into account to calculate the addition volume.

All the supplements were prepared using Milli-Q water. The BSA solution was filtered using an Acrodisc filter and stored at -20°C. The other solutions were filtered through an Acrodisc and stored at 4°C. After each specific supplementation, the pH of the culture medium was corrected with HCl 1M or NaOH 1M if necessary (colour indication by phenol red present in RPMI).

B. Fed-batch addition of glucose and glutamine to fermenter cultures

Fed-batch additions of glucose and glutamine to fermenter cultures were described in Chapter 4, where the details of the feeding regimes used are specified.

A concentrated stock solution of 50 mM glutamine and 14 mM glucose was prepared in Milli-Q water, filtered through an Acrocap filter and stored at -20°C. In the beginning of each fermenter run, a feeding bottle containing that solution, which was kept at 4°C throughout the run, was connected to the fermenter and additions were made accordingly to the feeding regime to use.

Table 2.2- Solutions prepared to supplement the serum-free culture medium for the statistical study [as specified in section 3.3].

	Low level *g/l*	**Concentrated stock**	**Volume per 100 ml** (ml)
BSA, Fraction V	5	x 40	1.25
Human Transferrin	0.005	x 500	0.1
Bovine Insulin	0.005	x 1000	0.2
	mM		
Sodium pyruvate	1	x 500	0.2
Putrescine	0.001	x 1000	0.1
Glucose	11	x 100	1
Alanine	0.10	x 200	0.5
Arginine	1.15	x 200	0.25
Asparagine	0.33	x 150	0.70
Aspartate	0.15	x 200	0.5
Cystine	0.021	x 200	0.5
Glutamine	2	x 100	0.5
Glutamate	0.14	x 200	0.5
Glycine	0.13	x 200	0.5
Serine	0.29	x 200	0.5
Methionine	0.10	x 200	0.5
Proline/Histidine/Hydroxyproline	0.17/0.097/0.15	x 200	0.5
Threonine/Valine/Isoleucine	0.13/0.17/0.38	x 200	0.5
Leucine/Tryptophan/Lysine	0.38/0.024/0.22	x 200	0.5
Phenylalanine/Tyrosine	0.09/0.013	x 200	0.5

note: all the supplements were the same as those used to supplement the basal RPMI, except for BSA which was obtained from Sigma in this study. The concentration of each supplement was increased by 100% in the culture medium, except for BSA, glutamine and arginine, which were increased by only 50%.

C. Low-BSA cultures

Serum-free medium containing low concentration of BSA (Chapter 5), was prepared using the same procedure as before (section 2.1.2.1), except that the protein stock solution was prepared with a lower level of BSA, but still as a x20 concentrated stock:

	Final concentration
Proteins (x20)	
Bovine serum albumin	1 g/l
Human transferrin	5 mg/l
Bovine insulin	5 mg/l

D. Lipids, Pluronic F68, fatty acid-free BSA and foetal calf serum supplementation

In Chapter 5 experiments are described in which the culture medium was supplemented with various concentrations and combinations of a lipid mixture, Pluronic F68, cholesterol, linoleic acid, oleic acid, choline and ethanolamine. In some cases fatty acid-free BSA (FAF-BSA) was also added individually to the culture medium, at a final concentration of 1 mg/ml.

A commercial water soluble lipid mixture, cholesterol-rich, was obtained from Sigma and used throughout these studies. This lipid emulsion is provided in sterile conditions and it was kept at 4°C.

Free fatty acids, oleic and linoleic acids, and cholesterol (all from Sigma) were prepared as fatty acid-free BSA (Miles Pentex, Fraction V) complexes at a ratio of 1:100 w/w. A concentrated stock (0.5 mg/ml) was prepared by dissolving them in absolute ethanol at a concentration of 20 mg/ml and, after filtering through Acrodisc filters, adding dropwise and under sterile conditions 25 µl aliquots per 1 ml of a FAF-BSA solution (50 mg/ml in PBS), which was also previously filtered through an Acrocap. The complexes were shaken for 1 h at

20°C protected from light and stored at 4°C.

Choline chloride and ethanolamine were obtained from Sigma and prepared in Milli-Q water as 100 x concentrated solutions. They were filtered through Acrocap filters and stored at 4°C.

Pluronic F68 was obtained from Sigma as a sterile 10% solution and kept at room temperature.

For individual supplementations, FAF-BSA (Miles Pentex Fraction V) was prepared as a 100x concentrated solution (100 mg/ml) in Milli-Q water, filtered through an Acrocap and stored at -20°C.

Foetal bovine serum was obtained from Gibco, Life Technologies (Uxbridge, Middlesex, UK) and used at a concentration of 8% (w/w). In those particular experiments, trace elements and proteins were excluded from the culture medium.

2.1.3- Cell culture methods

All the cell culture work was carried out in a laminar flow hood (Flow Laboratories) and the cell culture sterile disposable material was obtained from Fisons, Falcon or Greinier, unless otherwise indicated. When sterile glass or other material, e.g., sterile connectors, material was required, including the fermenters, they were sterilised in a Rodwell autoclave model Ensign (Hornchurch, Essex, England) for 45 minutes at 120°C.

2.1.3.1- Cell maintenance

Cell working stocks for the present investigations were prepared from a master stock existent at the University of Kent. Cells were allowed to grow for 5-6 passages, after which they were

harvested in the mid-exponential phase of growth. The cell culture medium was centrifuged at 1000 rpm in a MSE bench top centrifuge Model Chilspin 2 (Fisons) and the cell pellet resuspended in fresh RPMI containing 2.5% BSA (Miles Pentex Fraction V) and 10% DMSO (Sigma) at a density of 2-5 x 10^6 cells/ml, for cryopreservation. This freezing mixture containing the cells was then divided into 1.8 ml cryopreservation tubes (Nunc, Roskilde, Denmark) in 1 ml aliquots, placed in a polystyrene box and stored at -70°C for 2 h, after which they were tranferred to liquid nitrogen storage until required.

Cell revival was done by rapidly thawing one vial of frozen cells and placing the tube in a 37°C water bath until defrosted. The cells were then washed with culture medium to remove traces of DMSO, resuspended in 15 ml of fresh medium, put in a sealed 25 ml Erlenmeyer sterile culture flask and gassed with 5% CO_2 in air before incubation at 37°C in an orbital shaking incubator Model Infors HT (Rittergasse, Bottminger) at 100 rpm.

Scaling-up of the culture to obtain the required cell density to set up each experiment was done by subculturing the cells every 2-3 days in fresh culture medium, and going successively from the 25 ml culture flask to a 50 ml culture flask, then to a 250 ml spinner flask (Techne, Cambridge, UK) and finally to a 500 ml spinner flask (Techne). Spinner flasks were maintained at 37°C in a CO_2 incubator Model 1500 (Flow Laboratories) with a 5% CO_2 atmosphere and keeping the caps loose. The stirrer speed was 100 rpm.

2.1.3.2- Shake-flask and fermenter cultures

Initial cell concentration for both shake-flask experiments and fermenter cultures were in the range of 1.5-2 x 10^5 cells/ml, and the cell inocula were harvested at the mid-exponential phase of 500 ml spinner cultures. Cells with the same culture history (same number of passages and subculturing procedure) were used for all the experiments, and as such a new cell

working stock was revived for each set of culture conditions.

A. Shake-flask cultures

Shake-flask culture studies were made in sealed 250 ml Erlenmeyer culture flasks. The cell inoculum for each experiment was prepared by centrifuging at 1000 rpm the desired volume of culture medium, after which the cell pellets were ressuspended and pooled to a common bottle. Each individual flask was then seeded with equal inoculum volume. Following the same procedure as the cell culture maintenance, the headspace of each flask was purged with 5% CO_2 in air to maintain the pH at 7.2 - 7.4 and they were then incubated at 37°C in an orbital shaking incubator at 100 rpm. The flasks were sampled every day (or as required) and a cell count was carried out. The remaining sample was centrifuged at high speed in a MSE microcentrifuge Model Microcentaur (Fisons) and the supernatant was aliquoted into appropriate volumes and stored at -70°C until required for analysis. If required, cell pellets were also stored at -70°C.

B. Fermenter cultures

Fermenter culture studies were made in 2 l fermenters (LH Fermentation, Reading, UK) with approximately 1.7 l working volume, connected to Anglicon controllers (Brighton Systems Limited, Newhaven, East Sussex, UK) for temperature, pH and oxygen control. The air, CO_2 and N_2 flow rate into the fermenter were controlled by flow control valves built into the controller unit. The exit and inlet gas lines of the fermenters were filtered by means of 0.22 μm Microcap filter cartridges (Gelman Sciences) and the exit line had a cooled condenser before the filter unit. The fermenters were provided with flat turbine impellers and the stirrer speed was set at 60 rpm. All the fermenter lines were mount with silicone tubing. Sampling, inoculation, air input and output and nutrient feeding for fed-batch option, were carried out by

means of ports on the top plate. Temperature, pH and dissolved oxygen tension probes were placed in the top of the plate. Figure 2.1 shows a diagram of the fermentation and control process.

Temperature was measured by a resistance thermometer and heat was supplied by a heating resistance jacket. The controller was tuned to give no more than 0.1°C overshoot.

The pH was measured by a steam sterilizable pH electrode (Ingold Messtechnik AG, Industrie Nord, Urdorf, Switzerland) and the culture maintained at 7.2 under the action of the pH controller channel, by sparging CO_2 whenever the medium pH became alkaline (early stages of the culture). The production of lactate and CO_2 in later growth phases decreases the pH of the culture, but the increased oxygen demand at higher cell densities leads to increased sparging of air, which displaces CO_2 and thus reduced the need for base addition.

Dissolved oxygen was measured by a steam sterilizable polarographic dissolved oxygen electrode (Ingold) and the oxygen controller was tuned to keep the culture at 40% air saturation by sparging air. A slow flow of air through the fermenter headspace prevented CO_2 build up. A slow bleed of nitrogen into the sparge line was also kept to prevent siphoning of medium when there was little addition of air or CO_2.

Sterilisation of the fermenter was carried out with all the probes and lines in place, and with the fermenter filled with 1.7 l PBS solution. Unlike the oxygen probe, the pH probe was calibrated prior to sterilisation.

The cell inoculum was prepared at the desired concentration and working volume in 2 l bottles and the contents were siphoned into the fermenter by means of sterile connections, after emptying the solution used to fill the fermenter during its sterilisation by the same procedure.

Sampling was done by a sample port at the top plate of the vessel and the sample was treated by the same procedure as described for the shake-flask cultures.

For fermenter fed-batch cultures, the feeding solution was supplied from a 500 ml reservoir maintained at 4°C. The flow rate was controlled using a peristaltic pump (Watson Marlow, Falmouth, Cornwall, UK), with periodic checks on the flow rate using a graduated reservoir in line. The additions were made every four hours, with a duration of 2 to 4 minutes, depending on the volume required and on the pump flow rate; a timer was used to turn on the pump at the appropriate time. The volumes added to the fermenter were small (ca. 15 ml *per* day) to avoid dilution of the culture. Sampling was done as described for the batch cultures.

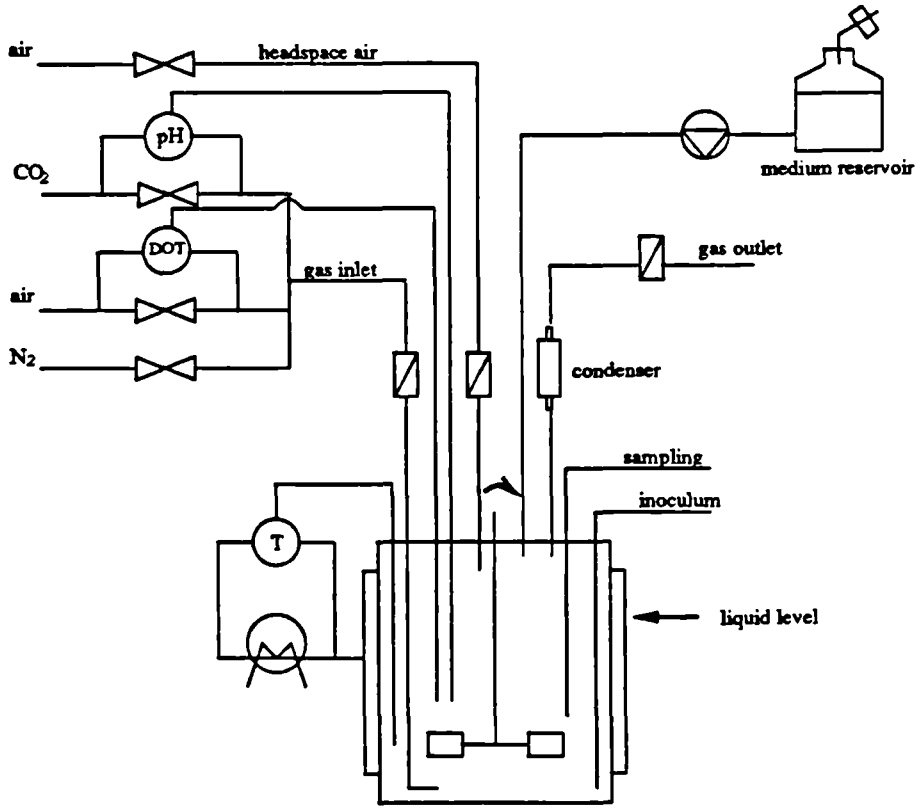


Figure 2.1- Process diagram for fermenter cultures under batch and fed-batch operation.

2.1.3.3- Plackett-Burman experimental set-up

A statistical experimental design, the Plackett-Burman method, was used in Chapter 3 and Chapter 5. Those experiments were carried out according to a design matrix, selected considering the number of variables to study (Tables 3.1 and 5.4). The matrix employed in Chapter 3 [Table 3.1] required 24 different culture conditions, corresponding to n=48 flasks, as each culture was set in duplicate. The matrix was organised in three sets of experiments, each of them including a control culture (flask 24 in the matrix), as follows: two sets of 9 cultures (18 flasks) and one set of 8 cultures (16 flasks). In each set, the growth and IFN-γ production data obtained for the duplicate control cultures were used to assess variations ocurring between the different experimental sets. Cell yield was adjusted for one data set, where the variation in growth was unusually high (ca.13%). The matrix used in Chapter 5 required a much smaller number of flasks (16) and so all the cultures were set up together. The components (variables) of each matrix were added to the corresponding basal medium according to its specified level in the matrix, after which the flasks were inoculated with aliquots of a common cell suspension.

2.2- Analytical Methods

2.2.1- Determination of CHO cell growth

In an Eppendorf tube, 100 µl of cell culture medium were mixed with 100 µl of trypan blue (Sigma) and cell counts were made in a Neubauer counting chamber, using a microscope Model ID 02 (Zeiss, West Germany). Cell viability was determined by dye exclusion as dead cells stain blue with this method.

2.2.2- IFN-γ analysis

2.2.2.1- ELISA assay for IFN-γ titre

IFN-γ titres were determined by a monoclonal antibody-based sandwich enzyme-linked immunosorbent assay (ELISA). All the buffers used in the assay are shown in Table 2.3.

Table 2.3- Composition of buffers used for the IFN-γ ELISA assay.

	Final concentration
Coating Buffer	
PBS tablets*	1/100 ml
20B8	10 µg/ml
Note: Adjusted to pH 8.0 using 5 M NaHO.	
Wash Buffer	
PBS tablets	1/100 ml
Casein§	0.05%
Tween 20§	0.1%
Note: warmed on hotplate to dissolve.	
Block Buffer	
PBS tablets	1/100 ml
Casein	0.5%
Tween 20	0.1%
Note: warmed on hotplate to dissolve.	
Substrate Buffer	
Glycine§	0.5 M
$ZnCl_2$¶	0.1 M
$MgCl_2$¶	0.1 M
Note: Adjusted to pH 10.4 using 5 M NaHO. Immediately prior to use, one phosphatase substrate tablet (Sigma 104) was added per 5 ml of substrate buffer.	

all the buffers were prepared with Milli-Q water. * Unipath Ltd, Basingstoke, Hampshire, UK; § Sigma; ¶ Fisons.

The assay was carried out in 96-well plates (Greinier), following the steps described in Table 2.4. The 20B8 antibody used to coat the assay plates was raised against recombinant IFN-γ from *Escherichia coli* (Celltech, Slough, UK), and a concentrated stock was kept at 4°C. The biotinylated antibody 20G7 was raised against *Escherichia coli*-derived IFN-γ (Celltech), recognising a different epitope on the IFN-γ molecule, and was prepared in wash buffer from a concentrated stock kept at 4°C. A concentrated stock of IFN-γ standards (Wellcome, Beckenham, UK) was kept at 4°C and 1/4 serial dilutions in wash-buffer were made to obtain a standard concentration range of 1200 to 160 IU. The samples were thawed and appropriate dilutions were made in wash buffer. For the determination of the intracellular IFN-γ, cell pellets were thawed and resuspended in 400 µl of wash buffer, after which the pellets were sedimented again by centrifuging at 7500 rpm for 5 minutes in a microcentrifuge. Appropriate dilutions were carried out using the supernatant obtained. Samples and standards were applied to the plate in triplicate. Extravidin-phosphatase was obtained from Sigma and prepared in wash buffer. The substrate reaction was carried out in the dark.

Table 2.4- Procedure for the ELISA assay for IFN-γ.

	Concentration	Time/temperature	µl/well
1. Coating- 20B8	10 µg/ml	Overnight- 4°C	50 µl
Wash x 2			
2. Blocking- block buffer		2 h RT	100 µl
Wash x 2			
3. Samples/ Standards	1200-160 (IU/ml)	1 h RT	50 µl
Wash x 3			
4. Second antibody- 20G7-biotin	5 µg/ml	1 h RT	50 µl
Wash x 3			
5. Extravidin-phosphatase	1/2000	1 h RT	50 µl
Wash x 4			
6. Substrate buffer		15 m RT	100 µl
7. Stop reaction- NaOH	5 M		20 µl

RT- room temperature.

Once the assay was completed, the plates were read at 410 nm in a Dynatech MR5000 plate reader (Dynatech, Billingshurst, West Sussex, UK). A calibration curve was constructed with the absorbance values obtained for the standards and the concentration of each sample was determined by interpolation.

2.2.2.2- Analysis of IFN-γ glycosylation

The IFN-γ glycosylation patterns were determined by three sequential steps: immunoprecipitation of the samples, SDS-page electrophoresis and silver staining.

2.2.2.2.1- Immunoprecipitation of IFN-γ

For each determination a 5 ml sample was immunoprecipitated using the 20B8 antibody raised against IFN-γ (Celltech). The buffers used for this assay are shown in Table 2.5.

Table 2.5- Buffers for immunoprecipitation of IFN-γ.

	Final concentration
Stock buffer	
20x MES*	1 M
Wash buffer 1	
MES	50 mM
Note: adjusted to pH 6.5 with 5 M NaOH	
Wash buffer 2	
Tris-HCl§	50 mM (pH 8.2)
EDTA§	5 mM
Nonidet P40¶	0.5%
Sample buffer	
Tris-HCl	62.5 mM
SDS§	2.5%
Bromophenol blue*	0.1%
Sucrose§	10%

all buffers prepared with Milli-Q water. * Sigma; § Fisons; ¶ BDH, Poole, UK.

In a 15 ml centrifuge tube, 0.25 ml of a 1 M MES solution and 10 µl of the 20B8 packed volume Reselute (Celltech) were added to 5 ml of thawed sample. The samples were placed in a rotating wheel and left at 4°C overnight, after which they were centrifuged at 3000 rpm to sediment the beads and the supernatant was aspirated. Wash buffer 1 (750 µl) was added to each tube and the beads gently resuspended. The suspensions were then transferred to Eppendorf tubes, which were centrifuged for 3 minutes at low speed (7500 rpm) in a microcentrifuge and the supernatant was aspirated. The beads were further washed twice with wash buffer 1 and twice with wash buffer 2, using the same procedure. After the final washing step, the beads were resuspended in 15 µl of sample buffer and boiled for 3 minutes to dissociate IFN-γ from the beads. Samples were then centrifuged at 7500 rpm for 5 minutes. The supernatant was loaded into pre-cast wells of a 14% polyacrylamide SDS Tris-glycine gel (Novex, San Diego, CA, USA), with 1.0 mm x 10 well and 1.0 mm thickness x 7.5 cm height x 8 cm width.

2.2.2.2.2- SDS-page electrophoresis

The 14% polyacrylamide SDS-PAGE gels were assembled into a Model XCell gel apparatus (Novex) and the electrode reservoirs were filled with glycine electrode buffer (Bio-Rad, Richmond, CA, USA), which consisted of a solution 0.025 M Tris-HCl, 0.192 M glycine and 0.1% SDS. Supernatant (15-20 µl) was loaded into the wells of the gel with an Hamilton syringe. A Bio-Rad SDS-PAGE protein standard, with molecular weight ranging from 14.5 to 97.4 kD was also applied to one well of the gel. The gels were run for approximately 2 hours at 125 volts.

2.2.2.2.3- Silver staining

The silver staining of the gels was done using the Silver Stain Plus kit obtained from Bio-Rad and unless otherwise indicated, all the reagents were provided by that supplier. Three steps

were involved in the procedure: fixing, washing and staining. All the necessary solutions were prepared as indicated in Table 2.6, according to the Kit indications, and Milli-Q water was used throughout all the assay.

Table 2.6- Reagents required for the Bio-Rad Silver Staining procedure.

	Volume (ml)
Fixative enhancer solution	
Methanol*	100
Acetic acid§	20
Milli-Q water	70
Fixative enhancer concentrate	10
Staining solution	
Milli-Q water	35
Siver complex solution	5
Reduction moderator solution	5
Image development reagent	5
Development accelerator reagent	50

Note: This solution was prepared while stirring rapidly the contents with a Teflon coated stirring bar and prepared before use.

* Aldrich; § Fisons. The components of each solution were added in the specified order. The amounts shown are sufficient for 2 gels.

Once the gel electrophoresis was completed, the gels were first placed in clean boxes in the fixative enhancer solution and provided with gentle agitation. The staining procedure was followed as described in Table 2.7.

Table 2.7- Silver staining procedure for mini-gels.

Step	Time
Fixative	30 m
Milli-Q water wash	3 x 30 m
Staining	10-20 m
Stop	10 m

After stopping the reaction, each band on the silver-stained gels was quantified by conversion into absorbance units with a Bio-Rad Model 1050 scanning densitometer connected to an Amstrad 1640 microcomputer. From the scans obtained, the area under each peak was integrated to allow the relative proportions of each IFN-γ variant to be estimated. A photograph of the gel bands corresponding to IFN-γ obtained with this method (lane 3), together with molecular weight markers (lane 1), is shown in Figure 2.2. Three major variants of IFN-γ were detected on the gel, also showing the microheterogeneity existent within each variant: 1) at the lower molecular weight range a cluster of non-glycosylated forms, 0N (ca. 15-17 kDa), 2) an intermediate cluster of bands corresponding to IFN-γ glycosylated at one site only, 1N (19-22 kDa) and 3) a top cluster of bands corresponding to IFN-γ glycosylated at the two potential sites, 2N (23-27 kDa). Lane 2 shows that the medium components do not interfere with this assay. The IFN-γ patterns shown in Figure 2.2 are consistent with those earlier reported (Curling *et al.*, 1990).

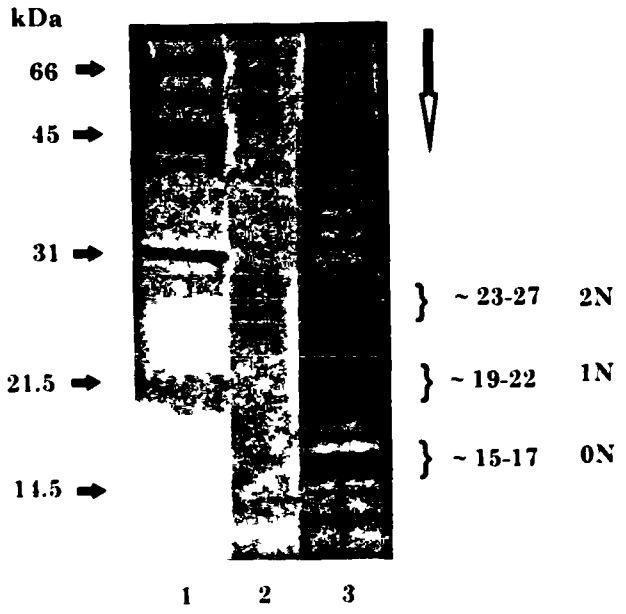


Figure 2.2- Analysis of IFN-γ variants produced by CHO cells. Immunoprecipitated samples were resolved by SDS/PAGE on a 14% polyacrylamide gel and detected by silver stain, as described in the text. The white arrow indicates the direction of migration. Lane 1- MW markers; lane 2- serum-free medium; 3- sample of a batch culture at ca. 100 h.

2.2.3- Metabolite analysis

2.2.3.1- Enzymatic determination of glucose

Glucose was determined using the o-dianisidine method (Sigma assay no. 510), which is based on the following coupled enzymatic reactions:

1) Glucose + H_2O_2 + O_2 ----------> Gluconic acid + 2 H_2O_2

2) H_2O_2 + o-dianisidine ----------> Oxidised o-dianisidine

(colourless) (brown)

All the reagents required for the assay were provided by the kit, unless otherwise indicated.

A. Reagents, samples and standards preparation

The following solutions were prepared for the assay:

1. 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 shaking. The solution was protected from the light and stored at 4°C (stable for 1 month).

2. Colour reagent solution- one vial of o-dianisidine dihydrochloride was reconstituted with 20 ml of Milli-Q water. The solution was kept at 4°C (stable for 3 months).

3. Combined enzyme-colour reagent solution- 100 ml of enzyme solution were mixed with 1.6 ml of 0-dianisidine dihydrochloride solution. The solution was kept at 4°C (stable for 1 month).

A glucose standard of 12 mM was prepared with D-glucose (Sigma) in Milli-Q water and 4 doubling dilutions were carried out to obtain 6, 3, 1.5 and 0.75 standards. Both standards and samples were diluted 1:10 with Milli-Q water.

B. Assay procedure

The assay was carried out in 1ml disposable cuvettes, to which 100 µl of each sample, standard and a blank (Milli-Q water), were first added. 1 ml of combined enzyme-colour reagent solution was added on the top of that and the contents were mixed well. The cuvettes were then incubated at room temperature for 45 minutes, after which the absorbance was read at 450 nm in a UV/VIS spectrometer Model UNICAM 8625 (Unicam Ltd, Cambridge, UK). The concentration of each sample was determined by construction of a standard curve.

2.2.3.2- Enzymatic determination of lactate

Lactate was determined by an enzymatic assay from Sigma (assay no.735). The assay relies on the action of lactate oxidase on lactate, which produces pyruvate and hydrogen peroxide. The hydrogen peroxide catalyses the oxidative condensation of chromogen precursors to produce a coloured dye with a maximum absorbance at 540 nm.

All the reagents required for the assay were provided by the kit, unless otherwise indicated.

A. Reagents, samples and standards preparation

The lactate reagent was prepared by adding 10 ml of Milli-Q water to each vial of lactate reagent, and the contents mixed with gentle inversion.

A lactate standard was obtained by preparing a 16 mM solution of L-lactic acid (Sigma), aliquoted into 1 ml vials and stored at -20°C. Serial doubling dilutions of that standard were carried out to obtain a standard concentration range of 16 to 1 mM. Any sample thought to be over 14 mM was diluted 1:2 in Milli-Q water.

B. Assay procedure

The assay was carried out in 1ml disposable cuvettes, to which 10 µl of either sample, standard or blank (Milli-Q water), were added. 1 ml of the Lactate reagent was added on the top of that and the contents mixed well. Cuvettes were incubated 5 to 10 minutes at room temperature, after which the absorbance was read in a spectrometer at 540 nm. The concentration of each sample was determined by construction of a standard curve.

2.2.3.3- 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.

A. Reagents, standards and sample preparation

The following reagents were prepared for this assay:

1. Sodium phenate

Phenol (Fisons)	2.5 g
5 M NaHO	6.24 ml
Milli-Q water	q.b. 100 ml

Note: stable for one month at 4°C.

2. Sodium nitroprusside stock

Sodium nitroprusside (Fisons)	0.3 g
Milli-Q water	30 ml

Note: aliquoted into 1ml vials and stored at - 20°C wrapped in silver foil.

3. Sodium nitroprusside working solution

Sodium nitroprusside stock	1 ml
Milli-Q water	100 ml

Note: prepared immediately prior to use.

4. Sodium hypochlorite

Sodium hypochlorite solution (Fisons)	0.71 ml
Milli-Q water	100 ml

Note: prepared immediately prior to use.

An ammonia standard was obtained by preparing a 5 mM ammonium chloride (Fisons) solution. Serial doubling dilutions were carried out in Milli-Q water to give a 2.5 to 0.325 standard concentration range.

B. Assay procedure

50 µl of sample, standard and blank were added to 3 ml disposable cuvettes. Then the 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 of the cuvettes were mixed well and incubated at room temperature for 30 minutes. The absorbance was read at 630 nm in a spectrometer. The concentration of each sample was determined by construction of a standard curve.

2.2.3.4- HPLC determination of amino acids

Amino acid concentrations were determined by reverse phase HPLC, using a reverse phase Spherisorb C18 column (Hichrom, Reading, Berks, UK), of orthophthalaldehyde (OPA) derivatised amino acids. In the presence of mercaptoethanol, OPA reacts with primary amino acids to form fluorescent derivatives. The following amino acids were detected by the assay: aspartate, glutamate, serine, glutamine, histidine, glycine, threonine, arginine, alanine,

tyrosine, methionine, valine, phenylalanine, isoleucine, leucine and lysine. Imines, such as proline and hydroxyproline, are not derivatised unless they are modified by pre-treatment with hyphochlorite. Cysteine and cystine are not derivatised using this method. A procedure similar to the one described by Seaver *et al.* (1984) was followed. The HPLC system consisted of a Model SIL 9A auto-sampler (Shimadzu, Hetton, UK), a Model Series 4 Liquid Chromatography solvent delivery system (Perkin-Elmer, Norwalk, CT, USA), a luminescence spectrometer (Perkin-Elmer) and a laboratory computing integrator Model LCI-100 (Perkin-Elmer). The column was calibrated using a standard amino acid mixture (Sigma AA-S-18) with the addition of glutamine and asparagine.

2.2.3.4.1- Sample and buffers preparation

The buffers required for this assay were prepared as described in Table 2.8.

Table 2.8- Buffers for HPLC determination of derivatised amino acids.

	Concentration
Buffer A- Phosphate Buffer	
di-Sodium hydrogen phosphate (12 H_2O)*	0.05 M
Sodium di-hydrogen phosphate*	0.05 M
Sodium acetate*	0.05 M
Methanol§	2%
Tetrahydrofuran§	2%
Buffer B- 70% Methanol	
Methanol	70%
Buffer C- 100% Methanol	100%
Buffer D- 100% Milli-Q Water	

* Fisons; § Aldrich. Buffers A and B were prepared using Milli-Q water.

Samples and standard (serum-free medium frozen at -70°C in 500 µl aliquots) were thawed and 400 µl aliquots were centrifuged through 10-KDa exclusion limit ultrafiltration membranes (Millipore, Harrow, Middlesex, UK) for approximately 15 minutes at high speed in a microcentrifuge. The filtrate was then diluted 1:25 with Milli-Q water and 200 µl of each dilution was placed in the auto-sampler vials and cap with septum in place.

2.2.3.4.2- Auto-sampler preparation

The vials containing the 200 µl aliquots of sample were loaded into the auto-sampler tray. The sampler was programmed in order to add 200 µl of fluoraldehyde (Pierce Chemical Co., Rockford, IL, USA) to each sample vial and then to inject 20 µl of each vial into the column. The line was purged twice with buffer A prior to the beginning of the run and between each sample injection.

2.2.3.4.3- HPLC assay

One litre of each buffer was placed in the respective buffers reservoirs, the system was degassed for 5 minutes and then purged with buffers C, D and A, in that order. The column was washed with buffer B for 15 minutes before starting the equilibration section of the chromatography program (Table 2.9). The fluorescent detector auto zero was set when a stable reading was achieved when running buffer A through the column. Sampler and integrator were programmed for the number of samples to be run.

The amino acid profile was obtained by the 40 minutes gradient shown in Table 2.9. The fluorescent OPA-amino acid derivatives were detected using a 334 nm excitation filter and 425 emission filter. The data was collected and analysed by the sample integrator. Using this method, there is a linear relationship between each chromatogram peak area and amino acid

concentration over the typical range of amino acids in the cell culture medium. The concentration of each amino acid was thus determined by dividing the area of the sample peak by that of the standard and multiplying by the concentration of the standard.

Table 2.9- Chromatography program for OPA amino acid assay.

Sector	Time (min)	Flow (ml/min)	Buffer A %	Buffer B %
Equilibrium	5	1.5	100	0
1	2	1.5	85	15
2	15	1.5	60	40
3	20	1.5	0	100
4	3	1.5	0	100

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 left.

2.3- Experimental data analysis

The culture performance throughout this study was expressed in terms of the following parameters: (1) CHO cell growth (specific cell growth rate, viable cell density and cell viability), (2) IFN-γ production (specific production rate and titre) and (3) IFN-γ glycosylation.

Most of the time, cell growth and IFN-γ production were not influenced by the same factors, thus enhanced cell growth was not always an indication of improvements in IFN-γ titre. As one of the major aims was set to be the improvement in the titre of IFN-γ obtained in culture, the results are presented in terms of volumetric productivity (IU/ml) rather than in terms of cell productivity (IU/10^6cells). In some instances, cell productivity is also presented as a parallel expression of distinct cell performance.

All the cultures described in each set of experiments were simultaneously set in duplicate, unless otherwise indicated. The experimental data presented in each Chapter represent mean values obtained for each culture condition, for which the associated standard error was calculated.

Cell growth and IFN-γ production parameters were calculated or expressed as follows:

1. Specific cell growth rate- μ

$$\mu = \frac{1}{x}\frac{dx}{dt}$$

x- viable cell concentration

The slope of the linear part of the curve $ln\ x = f(t)$ was taken as μ (usually up to ca. 60-75 h). Approximately four data points were used for each determination.

2. Specific metabolic rates- q_m

q_m- specific consumption/production of a particular metabolite (e.g., IFN-γ, glucose)

$$q_m = \frac{\Delta m}{x_{ave} \cdot t}$$

Δm- net consumption/production of a particular metabolite at time t

x_{ave}- average viable cell density from t_0 to t

For each set of experiments the time point t was chosen from the end of the exponential growth phase of the cells, varying between ca. 60 to 75 h from set to set.

3.Yield quotients

$$Y_{a/b} = \frac{\Delta a}{\Delta b}$$

Δa,Δb- net consumption/production of a/b at each specified time point

4. Viable cell yield and IFN-γ yield

When growth and production data are expressed in yield terms, they correspond to maximum titres obtained in culture.

3- STATISTICAL ANALYSIS OF THE CULTURE MEDIUM

3.1- Introduction

A wide range of biological products is produced by recombinant DNA technology using eukaryote or prokaryote host systems. In the last decade it has become clear that there are several advantages in using animal cells as hosts instead of bacteria or yeast, despite the perceived economical advantage of the latter systems. In general, animal cells have the ability to secrete proteins in a correctly folded functional state and with the correct post-translational modifications, such as glycosylation (Berman and Lasky, 1985; Bialy, 1987; Parekh *et al.*, 1989a). The optimisation of protein production in animal cell culture is an important objective as the product yields obtained with these systems tend to be low. Process optimisation may involve the study of many biochemical and physical parameters, including media formulation, culture parameters and culture systems. The nutritional environment of the cell may significantly alter the product yield, thus the manipulation of the medium components constitutes a potent way of improving the productivity.

The media used for animal cell culture generally are very complex and the significance of particular components for growth or product synthesis often is unknown. The basal media manufactured by various companies, e.g., Minimum Essential Medium (MEM), Dulbecko's Modified Essential Medium (DMEM), Roswell Park Memorial Institute (RPMI) medium contain essentially a mixture of inorganic salts, amino acids, glucose and vitamins, varying in the number and concentration of those nutrients. All these media have been empirically developed over a number of years. Traditionally, the main supplements to these formulations were bovine, foetal bovine or calf serum but now the use of more defined serum-free medium has become a more common method for the cultivation of animal cells, due to the disadvantages presented by serum (discussed in section 1.3.1). A mammalian cell culture

medium is now usually composed of a basal formulation, such as one of those refered to above, plus a protein rich-supplement usually including binding proteins, growth factors, adhesion factors and hormones, lipids and/or lipid precursors and several trace elements, e.g., selenium (Barnes and Sato, 1980). Optimisation of nutrient formulation of serum-free medium may be exploited to enhance culture performance, either in terms of cell growth and/or product synthesis. The growth of sublines of CHO cells, including the parental CHO-K1, is relatively easy to achieve in serum-free medium (Hamilton and Ham, 1977; Gasser *et al.*, 1985), but detailed studies of media to improve the productivity of recombinant proteins in CHO cells are scarce. The cell line used in this study, a CHO 320 producing human recombinant IFN-γ, has been previously adapted to a serum-free medium (Hayter *et al.*, 1989). The latter is based on RPMI and supplemented with glutamine, bovine serum albumin (BSA), human transferrin, bovine insulin, sodium pyruvate, alanine, putrescine, $FeSO_4$, $ZnSO_4$, Na_2SeO_3 and $CuSO_4$. Recently, Hata *et al.* (1992) reported an improvement in CHO cell culture medium for the production of human growth hormone by addition of N-acetylglucosamine, glutathione, putrescine, succinic acid, choline chloride, i-inositol and iron to alpha MEM medium supplemented with 1% dialysed foetal calf serum. In the present study, apart from N-acetylglucosamine and succinic acid, all the other components are already present in RPMI and putrescine is added to the basal medium used. There is still scope for substantial work towards the development of a defined serum-free CHO medium suitable for biological production applications. The development of such formulations has to be done for each specific line, as different CHO recombinants derived from a common parental strain vary significantly in terms of their metabolic requirements, even under identical cultivation conditions (Jayme, 1991).

The quality of the product obtained in culture is another important aspect to consider when analysing the cell culture system. IFN-γ is a glycoprotein, and the product obtained in culture is a mixture of three major variants: one form glycosylated at two sites (2N glycoform), another glycosylated only at one site (1N glycoform) while another form is not glycosylated.

Changes in the proportion of these forms during batch cultures has been previously reported (Curling *et al.*, 1990), which could possibly be related to deterioration in the culture conditions with time. The glycosylation patterns of IFN-γ have been also shown to be affected by glucose pulses in chemostat cultures (Hayter *et al.*, 1992). Changes in culture conditions have been shown to alter the glycosylation patterns of other proteins (see section 1.4.2), although studies on changes throughout the culture are scarce.

The importance of serum-free medium components on cell growth and production of recombinant IFN-γ was investigated in this study. The complexity of the medium led to the adoption of a statistical optimisation approach. The classical method of changing one medium variable at a time in order to optimise performance is impracticable. Selecting the possible significant variables is both time consuming and expensive, unless a logical basis for minimising the effort while maximising the probability that important variables are identified is employed. This need for efficient methods for screening large number of variables has led to the adoption of statistical experimental designs. Greasham and Inamine (1986), Bull *et al.* (1990) and Leslie (1992) have described some of the existing statistical designs, while their application to the formulation and improvement of culture media has been also discussed in the former cases. Statistical methods have been applied to microbial culture optimisation (De Meo *et al.*, 1985; Staheli, 1987) but only more recently to the optimisation of animal cell cultures e.g., optimisation of follicle stimulating hormone production by CHO cells (Gebert and Gray, 1990) and optimisation of Factor VIII expression by CHO cells (Ganne and Mignot, 1991). In both of the latter cases, significant improvements in production were achieved. A fractional factorial experimental method has also been used for the identification of key variables, and interactions between variables, affecting hybridoma cell growth and monoclonal antibody production (Gaertner and Dhurjati, 1993). In this study, a statistical design based on the Plackett-Burman (Plackett and Burman, 1946) procedure was chosen. This methodology provides an efficient way of screening a large number of variables and identifying the most

important ones. Its usefulness has been earlier illustrated by a case history involving the preparation of a new catalyst (Stowe and Mayer, 1966). It allows the investigation of up to N-1 variables with N experiments and designs of up to 100 experiments were proposed by the authors. Greasham and Inamine (1986) recommend the use of this method when more than five independent variables are to be investigated.

In this present research, 20 different variables, including low molecular weight nutrients such as glucose and amino acids and also protein supplements used in the serum-free medium, were tested in only 24 experiments. The implementation of the Plackett-Burman statistical design and its application to the optimisation of the cell culture media for CHO cell growth and IFN-γ production is exemplified. The effect of the outcome formulation on the glycosylation pattern of IFN-γ was also evaluated. Follow-up experiments were based on the identification of important nutrient variables with the Plackett-Burman method, with a view to having better understanding of cell behaviour and further improvements of cell culture performance.

3.2- CHO cell growth and IFN-γ production in serum-free medium

The serum-free medium previously developed at the University of Kent for this particular cell line was used as the basis for the optimisation studies. This medium was selected based on the range of media available in the literature for culturing cells in serum-free media, where little optimisation has been attempted. CHO cell growth and interferon-γ production kinetics in this medium were first characterised (Figure 3.1). Typically, cells reach maximum viable cell concentrations of 8-9 x 10^5 cells/ml after 100 hours. IFN-γ is produced only during cell growth phase, reaching concentrations of approximately 8000 IU/ml and maximum specific production rates of approximately 200 IU/10^6cells/h are usually obtained during exponential growth of the cells. Very similar behaviour has been reported previously with these cells

(Hayter *et al.*, 1991a). In chemostat cultures IFN-γ production rate also seemed to be a function of cell specific growth rate (Hayter *et al.*, 1993), suggesting that production is growth associated. However, this is not yet clear as production of IFN-γ by non-proliferating cells has also been reported (Hayter *et al.*, 1991a).

These CHO 320 cells are sensitive to the culture conditions, and the quality of some of the medium components, which may vary from batch to batch, namely proteins, can influence the behaviour of the culture. In each set of experiments a positive control culture, grown under the existent serum-free medium conditions, was included to compensate for any variability that might occur. The results obtained in each set of experiments are related to this control culture, unless otherwise indicated.

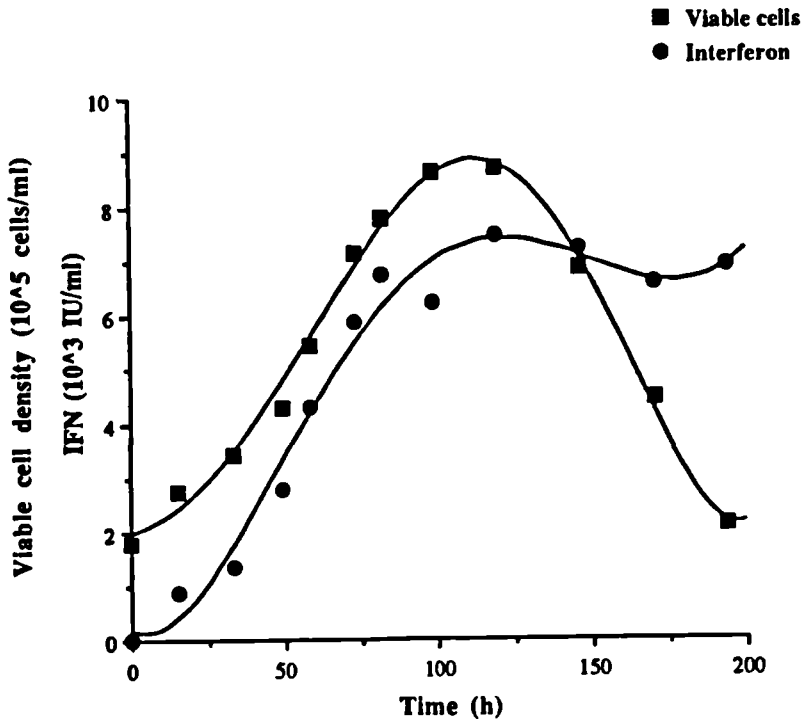


Figure 3.1- Kinetics of CHO cell growth and IFN-γ production in stirred batch culture.

3.3- Statistical screening of the medium components: the Plackett-Burman design

3.3.1- Experimental design

The Plackett-Burman design allows the investigation of up to N-1 variables with N experiments. In practice, all the experiments are carried out according to a design matrix, which is based on the number of variables to be studied. The matrix applied to the present study is shown in Table 3.1. Each row represents a different experiment and each column represents a different variable. Three variables (E, O and X in Table 3.1) are designated as "dummy variables", as they are not real variables and no change is made to them, but they are used to estimate the experimental error. The number of variables under study is then reduced to N-4. Each independent variable is tested at two levels, a high (+) and a low (-) level, which in the present investigation means two different nutrient concentrations.

The following independent variables were selected for analysis: glucose, BSA, insulin, transferrin, putrescine, sodium pyruvate and all of the amino acids present in the culture medium. During the studies of CHO cell growth and IFN-γ production, unlike all the other components, BSA from different suppliers has been used. It is important, therefore, to point out that the BSA used in the Plackett-Burman matrix experiments and that used to supplement the base medium were not identical. The implication of the source of material on culture performance is further discussed below. Effects of the amino acids in the major pathways were tested independently (asparagine, aspartate, glutamate, glutamine, serine, glycine and alanine). Arginine was also studied separately because its concentration in the medium is higher than the other amino acids, and it is not extensively used during growth (Hayter *et al.*, 1991a). Methionine and cystine were also considered independently. The remaining amino acids were tested in sub-groups according to their metabolic relationships, i.e., (a) tyrosine plus phenylalanine, (b) threonine, valine plus isoleucine, (c) leucine, tryptophan plus lysine and (d)

proline, histidine plus hydroxyproline. In each case a high and a low concentration of each nutrient or subgroup of nutrients was tested.

Table 3.1- Plackett-Burman matrix for the study of 23 variables with 24 experiments.

		VARIABLES																							
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	W	X	Y
	1	+	+	+	+	+	-	+	-	+	+	-	-	+	+	-	-	+	-	+	-	-	-	-
	2	+	+	+	+	-	+	-	+	+	-	-	+	+	-	-	+	-	+	-	-	-	-	+
	3	+	+	+	-	+	-	+	+	-	-	+	+	-	-	+	-	+	-	-	-	-	+	+
	4	+	+	-	+	-	+	+	-	-	+	+	-	-	+	-	+	-	-	-	-	+	+	+
	5	+	-	+	-	+	+	-	-	+	+	-	-	+	-	+	-	-	-	-	+	+	+	+
	6	-	+	-	+	+	-	-	+	+	-	-	+	-	+	-	-	-	-	+	+	+	+	+
F	7	+	-	+	+	-	-	+	+	-	-	+	-	+	-	-	-	-	+	+	+	+	+	-
L	8	-	+	+	-	-	+	+	-	-	+	-	+	-	-	-	-	+	+	+	+	+	-	+
A	9	+	+	-	-	+	+	-	-	+	-	+	-	-	-	-	+	+	+	+	+	-	+	-
S	10	+	-	-	+	+	-	-	+	-	+	-	-	-	-	+	+	+	+	+	-	+	-	+
K	11	-	-	+	+	-	-	+	-	+	-	-	-	-	+	+	+	+	+	-	+	-	+	+
	12	-	+	+	-	-	+	-	+	-	-	-	-	+	+	+	+	+	-	+	-	+	+	-
	13	+	+	-	-	+	-	+	-	-	-	-	+	+	+	+	+	-	+	-	+	+	-	-
	14	+	-	-	+	-	+	-	-	-	-	+	+	+	+	+	-	+	-	+	+	-	-	+
	15	-	-	+	-	+	-	-	-	-	+	+	+	+	+	-	+	-	+	+	-	-	+	+
	16	-	+	-	+	-	-	-	-	+	+	+	+	+	-	+	-	+	+	-	-	+	+	-
	17	+	-	+	-	-	-	-	+	+	+	+	+	-	+	-	+	+	-	-	+	+	-	-
	18	-	+	-	-	-	-	+	+	+	+	+	-	+	-	+	+	-	-	+	+	-	-	+
	19	+	-	-	-	-	+	+	+	+	+	-	+	-	+	+	-	-	+	+	-	-	+	-
	20	-	-	-	-	+	+	+	+	+	-	+	-	+	+	-	-	+	+	-	-	+	-	+
	21	-	-	-	+	+	+	+	+	-	+		+	+	-	-	+	+	-	-	+	-	+	-
	22	-	-	+	+	+	+	+	-	+	-	+	+	-	-	+	+	-	-	+	-	+	-	-
	23	-	+	+	+	+	+	-	+	-	+	+	-	-	+	+	-	-	+	-	+	-	-	-
	24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

(+) represents the high level of the particular variable and (-) the low level of the same variable.

The low level was taken as that contained in the base medium while the high level was established at double these concentrations except for the following components: BSA, 1.5 times because its initial level is relatively high; glutamine, 1.5 times due to possible inhibitory effects caused by ammonia production; arginine, 1.5 times because its initial concentration is higher than the other amino acids. The concentrations of each selected variable are given in Table 3.2.

Table 3.2- Components under study and their corresponding concentrations.

Variable	Low level	High level
	g/l	*g/l*
A - BSA	5	7.5
B - Transferrin	0.005	0.01
C - Insulin	0.005	0.01
	mM	*mM*
D - Sodium pyruvate	1	2
E - Dummy		
F -Putrescine	0.001	0.002
G - Glucose	11	22
H - Alanine	0.10	0.20
I - Arginine	1.15	1.725
J - Asparagine	0.33	0.66
K - Aspartate	0.15	0.30
L - Cystine	0.021	0.042
M - Glutamine	2	3
N - Glutamate	0.14	0.28
O - Dummy		
P - Glycine	0.13	0.26
Q - Serine	0.29	0.58
R - Methionine	0.10	0.20
S - Proline/Histidine/Hydroxyproline	0.17/0.097/0.15	0.34/0.19/0.15
T - Threonine/Valine/Isoleucine	0.13/0.17/0.38	0.26/0.34/0.76
W - Leucine/Tryptophan/Lysine	0.38/0.024/0.22	0.76/0.048/0.44
X - Dummy		
Y - Phenylalanine/Tyrosine	0.09/0.013	0.18/0.26

All the experiments were performed in shake-flasks in duplicate according to the design matrix (Table 3.1). The parameters evaluated for each experiment were specific growth rate, which was calculated from the linear part of the exponential growth curve, maximum viable cell production and maximum IFN-γ titre. The results as required by the statistical analysis are

shown in Table 3.3.

Table 3.3- Cell growth and IFN-γ production data for Plackett-Burman analysis.

Experiment	μ (h^{-1})	**Cell titre** (10^5 cells/ml)	**Interferon-γ titre** (IU/ml)
1	0.022 ± 0.000	8.9 ± 0.2	9276 ± 340
2	0.030 ± 0.002	11.2 ± 0.3	8140 ± 620
3	0.025 ± 0.000	9.7 ± 0.2	8048 ± 786
4	0.033 ± 0.001	12.2 ± 0.4	12608 ± 108
5	0.024 ± 0.000	9.0 ± 0.1	8581 ± 850
6	0.026 ± 0.001	7.8 ± 0.4	7597 ± 675
7	0.023 ± 0.002	9.0 ± 0.5	10158 ± 705
8	0.017 ± 0.000	6.9 ± 0.2	6703 ± 365
9	0.030 ± 0.000	11.6 ± 0.3	8223 ± 438
10	0.031 ± 0.001	12.3 ± 0.1	12973 ± 565
11	0.030 ± 0.001	11.2 ± 0.1	8354 ± 435
12	0.032 ± 0.001	11.0 ± 0.1	6815 ± 288
13	0.031 ± 0.002	10.8 ± 0.3	11495 ± 500
14	0.023 ± 0.000	7.7 ± 0.4	11318 ± 620
15	0.030 ± 0.002	11.9 ± 0.2	10108 ± 370
16	0.019 ± 0.001	6.5 ± 0.2	6366 ± 400
17	0.022 ± 0.000	6.1 ± 0.1	4493 ± 210
18	0.032 ± 0.001	11.2 ± 0.3	7142 ± 250
19	0.025 ± 0.001	9.6 ± 0.2	11131 ± 625
20	0.021 ± 0.001	6.5 ± 0.3	6463 ± 475
21	0.032 ± 0.000	9.0 ± 0.4	6517 ± 305
22	0.028 ± 0.001	5.9 ± 0.3	6372 ± 385
23	0.023 ± 0.000	7.8 ± 0.2	6205 ± 745
24	0.028 ± 0.001	9.5 ± 0.4	7500 ± 620

IFN-γ titres are the maximum titres achieved in culture, which corresponded to the same time point in all the experiments.

In this study, experiment 24 corresponds to the control culture, the original formulation for the serum-free medium. The results clearly show that both cell growth and IFN-γ production were influenced by different components of the cell culture medium. In some experiments, there was an increase of approximately 20-30% in cell titre (e.g., experiments 2, 4, 9, 10, 11, 15, 18), which also corresponded to higher initial specific growth rates. IFN-γ production seemed to be affected by different components, as not always the highest titres corresponded to the highest cell titres (e.g., experiments 7, 14, 19) nor did higher cell numbers correspond to the highest IFN-γ titres (e.g., experiments 2, 9, 11, 12, 18). In some cases, significant increases in IFN-γ titres were obtained over the control culture (e.g, experiments 1, 4, 7, 10, 13, 14, 15). The more pronounced reductions in IFN-γ titre in relation to the control culture mostly coincided with lower cell numbers (e.g., experiments 8, 16, 17, 20, 21, 22, 23). To draw any conclusion on which components are responsible for this behaviour from this analysis alone would be difficult, as there are several parameters changing at the same time. In order to calculate the effect that each particular component has on each parameter, a statistical analysis is applied to the results.

3.3.2- Experimental effects of the study variables

Statistical analyses were made to identify those medium variables that had a significant effect on CHO cell growth and IFN-γ production. First, the effect of each variable was determined as the difference between the average value of the response for the 12 experiments at the high level (+) and the average value for the 12 experiments at the low level (-):

$$E_A = \frac{R \text{ at } (+)}{12} - \frac{R \text{ at } (-)}{12} \qquad (3.1)$$

where E_A is the effect of the variable, and R is the measured response.

The variance of the experimental error was determined as the average square of the dummy effects (E_d):

$$V_E = \frac{\Sigma (E_d)^2}{n} \qquad (3.2)$$

where V_E is the variance of the effects and n is the number of dummy variables.

The standard error of the effects was determined as the square root of the variance:

$$S.E._{eff.} = \sqrt{V_E} \qquad (3.3)$$

where $S.E._{eff.}$ is the standard error of the effects.

Finally, the significance level of each variable effect was determined using the student t-test:

$$t\text{-value} = \frac{E_A}{S.E._{eff.}} \qquad (3.4)$$

These analyses allow the evaluation of the probability of finding the observed effect purely by chance. Confidence levels are accepted only above the 70% level. Thus, it is possible to rank the variables that significantly affect the system, either positively or negatively.

These statistical analyses were applied to the results obtained. The effect of each variable on the value of each parameter together with its associated t-value and level of significance was determined, and is shown in Table 3.4. The effect of the dummy variables should ideally be zero. A deviation from zero is assumed to be a measure of the lack of experimental precision and analytical error in measuring the response but it could also reflect interaction between variables, which is not accounted for by the Plackett-Burman analysis. The effect of the dummy variable X is large compared with the others, being among the significant variables. However, this variable was still used for the calculation of the variance, as a measure of the uncertainty of the results.

Table 3.4- Components and their effects on cell growth rate, viable cell production and IFN-γ titre.

	μ (h^{-1})			Viable cell production (10^5 cells/ml)			Interferon titre (IU/ml)		
Variable	a	b	c	a	b	c	a	b	c
BSA	0.00009	0.08		1.075	1.609	**70%**	0.337	5.476	**99%**
Transferrin	-0.00004	-0.04		0.658	0.986		-0.059	-0.967	
Insulin	-0.002	-2.263	*80%*	-0.508	-0.761		-0.179	-2.906	*90%*
Pyruvate	0.00008	0.072		-0.358	-0.536		0.102	1.659	**80%**
Dummy (E)	0.0007			-0.075			0.013		
Putrescine	-0.0001	-0.104		-0.542	-0.811		-0.049	-0.801	
Glc	0.0001	0.104		-0.125	-0.187		0.066	1.075	
Ala	0.0005	0.504		-0.075	-0.112		-0.125	-2.027	*80%*
Arg	-0.001	-1.416	*70%*	-1.025	-1.534	*70%*	-0.203	-3.308	*95%*
Asn	-0.001	-1.32	*70%*	-0.042	-0.062		0.018	0.294	
Asp	-0.002	-1.815	*80%*	-0.925	-1.385	*70%*	-0.084	-1.369	*70%*
Cys	-0.002	-1.911	*80%*	-1.425	-2.133	*80%*	-0.067	-1.086	
Gln	0.0002	0.216		0.175	0.262		0.024	0.391	
Glu	-0.000008	-0.008		-0.025	-0.037		0.102	1.652	**80%**
Dummy (O)	0.0008			0.175			0.078		
Gly	0.007	6.742	**99%**	2.125	3.181	**90%**	0.043	0.704	
Ser	-0.002	-2.295	*80%*	-0.708	-1.06		-0.128	-2.076	*80%*
Met	-0.001	-1.336	*70%*	0.608	0.911		0.112	1.817	**80%**
Pro/His/Hdp	-0.0001	-0.12		0.358	0.536		0.145	2.358	**80%**
Thr/Val/Iso	-0.0009	-0.824		-0.592	-0.886		-0.1	-1.63	*70%*
Leu/Try/Lys	-0.002	-2.007	*80%*	-1.275	-1.909	*80%*	-0.015	-0.243	
Dummy (X)	0.001			1.142			0.071		
Phe/Tyr	0.0005	0.504		0.992	1.485	**70%**	0.15	2.436	**80%**
	V=0.0000011; SE=0.00104			V=0.446; SE=0.668			V=0.00378; SE=0.0015		

a- effect of the variable; b- t-value; c- significance level. For cell and IFN-γ production the effects are related to the ratio between each experiment and the control (experiment 24). Positive variables are shown in bold; negative variables are shown in italics.

Glycine was solely identified as having a stimulatory effect on the specific growth rate, while for cell production the effects of BSA, phenylalanine and tyrosine also were identified as significant. The results showed that BSA was also important for IFN-γ production. Sodium pyruvate, glutamate, methionine, proline, histidine, hydroxyproline, tyrosine and phenylalanine were also identified as important for IFN-γ production. Other nutrients, such as insulin, arginine, aspartate and serine were identified as being inhibitory for both cell growth and IFN-γ production. The fact that different medium components were identified as important for cell growth and recombinant protein production seems to indicate that the latter is not directly growth associated.

Glycine was identified as the most important component for cell growth, in terms of both specific growth rate and viable cell production. The fact that the carbon skeleton of glycine is used in the biosynthesis of purines (Stryer, 1981) may be one of the reasons for the positive effect of this component on cell growth. Also, it is noteworthy that glycine is synthesised from serine and tetrahydrofolate. Since these cells were grown in the presence of methotrexate the synthesis of tetrahydrofolate from dihydrofolate may be repressed. The production of glycine by the cells can be blocked due to a lack of tetrahydrofolate and the addition of glycine to the cell medium culture would then be required. In fact, CHO cells deficient in dihydrofolate reductase ($DHFR^-$) have been shown to require glycine in the culture medium in order to grow (Urlaub and Chasin, 1980). Previous studies on the effect of glycine on the growth of the cell line used in the present study have shown that glycine concentrations up to 1.0 mM stimulated cell growth while a further increase in concentration would cause growth inhibition (J.M. Tong and A.T. Bull, unpublished data). This observation is consistent with the positive effect found for this nutrient in the range studied i.e., up to 0.25 mM. Proline was identified as an important component for IFN-γ production. The parental CHO-K1 cells are proline auxothrophs (Kao and Puck, 1967) thus its positive effect may be related to the dependency of this cell line for proline. Some other amino acids were identified as enhancers

of IFN-γ production. This is not surprising as there is previous evidence that specific amino acid supplementation of the culture medium can increase protein production by animal cells (Luan *et al.*, 1987; Duval *et al.*, 1991; Lucki-Lange and Wagner, 1991).

Some amino acids were identified as inhibitory for cell growth. It has been shown that an increase in specific amino acid concentrations can lead to cell growth inhibition (Eagle 1955a,b). An increase in the amount of certain amino acids, namely alanine, serine and methionine, in the medium culture has been shown to inhibit the growth of CHO-K1 cells by antagonism of proline uptake (Curriden and Englesberg, 1981), as they are all transported by the same system (Shotwell *et al.*, 1981). The fact that serine and methionine were among the components identified as negative for cell growth may be a reflection of this situation. Alanine was not identified as a negative component, but previous work (J.M. Tong and A.T. Bull, unpublished data) has shown that CHO cell growth inhibition by alanine only occurs above 3 mM, far above the concentrations used in this study.

BSA showed positive effects on both cell growth and IFN-γ production. The fact that albumin may act as a carrier for metals, lipids and hormones (Barnes and Sato, 1980) may explain the positive effect on growth and production which was observed. BSA confers high viscosity to the culture medium and may also protect cells from mechanical shear damage, e.g., agitation and aeration, (Lambert and Birch, 1985; Hülscher and Onken, 1988; Papoutsakis, 1991a; Smith and Greenfield, 1992). Two other proteins were examined in this study, transferrin and insulin. Both proteins have been shown to enhance the growth of several cell lines in serum-free medium. Insulin plays an important role in glucose metabolism and biosynthesis of fatty acids (Mather and Sato, 1979) and recently it has been shown that the parental CHO-K1 cells have an insulin-sensitive facilitated diffusion system for glucose transport (Hasegawa *et al.*, 1990). Insulin is among the components identified as inhibitory for cell growth and production. Optimum concentrations ranging from 5 to 20 μg/ml have been found for the

growth of hybridomas (Darfler and Insel, 1982; Kovar, 1986) but concentrations higher than the optimum have been found to decrease its stimulatory effect on cell growth (Kovar, 1986). The effects of insulin on the growth of a CHO cell line have been investigated earlier and an optimum concentration of 2 μg/ml was found, although variable results were obtained (Gasser *et al.,* 1985). The highest insulin concentration used in this study may be above the optimum for this cell line, which may explain the negative effect found for this nutrient. The stimulatory effect of transferrin in the cell culture medium is most probably related to its iron binding properties (Aisen and Listowsky, 1980). Transferrin is essential for the growth and survival of most cell lines cultured in serum-free medium and its omission may lead to complete inhibition of cell growth (Kovar and Franek, 1984 and 1985). Optimum concentrations ranging from 1 to 10 μg/ml have been reported and concentrations higher than the optimum were also found to be effective without causing evident toxicity (Iscove and Melchers, 1978; Kovar and Franek, 1985). In the present study the fact that an increase in the medium concentration of transferrin did not show any effect on cell growth may indicate that the present concentration is within its typical optimum broad plateau. Previous studies on the effect of transferrin on the growth of a CHO cell line have also indicated that transferrin was a major growth-promoting component, having a broad optimum concentration range of 2 to 10 μg/ml (Gasser *et al.,* 1985). The effect of removing insulin or both insulin and transferrin from the culture medium has been previously studied with the present cell line and it was found that in both cases a dramatic reduction in cell growth rates and IFN-γ production rates was observed with a simultaneous reduction in glucose uptake rate (Tomlinson, 1991).

3.4- Cluster effect of the positive and negative groups of variables

3.4.1- Cell growth and IFN-γ production: titre and product quality

In order to assess the results obtained, the common positive variables found with the Plackett-Burman design were grouped together and their cluster effect was tested in batch cultures. A similar procedure was followed for the negative variables. The components included in the positive group were glycine, BSA, sodium pyruvate, glutamate, and the groups phenylalanine/tyrosine and proline/histidine/hydroxyproline. The concentrations of these components were the corresponding high levels for the Plackett-Burman matrix. Insulin, arginine, alanine, asparagine, cystine, serine, and leucine/tryptophan/lysine were included in the negative group. A control experiment with all the variables at the normal level was run in parallel. Cell growth rate, cell production and IFN-γ titre were evaluated for each set of experiments (Table 3.5).

Table 3.5- Effects of the positive and negative variables on CHO cell growth and IFN-γ production.

	Positive Variables (+ II)	**Control (I)**	**Negative Variables (- II)**
Specific growth rate	0.030 ± 0.002	0.021 ± 0.001	0.018 ± 0.001
(h^{-1})	(+43%)		(-14%)
Viable cell production	11.3 ± 0.2	8 ± 0.5	7.1 ± 0.3
(10^5 cells/ml)	(+41%)		(-11%)
IFN-γ titre	9277 ± 1543	6408 ± 363	5878 ± 367
(IU/ml)	(+45%)		(-8%)

the control in these experiments signifies values obtained with the original growth medium; the values in parenthesis indicate percentage increase/decrease over the control cultures. Experiments were made in quadruplicate.

An increase in the level of the positive variables led to an improvement of about 40-43% in cell growth and 45% in the production of IFN-γ. An increase in the concentration of the negative nutritional variables did not produce an overall effect as marked as those of the positive variables on the same parameters, but an inhibitory effect was noticed. The results indicated decreases of 8 to 14% for the three parameters. Based on a significance t-test, the effects of both positive and negative variables were found to be statistically different from the control experiment (significance levels varying from 95 to 100% for positive effects and from 80 to 95% for negative effects). The statistical design technique used in this study was shown to be a very valuable one for process optimisation, as it was shown that the identification of important variables in the process under study could be made without using a factorial experimental procedure. In fact, a significant improvement of both cell growth and IFN-γ production was achieved based on the variables identified with the Plackett-Burman design.

An improved formulation was obtained, which was based only on parameters of cell growth and productivity but not product quality. The possibility that the change in the culture environment could affect the post-translational modification of the product was then investigated. The glycosylation patterns of the product obtained from control cultures and cultures supplemented with the positive variables were analysed. The profiles obtained were very similar (Figure 3.2). A gradual decrease in the proportion of the glycoform occupied at both sites (2N-glycoform), from ca. 70% at 40 hours to ca. 50% at 100 hours, with a simultaneous increase in the glycoform occupied at one site only (1N-glycoform), from ca. 30% at 30 hours to ca. 45% at 100 hours, was observed throughout the culture. In cultures containing the positive group of variables, a similar pattern was observed for the glycosylated forms, and the differences detected were too small to relate them to any change in the culture environment. In both situations, the proportion of non-glycosylated (0N-glycoform) form remained constant until late in culture, ca. 130 h, representing about 5% of the total product. Changes in the glycosylation patterns of IFN-γ, produced by the same CHO cells, during the

course of a culture have been previously noticed (Curling *et al.*, 1990), namely a decrease in the proportion of the 2N glycoform. Changes in the glycosylation of a monoclonal antibody by hybridomas throughout a fed-batch culture has been also reported (Robinson *et al.*, 1993). These authors found a greater percentage of antibody with incomplete, mannose terminated glycoforms at the H-chain glycosylation site at late times in the fed-batch culture.

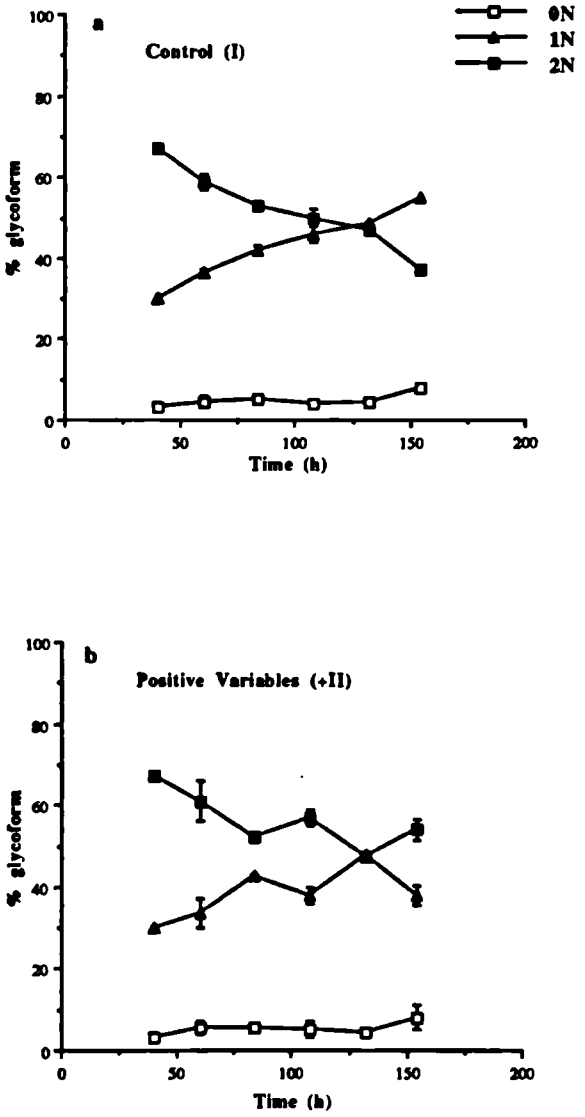


Figure 3.2- Glycosylation patterns of IFN-γ obtained from a) control cultures and b) cultures containing increased concentrations of the positive group of variables.

3.4.2- Analysis of the culture medium

In order to investigate the extent of nutrient depletion in the three different cultures (Control-I, Positive +II and Negative -II), glucose and amino acid analysis were carried out. The positive effect observed could be due to overcoming any nutrient limitation. It was also possible that changes in cell metabolism were occurring when the concentration of the positive or negative group was increased in the culture medium. This could provide part of the explanation for the stimulating effect of the positive group of variables.

Cell growth curves for the original medium and medium containing increased concentrations of the positive (+II) and negative (-II) group of variables are presented in Figure 3.3. The metabolism of glucose and amino acids during the culture for the same cultures is shown in Figure 3.4a-i. Cystine, proline and hydroxyproline are not detected by the HPLC assay and no data are available for these nutrients; data for histidine are not presented, as the peak for this amino acid is not very well separated from other components. Here, the interest is in examining the results in terms of nutrient depletion and differences between the cultures. A more detailed examination of the amino acid metabolism is described later in Chapter 4. The results are to be compared with the control culture (I), i.e., the original medium. Analysis of the control culture showed that only glutamine and glucose were exhausted from culture and the depletion of glutamine coincided with the onset of the stationary phase of the cells (Figure 3.4a,b). Asparagine was also consumed rapidly by the cells (Figure 3.4c). The essential amino acids valine, isoleucine, phenylalanine, methionine, leucine, lysine, tyrosine and threonine were utilised throughout the cultures, but were not exhausted at the end of the culture (Figure 3.4d-g). Arginine levels remained constant during the culture (Figure 3.4b). The consumption patterns for glycine and serine (Figure 3.4h) seemed to be closely related. While there was an initial increase in serine and a decrease in glycine levels, this pattern was reversed later in culture, ca. 75 h, coinciding with glutamine depletion. There was a net production of alanine

(Figure 3.4i) and although a net production of glutamate (Figure 3.4i) was also noticed in the early stages of the culture, its concentration slightly decreased as glutamine was depleted from culture. The patterns obtained for supplemented cultures were similar to those found for the control cultures. A few differences were noticed in terms of net production or consumption, but these were related to the higher or lower level of the particular nutrient in the beginning of the culture (serine, glycine, arginine, asparagine, tyrosine, phenylalanine, leucine, lysine). Only glutamate (Figure 3.4i) showed a higher net accumulation in negative cultures, as compared to the control containing the same initial concentration. In none of the cultures did the levels of glycine and alanine reach the inhibitory levels referred in the section above.

Differences in glucose and glutamine consumption were seen in both supplemented cultures, +II and -II. The initial specific consumption rates for these components in each of the cultures is shown in Table 3.6. Lower values were obtained for cultures supplemented with the positive group of variables and higher values for cultures supplemented with the negative group of variables. In the former case, this seems to indicate a more efficient utilisation of glucose and glutamine when the concentration of the positive nutrients is increased in the culture medium, which could account for the improved yield of cells on those nutrients which may become limiting. It is most likely that cells respond differently to different culture environments.

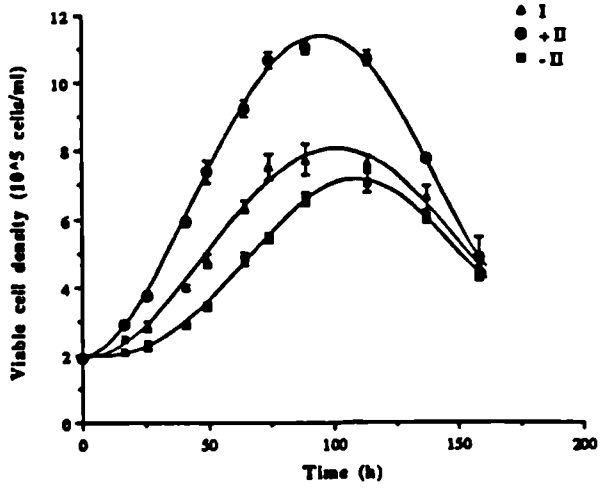


Figure 3.3- Cell growth in control batch cultures (I), and cultures containing the positive group of nutrients (+II) or negative group of nutrients (-II).

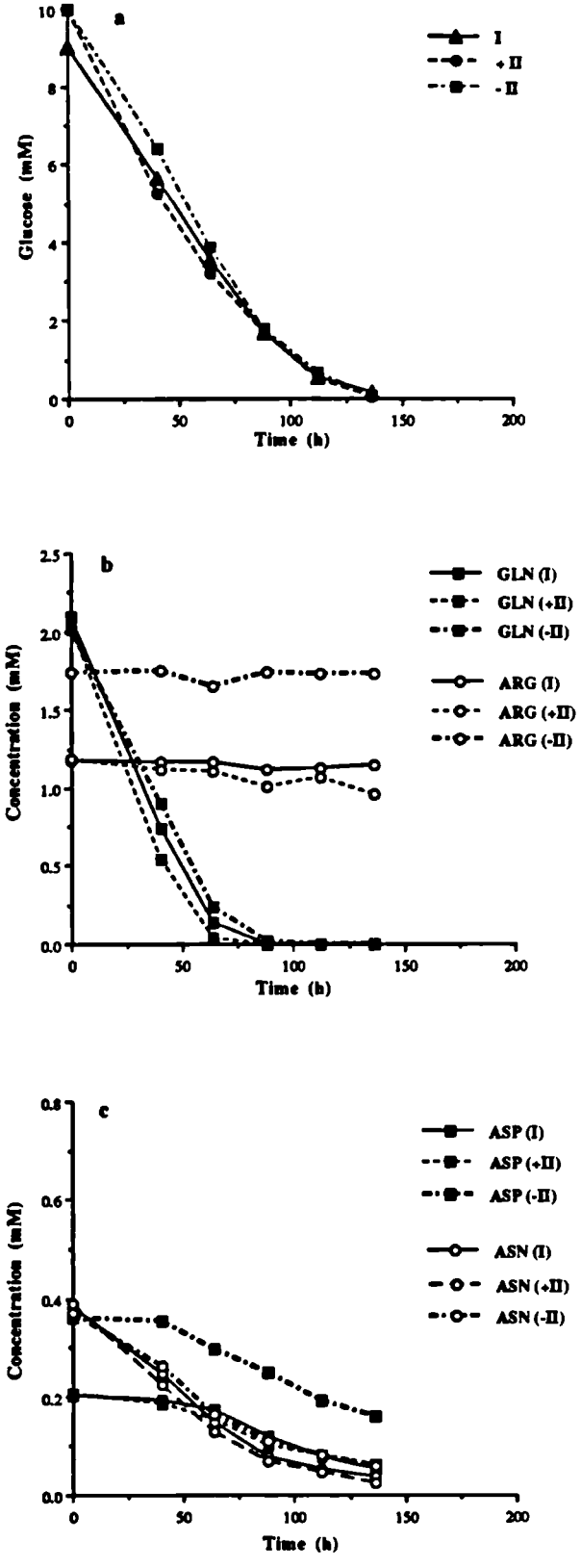


Figure 3.4- Glucose and amino acid consumption/production in control batch cultures (I), and cultures containing the positive group of nutrients (+II) or negative group of nutrients (-II). a) glucose; b) glutamine and arginine; c) aspartate and asparagine.

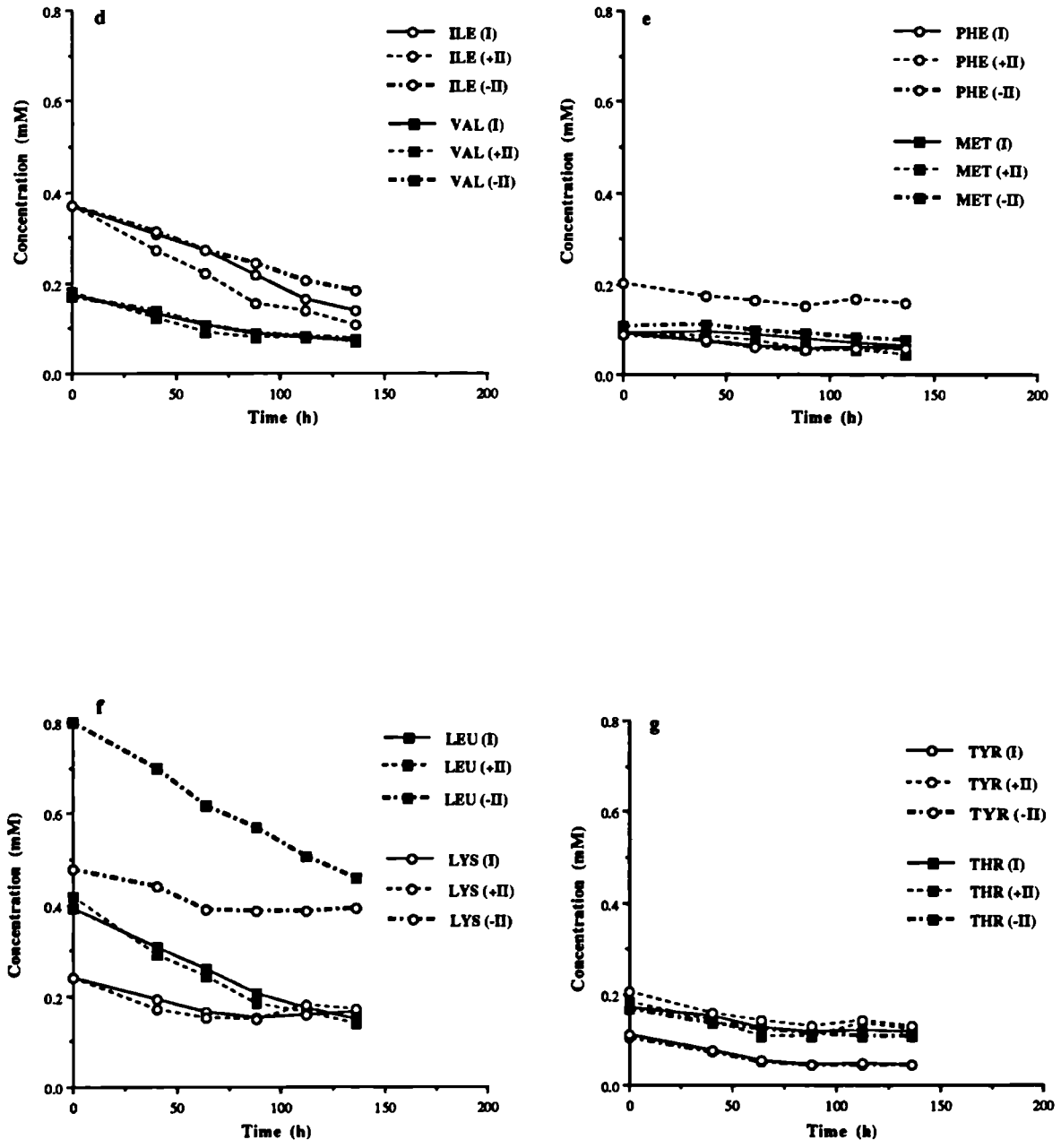


Figure 3.4- Contd. d) isoleucine and valine; e) phenylalanine and methionine; f) leucine and lysine; g) tyrosine and threonine.

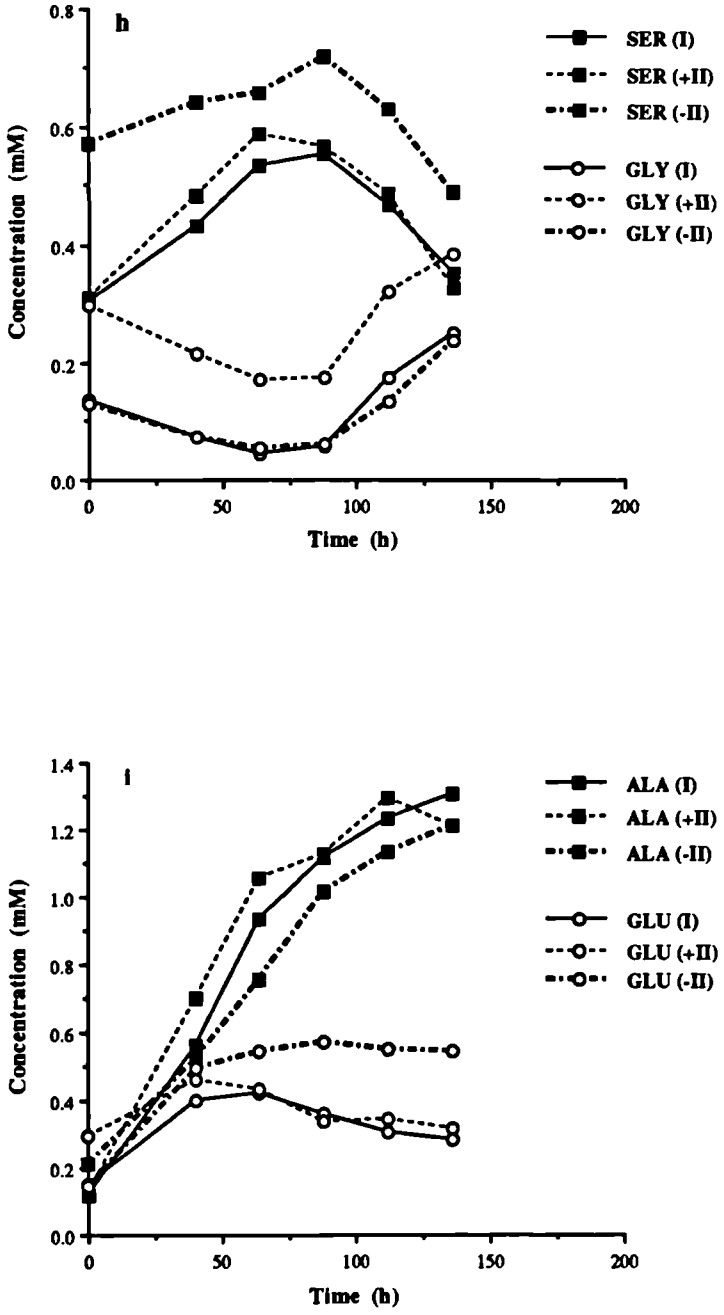


Figure 3.4- Contd. h) serine and glycine; i) alanine and glutamate.

Table 3.6- Initial consumption rates of glucose and glutamine in batch control cultures (I) and batch cultures containing the positive (+II) or negative (-II) groups of variables.

	Control (I)	Positive variables (+II)	Negative variables (-II)
Glutamine nmol/10^6cells/h	75.6	56.2	82.1
Glucose µmol/10^6cells/h	0.220	0.187	0.270

Values determined from the exponential growth phase of the cells.

Ammonia and lactate are two major end-products of cell metabolism, arising mainly from glutamine and glucose metabolism respectively. Both were monitored in these cultures and similar final titres were found (Table 3.7); the accumulation of ammonia and lactate attained the same levels in culture. The final concentrations achieved are not inhibitory to the system as previous studies have shown that these cells were not inhibited by ammonia concentrations of up to 2 mM and lactate concentrations of up to 17.5 mM (Hayter *et al.*, 1991a).

Table 3.7- Accumulation of lactate and ammonia in batch control cultures (I) and batch cultures containing the positive (+II) or negative (-II) groups of variables.

	Control (I)	Positive variables (+II)	Negative variables (-II)
Ammonia (mM)	1.72	1.68	1.74
Lactate (mM)	14.1	13.1	13.9

Maximum titres in culture are shown.

3.4.3- The independent effect of BSA

BSA was identified as one of the most influential components in the culture medium. It is a very poorly defined component and also an expensive one, and an increase in its concentration is not desirable. In order to determine the actual contribution of BSA to CHO cell growth and IFN-γ production the stimulatory variables were further divided into two groups: (1) the amino acids plus sodium pyruvate group and (2) BSA, and their separate effects were determined (Table 3.8).

Table 3.8- Independent effect of the two positive group variables (amino acids plus pyruvate, and BSA) on CHO cell growth and IFN-γ production.

	Control (I)	**A** (II-A)	**B** (II-B)	**A+B** (II)
Specific growth rate (h^{-1})	0.022 ± 0.001	0.031 ± 0.001 (41)	0.027 ± 0.001 (23)	0.032 ± 0.001 (45)
Viable cell production (10^5 cells/ ml)	8.6 ± 0.3	12.2 ± 0.2 (42)	11.2 ± 0.5 (30)	12.6 ± 0.2 (47)
IFN-γ titre (IU/ml)	7775 ± 310	8495 ± 420 (10)	9213 ± 350 (18)	9915 ± 390 (28)

II-A. supplementation with amino acids plus sodium pyruvate. II-B. supplementation with BSA.
The values in parenthesis indicate percentage increases in the measured parameters above the control culture (I).

The results showed that the positive effect found for the amino acids and sodium pyruvate group on cell growth was independent of the BSA concentration (Table 3.8) but BSA alone also caused a significant but not so pronounced positive effect. In terms of IFN-γ production, a more pronounced effect was achieved if both groups were increased in the medium, and BSA alone was found to be more effective than the other positive group. This reinforced the

importance of BSA in the culture medium.

Subsequent experiments with different types of BSA indicated that the positive effect found for this medium component is probably due to differences in the source of BSA rather than to the actual amount present in the culture medium. As was mentioned earlier, BSA from two different suppliers, Miles Pentex for the basal medium and Sigma for the supplementation studies, was used in this statistical study. In fact, when the same type of BSA as the basal culture medium, Miles Pentex, was used to increase its initial concentration, no improvements on either cell growth (Figure 3.5) and IFN-γ production (Table 3.9) were achieved. This shows that different sources of BSA have different effects on this particular cell culture system but a parallel with other cell systems can not be assumed.

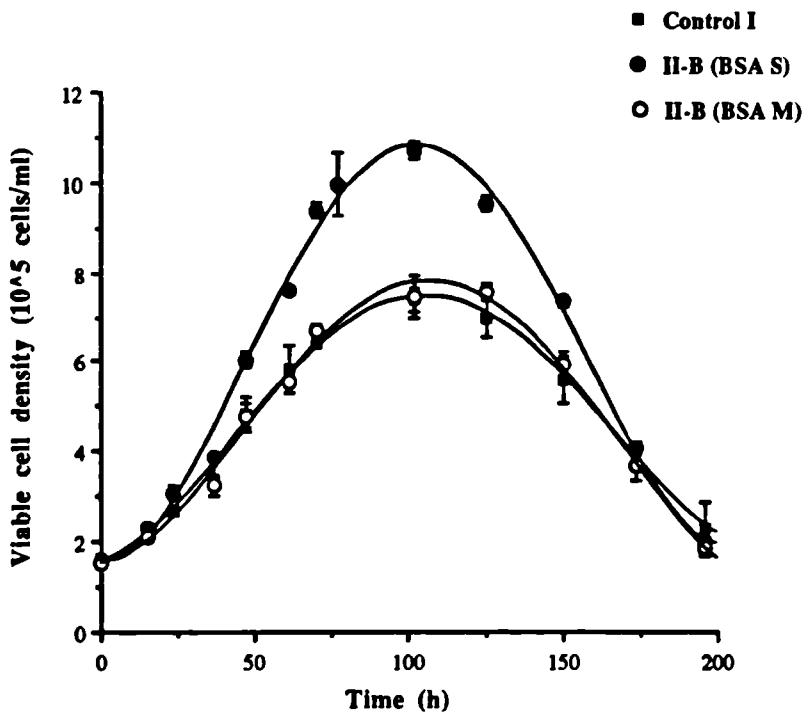


Figure 3.5- Effect of supplementing the culture with different sources of BSA on CHO cell growth. S= Sigma; M= Miles.

Table 3.9- Effect of different BSA preparations on IFN-γ production.

	IFN-γ titre (IU/ml)
Control (I)	7530 ± 400
II-B. BSA S	9410 ± 450
II-B. BSA M	7650 ± 350

II-B. supplementation with BSA. S- Sigma; M- Miles Pentex.

The results clearly indicate that the variability in BSA quality has to be taken into consideration. Most likely, the fractionation method for obtaining BSA affects its quality by either removing essential components it binds or not removing components that can be toxic to the cells. Jayme (1991) has reported different cell performance of culture media containing either chromatographically purified BSA or BSA obtained from Cohn fraction V (Cohn *et al.*, 1946), which primarily involves solvent precipitation. A much superior hybridoma cell culture performance was obtained with the former. It was suggested that solvent precipitation might have stripped essential lipids from the BSA, and although no correlation was found in terms of total titratable free fatty acids, a distinction in the HPLC lipid profile of both BSA preparations was clearly observed. With the present culture system however, CHO cell growth was very poor when a chromatographically purified BSA was used to substitute the original BSA (PM Hayter, personal communication) yet cells perform equally well, both in terms of growth and production, in fatty acid-free BSA, although not all the lipids are removed. The latter is obtained using a different purification procedure, a preliminary Cohn fractionation and heat shock fractionation and further charcoal and solvent treatment (Bovine albumins, Bayer Diagnostics, Pentex), and it is possible that toxic components for the cells are more efficiently removed using these methods. In fact, free fatty acids in the culture medium have been reported to have toxic effects on cell growth (Kovar and Franek, 1986). The BSA used to supplement the medium for the Plackett-Burman experiments is obtained by initial fractionation by heat shock, followed by charcoal treatment and extensive dialysis to reduce

low molecular weight substances (Sigma). The original BSA used in the culture medium is purified using a mixture of two methods, Cohn fractionation and heat shock fractionation, with no further treatment (Bovine albumins, Bayer Diagnostics, Pentex). Thus, since different purification methods are employed, the quality of these BSA preparations may be very different, and CHO cells appear to behave in accordance to the purification employed. Also, the level of endotoxins of these preparations is not controlled, and varies considerably from batch to batch, and this can also be problematic for cell growth.

Based on these considerations, the role of BSA in the culture medium seemed to require a more detailed investigation (Chapter 5), and was therefore excluded from the positive group of variables.

3.5 - The stimulating effect of the positive variables on CHO cell growth

Nutrient analysis provided a possible explanation for the stimulating effect of the positive group of variables as related to a change in glucose and glutamine metabolism, and the effect did not seem to be due to the replenishment of exhausted nutrients. Further studies were carried out in order to elucidate the mechanism behind the stimulating effect.

3.5.1 - Effect of methotrexate on CHO cell growth

From the statistical analysis, glycine was the component that had the most significant effect on cell growth. The use of the carbon skeleton of glycine in the biosynthesis of purines was one of the possible explanations for its positive effect on cell growth. A conditional factor could be related to this: since the cells were grown in the presence of methotrexate (MTX), it was possible that the synthesis of glycine was inhibited. Figure 3.6 shows the metabolic pathway for glycine synthesis.

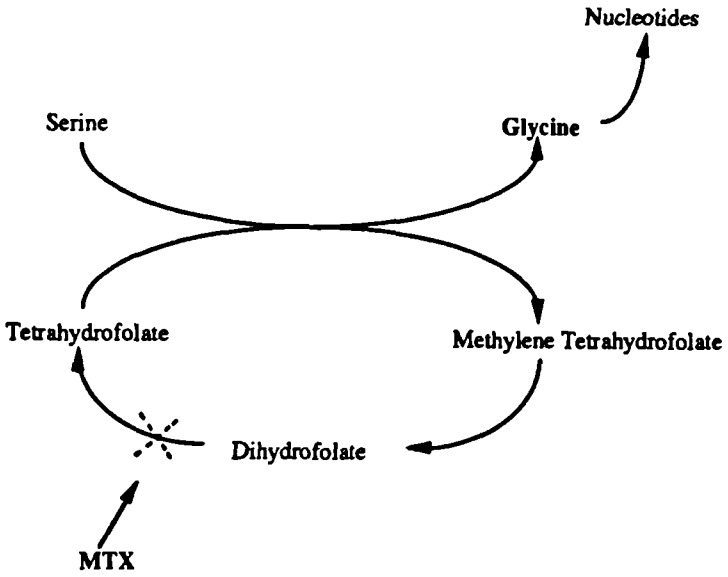


Figure 3.6- Synthesis of glycine from serine and tetrahydrofolate.

The presence of methotrexate in the culture medium may depress the synthesis of tetrahydrofolate from dihydrofolate and the production of glycine by the cells can be blocked due to a lack of tetrahydrofolate. Furthermore, CHO cells deficient in DHFR have been reported to require glycine and thymidine for growth (Urlaub and Chasin, 1980). In order to investigate if that initial effect was due to an overcoming of any possible MTX limitation by an increase in glycine concentration, MTX was omitted from the culture medium (I - no MTX) and cell growth under that conditions was compared against the initial supplementation with the positive group (II) (Table 3.10). The nutrients included in the positive group were sodium pyruvate, glycine, glutamate, phenylalanine/tyrosine and proline/hydroxyproline/histidine.

Table 3.10- Effect of MTX and the positive group on CHO cell growth.

Experiment	Specific growth rate (h^{-1})	Viable cell titre (10^5cells/ml)
I	0.019 ± 0.001	8.2 ± 0.4
II	0.024 ± 0.001 (26%)	11.6 ± 0.1 (41%)
I (no MTX)	0.021 ± 0.000 (10%)	9.8 ± 0.2 (19%)

I- control culture; I (no MTX)- control culture without MTX;

II- supplementation with the positive group of variables (except BSA).

The values in parenthesis indicate percentage increases above the control culture.

An increase in viable cell number and, to a lesser extent, in specific growth rate were obtained when MTX was not present in the culture medium. However, the effect was not the same as supplementing the culture medium with the positive group of variables. This result suggests that overcoming any possible limitation, caused by the presence of MTX, by increasing the concentration of glycine may account for part of the effect observed. This may partially explain why the initial addition of glycine to the cell culture medium would be beneficial. However, no precise conclusion can be drawn as methylene tetrahydrofolate is also involved in other nucleotide biosynthetic pathways, namely the methylation of deoxyuridylate to deoxythymidylate (Stryer, 1981), and the improvement in cell growth in the absence of MTX could also be related to this fact.

3.5.2- Fed-batch cultures with the positive group of components

In an attempt to further understand the reasons underlying the growth stimulating properties of the positive group of variables, the effect of different feeding strategies of those nutrients on

cell growth was investigated, with a view to improving the culture conditions. The nutrients included in this group were the same as in section 3.5.1 (positive group except BSA). Additions of these nutrients to shake-flask cultures were made as indicated in Table 3.11.

Table 3.11- Scheduled feeding of the positive group of variables to stirred batch cultures.

	Experiment					
Time (h)	I	II	III	IV	V	VI
0	-	+	-	-	-	*
24	-	-	+	-	-	*
48	-	-	-	+	-	*
72	-	-	-	-	+	*

(+) indicates addition at the high level (1 x 100% increase);

(*) indicates addition at 25% of the high level (100% in 4 x 25% aliquots).

The effect of the different time additions on CHO cell growth is shown in Table 3.12. The stimulating growth effect was marked if the concentration of those variables was increased at the beginning of the culture, with approximately 38% increase in both specific growth rate and viable cell titre (II). Intermittent feeding, with only a 25% increase in the initial concentration of these nutrients (VI) was slightly less effective, but still showing ca. 25% increase of the same parameters above the control culture. A small improvement in cell yields, ca. 10%, was apparent when the additions were made later in culture, but it is arguable whether that difference is significant. All the cultures entered the death phase at approximately the same time, ca. 100 h.

Table 3.12- Effect of different feeding strategies of the positive group on CHO cell growth.

Experiment	Specific growth rate (h^{-1})	Viable cell titre (10^5cells/ml)
I	0.016 ± 0.001	6.6 ± 0.3
II	0.022 ± 0.001	9.1 ± 0.3
III	0.018 ± 0.001	7.5 ± 0.2
IV	0.016 ± 0.001	7.2 ± 0.5
V	0.017 ± 0.001	7.2 ± 0.4
VI	0.020 ± 0.002	8.5 ± 0.5

The fact that cell growth stimulation was only pronounced if the concentration of the positive group was increased at the beginning of the culture suggested that the effect was not due to limitation by those nutrients; any effect resulting from additions made later in the culture might have been expected if this was the case. The increase in cell yields is probably due to an initial stimulating effect on specific cell growth rate rather than to the higher availability of those nutrients at any other time of the culture. Regular additions (starting after 2 days) of the essential amino acids to glutamine fed-batch cultures has been shown to promote a marked increase in hybridoma cell growth and monoclonal antibody production (Duval *et al.*, 1991). However, those amino acids were not only rapidly consumed by the cells, but some of them were depleted rapidly from batch cultures. Luan *et al.* (1987) also reported prolonged viability of hybridoma cells and increased monoclonal antibody production by additions of essential amino acids towards the end of a batch culture, but no amino acid quantification was presented. In the work reported by Duval and co-workers (1991) there was an obvious nutrient limitation, but in the present study amino acid analysis did not show any depletion of the detectable components included in that group. At this point it might be reasonable to question if there is any optimal concentration range of any component and if values falling outside that range do not stimulate cell growth equally well. Similarly, it seemed to be more advantageous

to keep the concentration of the positive variables at a high level in the beginning of the culture and to investigate some other possibilities to extend cell growth and IFN-γ production.

3.6- Concluding remarks

In this study, an early investigation into the effects of 20 nutritional variables selected from a developed serum-free culture medium was undertaken with a statistically designed experiment. If this approach was not adopted, it would be necessary to change each of those nutrients independently in order to determine their effect on CHO cell growth and IFN-γ production. A complete factorial design for the screening of that number of variables at two different levels would require 2^{20} experiments; in other words, it would not be feasible. Such a complete factorial experiment would account for all the possible interactions between variables, but most of the time high-order interactions tend to be very small. The Plackett-Burman design was used for the purpose of identifying important variables for both CHO cell growth and IFN-γ production, while quantifying their effect on the same parameters. That extraordinarily high number of experiments was thus reduced to 24.

Based on the positive variables identified with the Plackett-Burman design a significant improvement of CHO cell growth and IFN-γ production (approximately 40%) was achieved when the concentration of the nutrients BSA, glycine, glutamate, sodium pyruvate, tyrosine/phenylalanine and proline/hydroxyproline/histidine, was increased in the culture medium. The effect was only marked if the increase was made in the beginning of the culture and it did not seem to be related to nutrient depletion; there was some evidence that changes in glucose and glutamine metabolism contributed to that effect. The glycosylation pattern of IFN-γ was not affected by an increase in the initial concentration of the positive nutrients in the culture medium.

Following the identification of key independent variables with the Plackett-Burman procedure, it would seem reasonable to establish their optimum concentrations, and their interactions. However, no approaches of this kind were taken in this study, because it was decided that other points also raised from that statistical analysis merited further investigation. First, it was evident that glutamine and glucose were strong candidates for cell growth limitation and simply increasing their initial concentration in the medium was not an efficient methodology; the question arose of how to efficiently provide the cells with both of these nutrients (Chapter 4). Second, BSA was shown to be a very important component of the medium, but the complex behaviour of CHO cell growth and IFN-γ production towards different sources of BSA and the awareness of the implications of its presence in the culture medium called for a more detailed analysis of its role(s), rather than a search for its optimum concentration (Chapter 5).

4- FED-BATCH FERMENTER CULTURES

4.1- Introduction

Based on the Plackett-Burman analysis the initial medium composition was modified to improve cell growth and IFN-γ production. In the previous Chapter, the positive group of variables was also further subdivided into BSA and amino acids plus sodium pyruvate. It was shown that the stimulating growth effect of sodium pyruvate plus amino acids was independent of BSA. Although BSA proved to be a major component *per se* for both CHO cell growth and IFN-γ production, it was not initially included in the positive group due to the variability it introduces into the culture medium. There are other reasons not to increase its concentration in the culture medium; it was further analysed as a separate component (see Chapter 5).

The conditions of the culture medium deteriorate during a batch culture and cell growth usually becomes limited by nutrient depletion or by the accumulation of toxic components, which may become the bottleneck of the process. Analysis of nutrient consumption in the previous Chapter has shown that glutamine was depleted early in the culture, after approximately 75 h, while glucose was also used extensively and depleted in the later stages; both conditions could be related to the onset of the stationary phase. Final concentrations of ammonia and lactate obtained in batch culture, ca. 1.7 mM and 13 mM respectively, should not be inhibitory to the cells as 2 mM ammonia and 17.5 mM lactate have shown no effect on the growth of this cell line (Hayter *et al.*, 1991a). However, cell growth limitation by either glucose and/or glutamine starvation was a possibility to consider. Simply increasing the initial concentration of these nutrients in the culture medium is often unsuccessful due to their inefficient utilisation and an increase in the accumulation of inhibitory metabolites. This strategy has been investigated for this cell line by increasing initial glutamine and glucose

concentrations to 4 mM and 22 mM respectively (Hayter *et al.*, 1991a), and no improvements on cell growth or IFN-γ production were obtained; nutrient utilisation seemed to be less effective, as indicated by the higher glucose and glutamine uptake rates. Higher initial concentration of glutamine also lead to an increase in ammonia production. Glucose and glutamine were not identified as positive variables by the Plackett-Burman analysis although they were depleted early in culture; this may be also related to inefficient utilisation of these nutrients at higher initial concentrations. As discussed in section 1.3.2.1, ammonia and lactate are major products of glutamine and glucose metabolism, respectively. Controlled feeding of these components in order to reduce ammonia and lactate accumulation has been previously reported (Glacken *et al.*, 1986; Glacken, 1988; Ljunggren and Häggström, 1990). A significant reduction in ammonia production with a simultaneous increase in cell yield of MDCK cultures has been achieved by controlled feeding of glutamine, which was maintained at low levels throughout the culture (Glacken *et al.*, 1986).

The nutritional environment of the cells may affect the glycosylation patterns of a recombinant protein. A decrease in the proportion of the 2N-glycoform of IFN-γ was previously observed during batch culture (section 3.4.1). This shift follows the decline in glucose levels and rapid depletion of glutamine from culture. Glucose-limited chemostat cultures of these cells have shown an increase in the proportion of the 2N form of IFN-γ following transient periods of excess glucose (Hayter *et al.*, 1992), suggesting that glycosylation could be affected by the availability of this nutrient. However, studies from batch cultures with high initial concentration of glucose (22 mM) did not have the same effect on protein glycosylation (Hayter *et al.*, 1991b; Curling *et al.*, 1990) indicating that factors other than the availability of glucose *per se* could be affecting the glycosylation pathway. It has also been reported that CHO cells utilise an alternate glycosylation pathway when deprived of glucose, and this was thought to be due to depletion of nucleotide sugar precursors, leading to the synthesis of glycoproteins with truncated oligosaccharides (Rearick *et al.*, 1981). This is supported by the

early suggestion that the major function of aerobic glycolysis is to maintain high amounts of glycolytic precursors for macromolecule synthesis (Hume *et al.*, 1978).

The glycosylation biosynthetic pathway has been already described in section 1.4.1. It starts with the synthesis of a lipid-linked oligosaccharide, containing glucose, mannose and N-acetylglucosamine. The oligosaccharide processing reactions in the Golgi compartments yield complex oligosaccharide structures and use uridine-diphosphate (UDP), guanosine diphosphate (GDP) and cytidine monophosphate (CMP) as co-substrates (e.g. UDP-N-acetylglucosamine, CMP-sialic acid). It is possible that the supply of glycosylation precursors, such as aminosugars and nucleotides, are a limiting step for that pathway. Both glucose and glutamine are involved in the biosynthesis of these precursors. Ribose-5-phosphate, primarily formed by the pentose-phosphate pathway, and the amino group of glutamine are both necessary for the synthesis of purine and pyrimidine nucleotides. Glucose and/or glutamine depletion from the culture medium may thus constitute a block for the efficient glycosylation of the protein and it is a subject that needs to be addressed.

In this Chapter, glucose and glutamine fed-batch cultures were used in order to avoid the depletion of these nutrients from the culture, while keeping them at a concentration which would not allow excessive production of inhibitory metabolites. Cell growth, cell metabolism and IFN-γ production under these conditions were investigated. At the same time, the possibility that glucose and/or glutamine availability in culture affects the glycosylation of IFN-γ was evaluated.

4.2- Cell growth and IFN-γ production in fermenter cultures

Cell growth and IFN-γ production in fermenter cultures were first characterised. The analysis was done with a control culture (I) using the original growth medium and a culture with increased concentrations of the positive variables (II). The effects of the group of positive variables, including BSA, sodium pyruvate and amino acids, was thus tested under conditions of controlled dissolved oxygen and pH. Again, a significant improvement of CHO cell growth and IFN-γ production was achieved (Figure 4.1a,b) when compared with the control experiment, although the increase in IFN-γ titres was lower than what was previously observed in shake-flask cultures (ca. 25-30%). In both cases, a decrease in IFN-γ production was noticed before the cells reached the stationary phase.

4.3- Glucose and glutamine fed-batch cultures

The effect on cell growth and IFN-γ production of feeding glucose and glutamine to fermenter cultures was then investigated. These studies used as the basal medium the original growth medium (I) supplemented with the positive group, sodium pyruvate and amino acids, excluding BSA for the reasons mentioned previously; this latter became the control culture for the different feeding strategies used in this Chapter (culture II). In the following sections, the profiles of glucose and glutamine under the different culture conditions are first characterised, after which cell growth and IFN-γ production in relation to titre and quality are addressed. Further considerations on the behaviour of the cultures in relation to glucose and amino acids metabolism are also made.

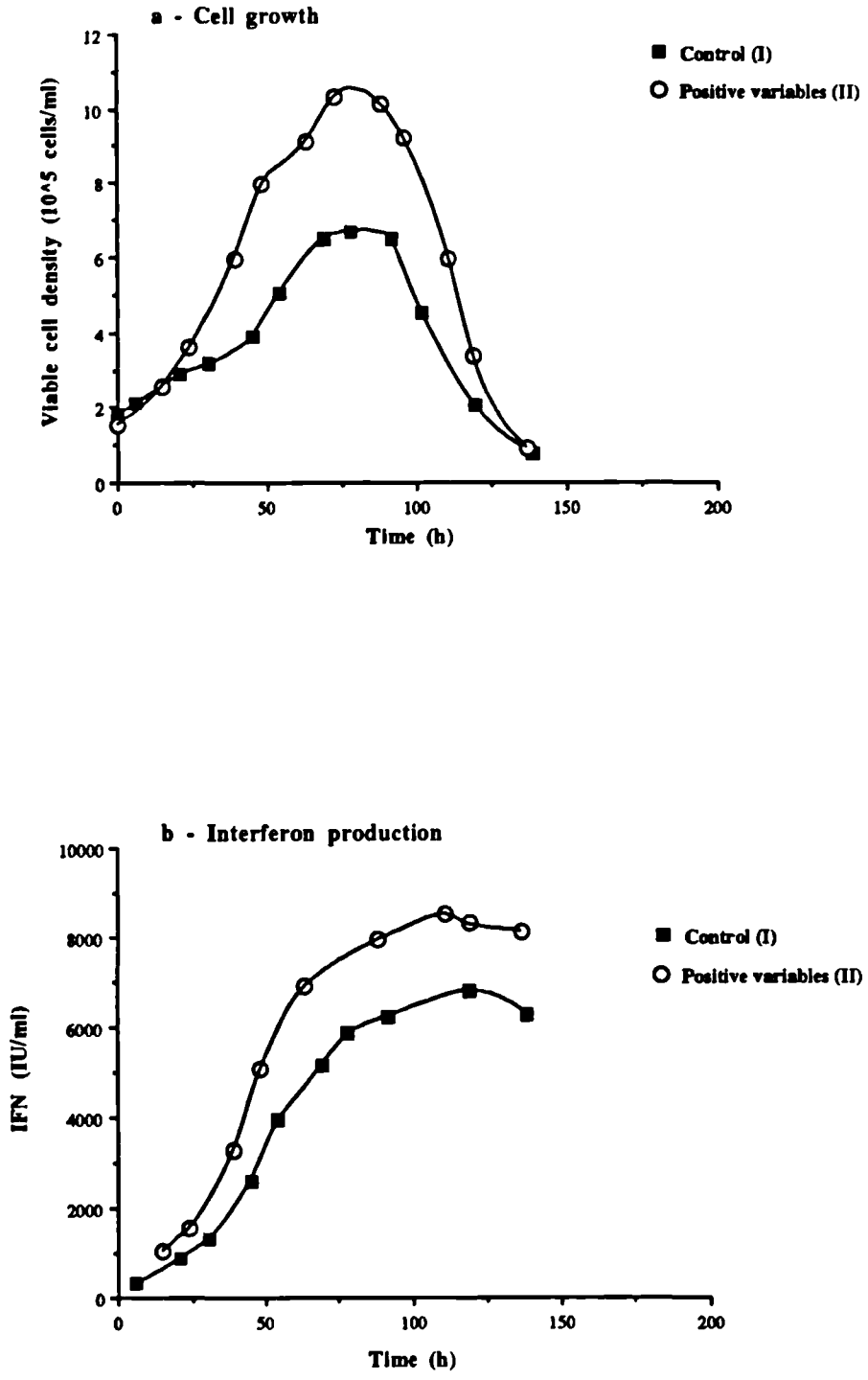


Figure 4.1- a) Cell growth and b) IFN-γ production in control fermenter cultures and cultures containing the positive group of variables.

4.3.1- Glucose and glutamine profiles

Fed-batch additions of glucose and glutamine were performed in fermenter cultures, with controlled pH and dissolved oxygen. An initial estimate for the feeding rates was based on the nutrient analysis performed and reported in Chapter 3. The feeding regimes used are shown in Table 4.1. Feeding rates were calculated based on the consumption rates for glucose and glutamine observed in the experiments earlier described (section 3.4.2). The amount added to the first fed-batch culture (FF1) was estimated in order to keep the concentration of those nutrients above 5 mM and 0.5 mM, for glucose and glutamine respectively, considering an average population of approximately 7.5 x 10^5 cells/ml. The additions were made 6 times in each 24 h period, in order to avoid large variations in the concentration profile. The profiles obtained for this culture were then used to set up the other cultures (FF2 and FF3). Initial concentration of glucose was the same in all the runs (ca. 11mM), while initial glutamine was set at two different concentrations, 2 and 0.5 mM. The latter was used to compensate the expected increase in ammonia production by feeding glutamine to the 2 mM cultures. All the cultures had increased concentrations of the positive group of variables at the beginning of the culture.

Table 4.1- Feeding regime of glucose and glutamine to fermenter cultures.

	Initial concentration (mM)		Increment (mM/addition)	
	GLU	**GLN**	**GLU**	**GLN**
II	11	2	-	-
FF1	11	2	0.44[a]	0.156[a]
			0.22[b]	0.078[b]
FF2	11	0.5	0.30[c]	0.109[c]
FF3	11	0.5	0.22[c]	0.078[c]

a- from 21 to 84 h; b- from 84 h; c-from 4 h.

The concentration profiles of glucose and glutamine for the different fermenter modes are shown in Figures 4.2a,b,c and 4.3a,b,c, where the actual and potential (amount present in culture if there was no consumption) concentrations during culture are shown. The potential concentration profiles show the different quantities of glucose and glutamine that each culture received. As all the cultures started with the same level of glucose, the total amount (potential) in a culture was dependent only on the different feeding rates and did not vary much, 18 to 20 mM, among the cultures (Figure 4.2a,b,c). On the other hand, different initial glutamine concentrations were used, 2 mM in FF1 as in the control culture and 0.5 mM in FF2 and FF3, and as a result the total amount added to the later ones, 3.5 and 4.5 mM respectively, was lower than FF1, 5.5 mM, but still higher than the control culture (Figure 4.3a,b,c). Apart from the control culture (II), to which no additions were made, glutamine was never depleted from the culture, while glucose was always kept above 5 mM. At 100 h the level of glucose in all the fed cultures was approximately 7.5 mM, as compared to 2.5 mM in the control culture (Figure 4.2). At the same time point, glutamine was depleted from the control culture, while the levels for the fed cultures were ca. 1mM, 0.6 mM and 0.3 mM for FF1, FF2 and FF3 respectively (Figure 4.3). The cumulative consumption of glutamine and glucose is shown in Figure 4.4a,b,c. After approximately 70 h, all the glutamine of the control batch culture (II) was consumed, while the consumption for FF1 at the same point was higher, ca. 2.7 mM, and for FF2 and FF3 lower, ca. 1.7 and 1.5 respectively. Consumption of glucose at the same point was just below 5 mM for the batch culture, and varying from 5.5 to 7.5 for FF1, FF2 and FF3 (increasing order). In all the fed-batch cultures, significant consumption of glutamine and glucose continued until viable cell titre reached a low value.

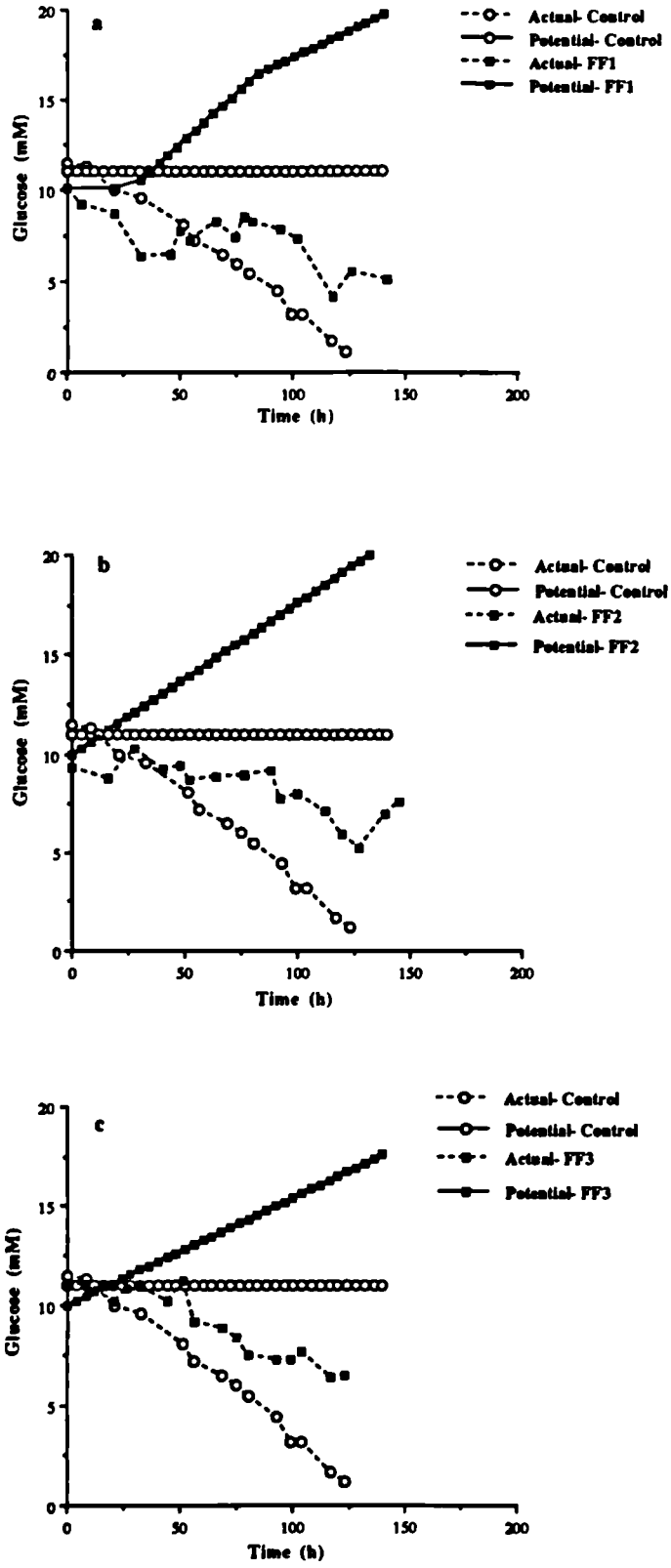


Figure 4.2- Glucose concentration profile in fed-batch cultures a) FF1, b) FF2 and c) FF3. Growth curves for the same cultures are found in Figure 4.5.

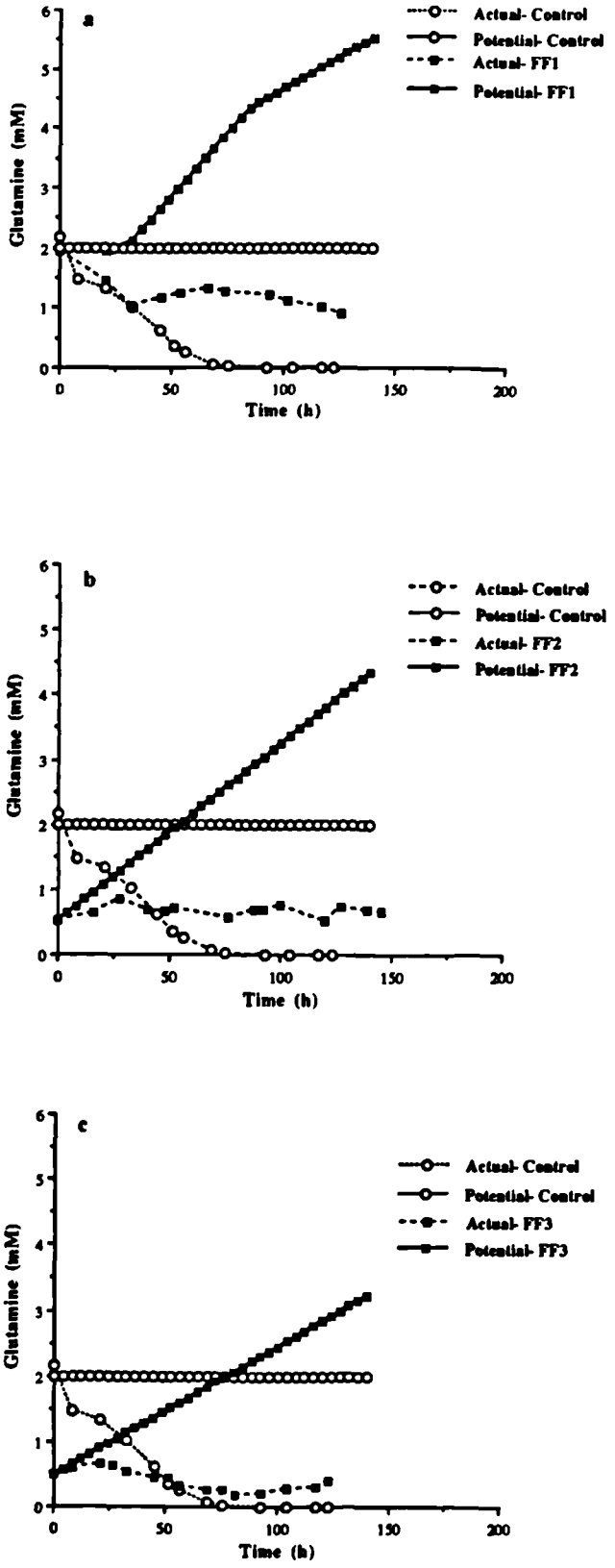


Figure 4.3- Glutamine concentration profile in fed-batch cultures a) FF1, b) FF2 and c) FF3.

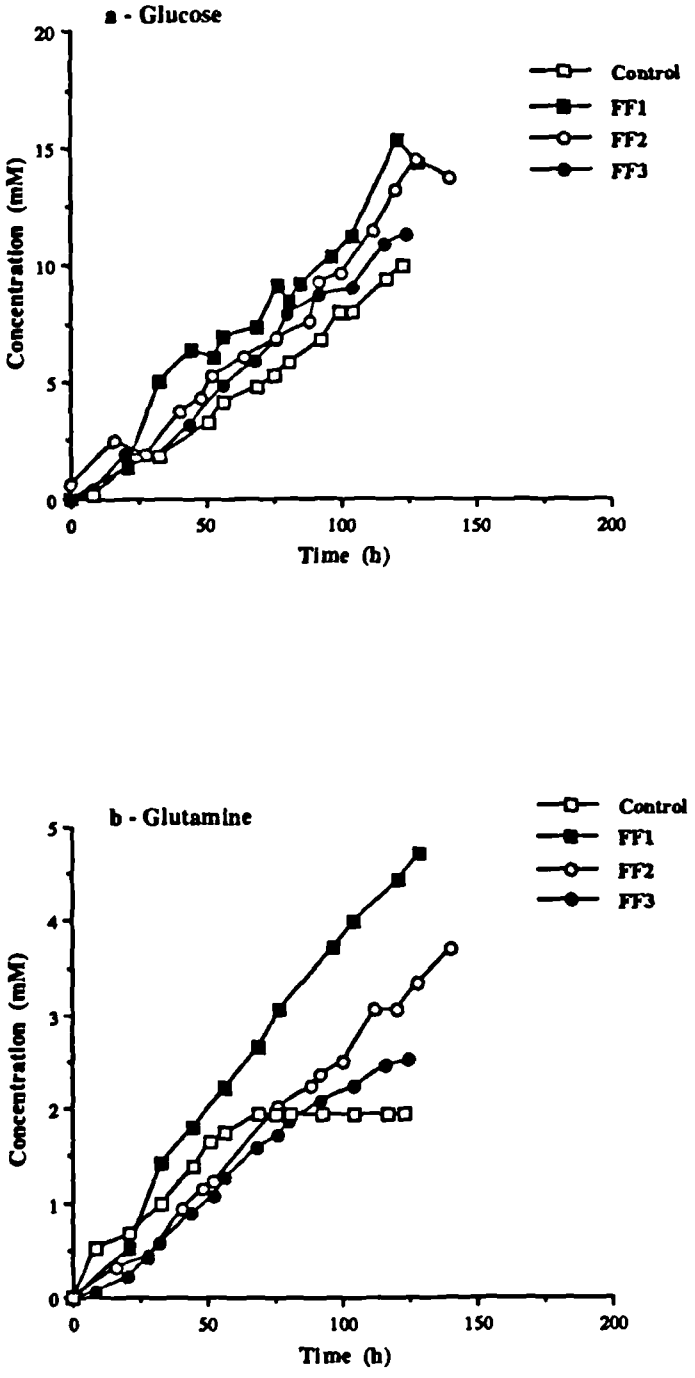


Figure 4.4- Cumulative consumption of a) glucose and b) glutamine in fed-batch cultures.

4.3.2- Cell growth and IFN-γ production

Cell growth and IFN-γ production obtained in the fermenter cultures are shown in Figure 4.5a,b,c. Cell growth in culture II was superior to that obtained in the control culture (I) but lower than obtained when BSA was also present, as was described in the previous section (see Figure 4.1a). In terms of IFN-γ production, there was still an increase over the control culture and titres were not much lower than what had been achieved with extra supplementation of BSA in fermenter culture (Figure 4.1b). Feeding of glucose and glutamine did not succeed in extending the growth phase of the cells (Figure 4.5a) and thereafter cells died at about the same rate as was observed in control cultures (Figure 4.5b). In cultures with lower initial glutamine concentration (FF2 and FF3) a slight increase in viable cell concentration (ca. 12%) and a small delay in the onset of the stationary phase was noticed. Initial specific growth rates were all similar (Table 4.2). IFN-γ production patterns were also very similar under the different modes (Figure 4.5c); the levels achieved were all of the same order of magnitude and little difference in production rates was observed (Table 4.2), although a slight increase was noticed for FF3 (ca. 11%). IFN-γ production was parallel to cell growth and as in batch cultures it stopped simultaneously with cell growth, also suggesting that production is growth associated.

Table 4.2- Specific cell growth and IFN-γ production rates in fermenter cultures.

	μ (h^{-1})	q_{IFN} ($IU/10^6 cells/h$)
II	0.028	188
FF1	0.028	193
FF2	0.029	200
FF3	0.030	209

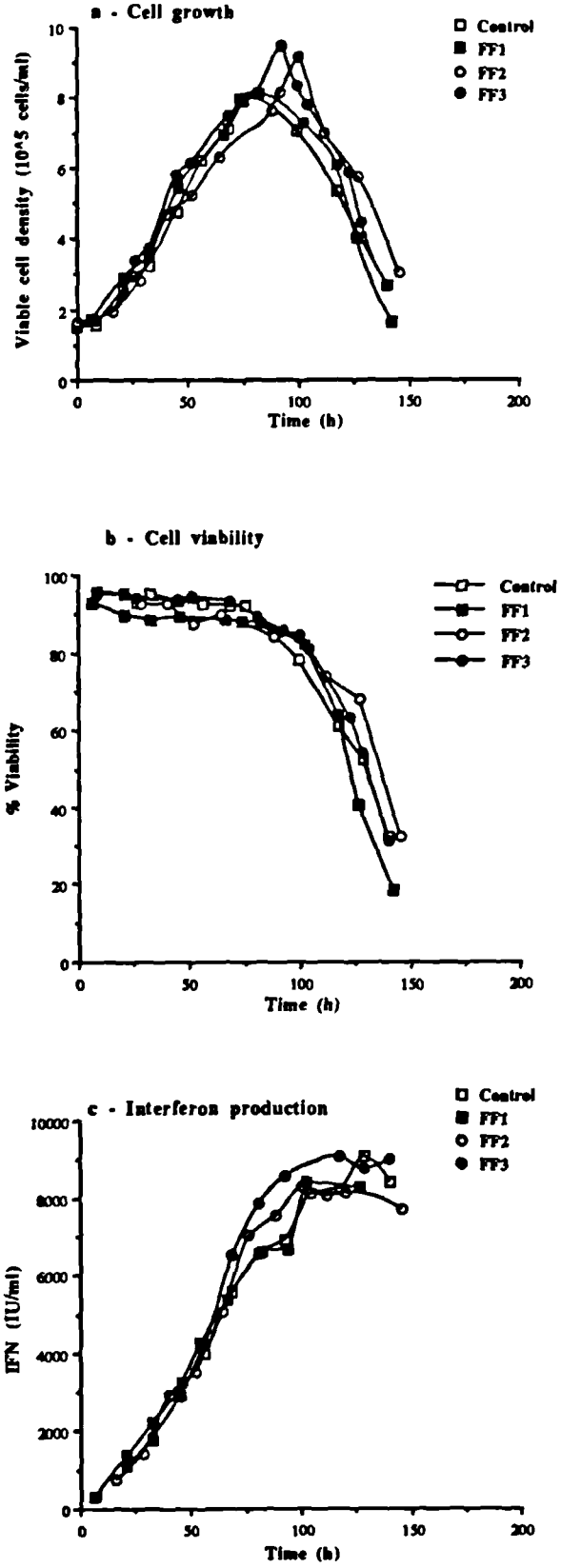


Figure 4.5- a) Cell growth, b) cell viability and c) IFN-γ production in fed-batch cultures.

Cell growth is affected by the availability of glucose and glutamine in the culture medium and with this cell line, a decrease of the glutamine medium concentration from 2 mM to 1 mM reduced cell growth by ca. 25%, and cells were not able to grow in the absence of glutamine (Hayter *et al.*, 1991a). The fact that both glucose and glutamine were available throughout the fed-batch cultures did not result in any net positive effect on cell growth and IFN-γ production, thus other factors have to be implicated in arresting growth and production. Butler and Spier (1984) reported an increase in BHK cell growth on increasing initial glutamine concentration from 1 mM to 4 mM but a further increase to 8 mM became inhibitory to cell growth. This seemed to be related to an increase in ammonia concentration from 2.2 mM to 3 mM. Daily additions of glucose and glutamine to hybridoma cultures have also been reported to extend hybridoma cell viability and to increase antibody production by at least 60% (Miller *et al.*, 1988; Reuveny *et al.*, 1986). In both cases the increase in antibody production was due to an increase in the number of viable cells. Cell death was suggested to be due to an increase in ammonia levels in those cultures.

Cell growth inhibition by ammonia and/or lactate was a possibility to consider. Figure 4.6a,b shows the accumulation of both metabolites during the cultures. The final levels of ammonia and lactate in the cultures fed with glutamine and glucose were higher, reflecting the continuous utilization of both glutamine and glucose during the whole culture period (Figure 4.4a,b,c). Approximately 3.5 mM ammonia was present by the end of culture FF1 and 2.5 mM by the end of FF2 and FF3, as compared to 1.7 mM in the control culture, level which stopped to increase after glutamine depletion (ca.75 h). Lactate levels reached ca. 18mM in all the fed-batch cultures, compared to ca. 14 mM in the control culture. The level of both ammonia and lactate at the point when growth stopped in each run is shown in Table 4.3.

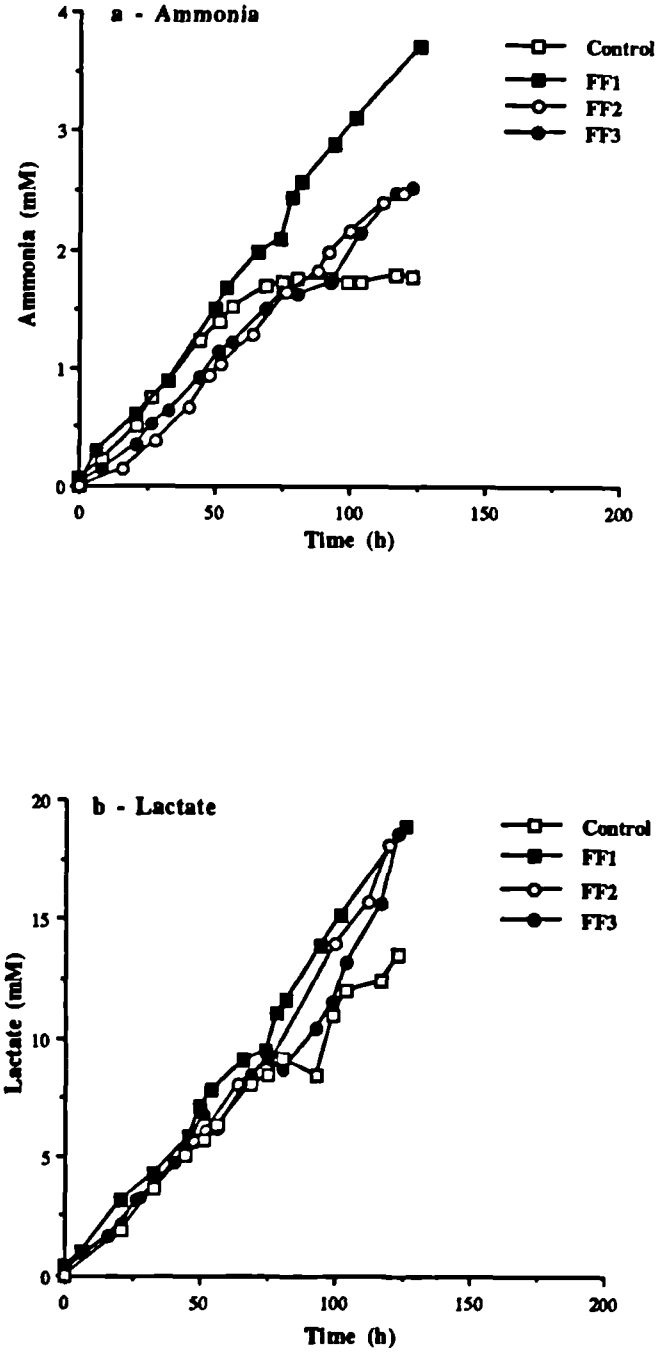


Figure 4.6- Accumulation of a) ammonia and b) lactate in fed-batch cultures.

Table 4.3- Ammonia and lactate levels at cessation of cell growth in fermenter cultures.

	Ammonia (mM)	Lactate (mM)
II	1.72	8.48
FF1	2.56	11.6
FF2	2.15	14.0
FF3	1.73	10.4

a- concentration in culture when maximum cell titre were achieved.

The ammonia level present in culture in FF1 at the cessation of growth (2.50 mM) could have been inhibitory for cell growth but the same hypothesis can not be put forward for FF2 and FF3 (2.15 and 1.73 mM respectively). CHO cells have a quite high tolerance for lactate and none of the cultures achieved sufficiently high levels to cause growth limitation (Hayter *et al.*, 1991a). The decrease in cell count at high residual glucose and glutamine concentration observed in FF1 possibly could be due to ammonia accumulation but the same can not be said for FF2 and in particular for FF3, where ammonia levels were very low.

In order to detect any possible nutrient limitation, the percentage of each amino acid remaining in culture when cell growth stopped was determined. Amino acid analysis revealed that none of the detectable amino acids were exhausted from the culture at that point. Figure 4.7 shows the remaining percentage of essential amino acids and aspartate; a smaller percentage was found in FF3 and in the control culture for most of them, but no depletion was found. Alanine and glutamate were produced during the cultures and the consumption/production of serine and glycine depended on the feeding regime used, but they were also not depleted from culture. The consumption/production patterns of these latter amino acids are further discussed in this Chapter. Proline, hydroxyproline and cystine are amino acids not detectable by the

assay used and limitation by any of these can not be excluded. The first two are included in the positive group of variables and considering the results obtained in the previous Chapter (section 3.5.2), limitation by any of these is most unlikely. There are no data for cystine but specific consumption rate of this amino acid for two hybridoma cell lines have been reported to be of the same order of magnitude of the other essential amino acids (Duval *et al.*, 1991). As the values obtained in the latter study were all comparable to the ones presented in this section it is possible that there is also little or no cystine depletion in the present culture, although a direct comparison can not be made.

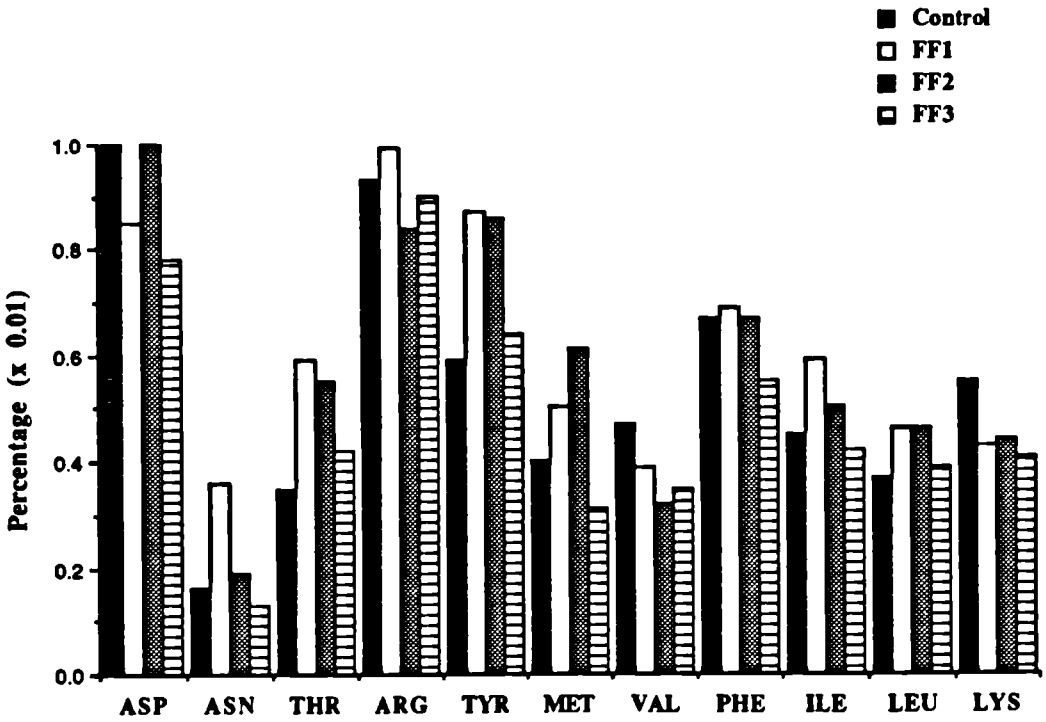


Figure 4.7- Percentage of each amino acid remaining in fed-batch cultures at the cessation of cell growth.

4.3.3- IFN-γ glycosylation patterns

The glycosylation pattern of IFN-γ obtained under each of the feeding conditions was determined. The proportions of each of the glycoforms is shown in Figure 4.8a-d. No major difference was detected between the cultures with high initial glutamine concentration (4.8a,b). The initial proportion of the non-glycosylated form in the control culture was 5 to 8% and only increased late in the culture (after 100 h). About 70% of the product was 2N-glycosylated by the beginning of the culture, but this decreased to about 50% after 100 h in the culture, with a simultaneous increase in the 1N-glycoform. No significant changes were found for the non-glycosylated form for FF1 and a similar trend was obtained for the 1N-glycoform and 2N-glycoform. A lower proportion of the 2N glycoform (ca. 50%) and a higher proportion of the non-glycosylated form (ca. 15%) was detected in the beginning of both FF2 and FF3, which had 0.5 mM initial glutamine concentration. The proportion of the non glycosylated form decreased during the fermentation to a steady value of approximately 5-7%, after ca. 75 h in culture with a simultaneous increase in the proportion of the 1N-glycoform. The proportion of each of the glycoforms in the beginning of the culture did seem to be a function of the initial concentration of glutamine. Both in FF2 and FF3, with 0.5 mM initial glutamine concentration, the proportion of the non-glycosylated form in the beginning of the culture was higher while the proportion of the 2N-glycoform was lower than in a typical batch culture or in the fed-batch culture which had the same initial glutamine concentration, 2 mM (FF1). The difference between these runs was only the concentration of glutamine in the beginning of the culture, as glucose concentration always commenced at the same initial level. Glutamine is an important nitrogen donor for several biosynthetic pathways, such as nucleotide synthesis, and a reduced flux through glutaminolysis may reduce the supply of important precursors to some of the pathways. This may be what is happening with the glycosylation pathway. An initial glutamine concentration of 0.5 mM may not be enough to cope with the cell demand for biosynthetic precursors which rely on that component.

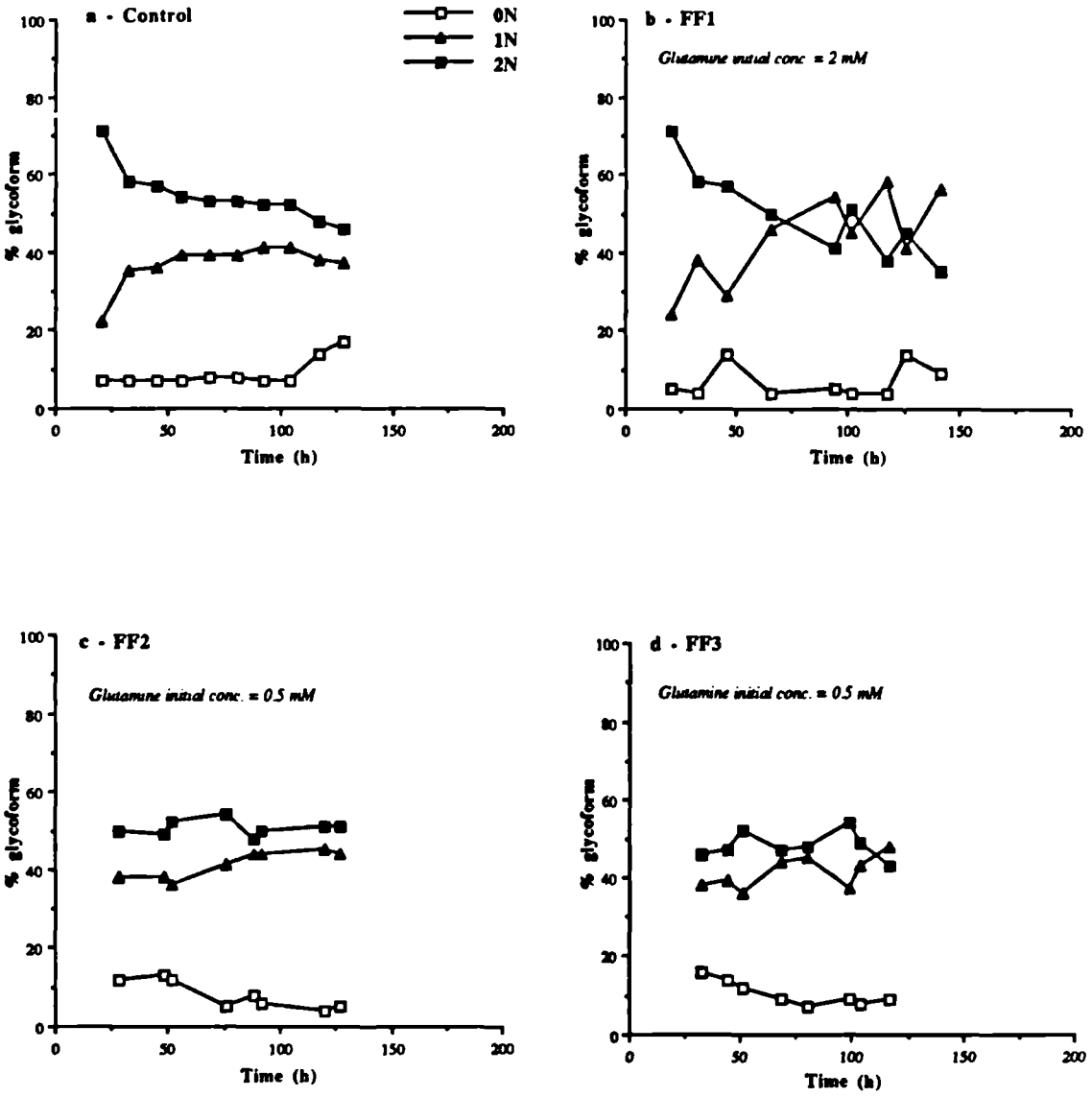


Figure 4.8- Glycosylation patterns of IFN-γ obtained from fermenter cultures with a,b) 2 mM initial glutamine and c,d) 0.5 mM initial glutamine concentration.

While the initial glutamine concentration seemed to affect the initial glycosylation profile of IFN-γ, the availability of glucose and glutamine throughout the culture did not prevent the typical gradual decrease in the proportion of the double glycosylated form of IFN-γ observed in batch cultures, as indicated by the patterns obtained in FF1. Indeed, after ca. 70 h, the IFN-γ patterns were similar in all the cultures independent of the glucose and glutamine available in the culture. Again there was evidence that the availability of glucose *per se* may not directly influence the glycosylation pathway. The results obtained here seem to support the suggestion that the major function of aerobic glycolysis or glutaminolysis is to maintain high amounts of glycolytic precursors for macromolecule synthesis. It is possible that there is an optimal range of concentrations for glucose and/or glutamine that allows a constant production of the glycosylated forms. In practice, it is not possible to keep glutamine at the initial level, 2 mM, throughout the culture due to inhibition problems from ammonia.

4.4- Metabolic flux changes in batch and fed-batch cultures

Feeding glucose and glutamine to the cultures may alter the metabolic pathways used by the cells. As discussed in section 1.3.2.1 there is a close relationship between the metabolism of glucose and glutamine and other nutrients which belong to the same metabolic pathway system or are indirectly affected by glucose or glutamine. The major routes for glucose and glutamine utilisation are illustrated in Figures 4.9 and 4.10. Production of glutamate, alanine, serine, aspartate and ammonia are closely associated with glutamine metabolism. Lactate, serine and alanine are related to glucose metabolism. Changes in cell metabolism under glucose and glutamine feeding were investigated by analysing the evolution of these components throughout the different cultures.

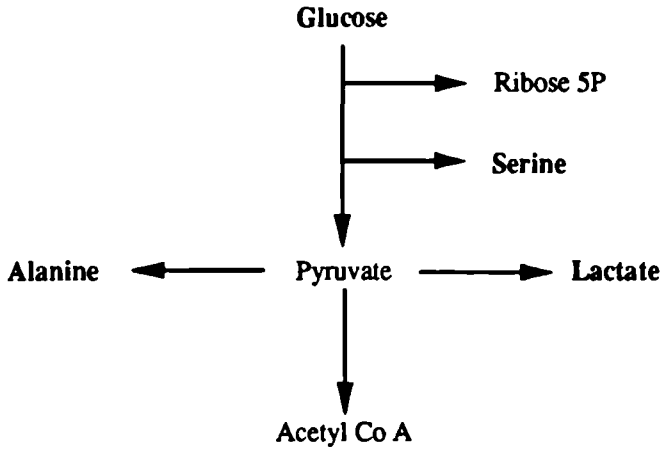


Figure 4.9- Major routes of glucose metabolism.

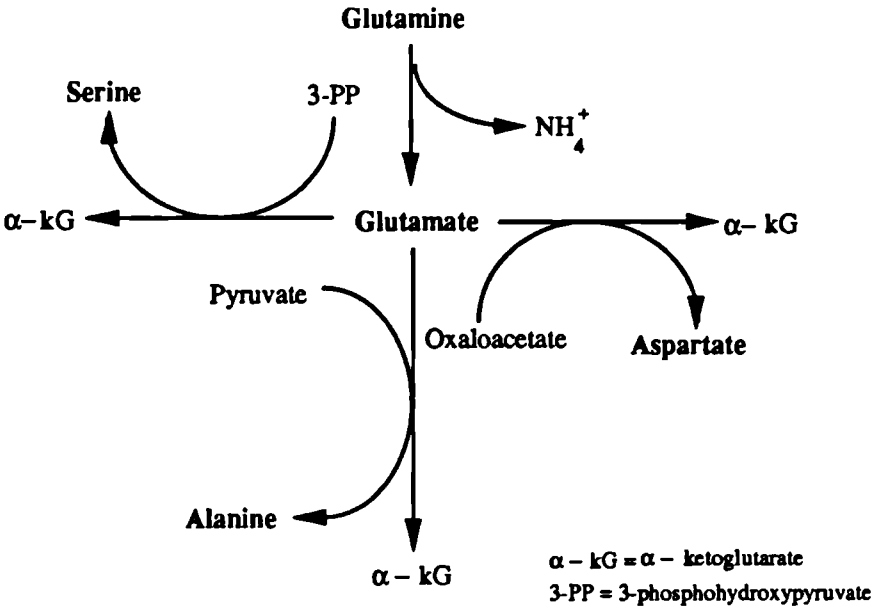


Figure 4.10- Major routes of glutamine metabolism.

4.4.1- Glucose and glutamine metabolism

The parameters determined to describe glutamine and glucose utilisation and the formation of ammonia and lactate are shown in Table 4.4. In the fed cultures consumption of glutamine (q_{gln}) and glucose (q_{glc}) were found to be a function of the glutamine and glucose level throughout the culture, with feeding strategy FF1 showing the highest rates. While an increase in the initial glutamine consumption rates was observed for FF1, a decrease in the same rate was observed in cultures with lower (0.5mM) initial glutamine concentration (FF2 and FF3). There was an increase in glucose consumption rate in both FF1 and FF2. Initial production rates of ammonia and lactate varied in the same way with glutamine and glucose consumption rates, respectively. The yields of lactate on glucose and ammonia on glutamine were similar from run to run.

The data obtained from these cultures allow a few considerations about the behaviour of the system. The increase in glutamine levels in FF1 led to an increase in both initial glutamine uptake rate and ammonia production rate while a decrease of glutamine levels in FF2 and FF3 resulted in a decrease of those rates. This behaviour is in accordance with what has been previously reported (Glacken *et al.*, 1986; Miller *et al.*, 1989b; Hayter *et al.*, 1991a; Ljunggren and Häggström, 1992) and at the point where cell growth stopped in the FF3 culture ammonia levels did not exceed those normally obtained in batch culture. Glucose levels were higher in all the fed-batch cultures, decreasing from FF1 to FF3 according to the feeding rates. For FF1 and FF2 an increase in both initial glucose uptake rate and lactate production rate was observed, but those values were similar for the FF3 and the batch cultures. An increase in glucose uptake rates at higher glucose concentrations has been also reported by other researchers (Zielke *et al.*, 1978; Reitzer *et al.*, 1979, Miller *et al.*, 1989a).

Table 4.4- Metabolic quotients for glucose and glutamine in fed-batch cultures.

		II	FF1	FF2	FF3
q_{gln} (nmol/10^6cells/h)	a	72.0	89.3	64.1	50.5
q_{glc} (μmol/10^6cells/h)	a	0.191	0.246	0.239	0.188
q_{amm} (μmol/10^6cells/h)	a	0.059	0.071	0.051	0.048
q_{lac} (μmol/10^6cells/h)	a	0.264	0.301	0.302	0.281
$Y_{amm/gln}$ (mol/mol)	a	0.77	0.79	0.79	0.86
	b		0.84	0.75	1.19
$Y_{lac/glu}$ (mol/mol)	a	1.47	1.26	1.27	1.40
	b		1.33	1.18	1.43

[a]values determined at the end of the exponential growth phase of the cells and [b] from that point until cell viability reached 50%.

4.4.2- Amino acid metabolism

The strong interplay between the various metabolic pathways of the cell was shown by the different amino acid profiles obtained under fed-batch conditions for those which are closely associated with glucose and glutamine metabolism. Significant differences were detected for glutamate, serine and glycine.

Amino acid consumption rates for the exponential growth phase of the cells are presented in Table 4.5. No major differences were detected during that period, except for serine and glycine, which will be examined separately. The essential amino acids showed similar consumption patterns, with leucine and isoleucine having the highest specific consumption rates. However, some of the amino acids seemed to be dependent on glutamine availability, showing lower rates of utilisation with increasing availability of glutamine (FF1 and FF2). Leucine, isoleucine, methionine, threonine and tyrosine are examples of this phenomenon.

Table 4.5- Amino acid initial consumption/production rates in fed-batch cultures.

	Control (II)	FF1	FF2	FF3
		(nmol/10^6cells/h)		
ASP	+0.35	-0.72	-0.70	+0.32
GLU	+6.59	+7.53	+6.52	+6.02
ASN	-10.8	-7.89	-7.85	-9.54
SER	+0.35	+9.32	+1.76	+1.44
GLY	+1.04	-2.15	-0.28	+0.77
THR	-3.12	-1.79	-2.21	-2.82
ARG	-0.69	-1.79	-3.59	-1.54
ALA	+26.0	+29.1	+30.1	+26.3
TYR	-2.77	-1.79	-1.29	-1.92
MET	-1.73	-1.08	-0.80	-1.54
VAL	-2.77	-3.59	-3.39	-2.59
PHE	-1.39	-1.79	-1.97	-1.60
ILE	-6.24	-4.30	-4.95	-5.15
LEU	-7.28	-5.38	-5.78	-6.11
LYS	-3.47	-3.23	-3.16	-3.81

values determined from the exponential growth phase; (+) production; (-) consumption.

The analysis of the complete time course revealed that some non-essential amino acids behaved differently, depending on the feeding regime used. In batch culture a net production of glutamate (Figure 4.11a), alanine (Figure 4.11b), and also serine early in the culture and glycine later in the culture was observed (Figure 4.12a,b,c). Production of alanine, glutamate and serine has also been previously reported for this cell line when grown in glucose-limited chemostat culture (Hayter *et al.*, 1992) and production of alanine, glutamate and glycine for other cell types (Butler and Thilly, 1982; Lanks 1987; Miller *et al.*, 1989a; Duval *et al.*, 1991). The glutamate level ceased to increase in batch culture after complete depletion of glutamine (ca.75 h), while its level steadily increased throughout the culture under fed-batch mode (Figure 4.11a). The main mechanism for the metabolism of glutamine seems to be via

phosphate dependent glutaminase, yielding glutamate and ammonia (Mckeehan, 1986). This net increase in glutamate is most likely a reflection of the continuous uptake of glutamine by the cells. Alanine and aspartate are also closely associated with glutamine metabolism (see section 4.4). Alanine was produced throughout all the cultures (Figure 4.11b), which may reflect transamination of pyruvate. The net production was similar in all of them, although the final levels seemed to be slightly higher under fed-batch mode, specially for FF2 and FF3. Ljunggren and Häggström (1992) also found that under glutamine limited conditions alanine production was substantially reduced. Aspartate profiles showed no significant difference between the different modes; significant proportions of nitrogen derived from glutamate have been also found in aspartate (Ardawi and Newsholme, 1982; Moreadith and Lehninger, 1984). This may explain the accumulation of aspartate in these cultures.

The serine and glycine profiles obtained under fed-batch conditions were different from what was obtained in the control culture (Figure 4.12a,b,c). Typically, serine is produced in the initial stage of the culture while glycine is consumed; this pattern is reversed later in culture, coinciding with glutamine depletion. Under fed-batch mode (FF1 and FF2) the serine level increased throughout the cultures, with a concurrent decrease in glycine level (Figure 4.12a,b); this pattern was most evident in FF1, where glutamine levels were kept at a higher level throughout the culture. Serine and glycine levels remained constant until the later stages (75 h) of the fed-batch process FF3, when a slight increase was seen (Figure 4.12c). As discussed earlier, it is possible that MTX inhibits the pathway for the production of glycine in the beginning of the culture, leading to a decrease in its level and an increase in serine level. The initial accumulation of serine may be also linked with glutamine availability. Serine is synthesised from 3-phosphoglycerate, and its production mechanism involves the transamination of glutamate to α-ketoglutarate. A high rate of serine metabolism has been reported for proliferating cells (Snell *et al.*, 1987) and glycine accumulation by the end of the culture may reflect that metabolism. The fact that under glutamine feeding the level of serine

continuously increased during the culture and that its net accumulation varied with glutamine consumption may suggest that its production is limited by glutamate availability. Glutamine pulses to glucose-limited chemostat cultures of this cell line have been shown to transiently increase the specific rate of serine production in culture (P.M. Hayter, personal communication), which is in accordance with the findings for fed-batch cultures. Transient increases of serine concentration after glucose and glutamine pulses to hybridoma chemostat cultures have also been reported (Miller *et al.*, 1989a,b). The present data do not allow any clear explanation, but the behaviour could also indicate less serine utilisation, as the highest accumulation of serine (FF1) corresponded to net reduction in glycine levels, while the lower accumulation of serine (FF3) corresponded to the highest net production of glycine. Changes in the metabolism of non-essential amino acids under glutamine limited fed-batch cultures has also been reported for myeloma cells (Ljunggren and Häggström, 1992). Under fed-batch glutamine limitation, glycine levels increased while serine was produced in the same way in batch and fed-batch cultures. These authors suggested that under the limited fed-batch culture cells use less serine or glycine. In the present study, in the fed-batch cultures net glycine production also increased as the levels of glutamine decreased but with a simultaneous decrease in the production of serine, which seemed to be linked with glutamine utilisation.

4.5- Concluding remarks

Providing the cell culture medium with glutamine, the depletion of which coincides with the end of growth and production phase, and with glucose, which is also depleted by the end of the culture, did not fail to prevent CHO cell growth and IFN-γ from ceasing. Inhibition did not seem to be caused by ammonia and lactate accumulation, which in the lower feeding regime did not attain higher levels that those normally found in a batch culture. In the fed-batch cultures, the initial glycosylation pattern of IFN-γ was found to be a function of the initial concentration of glutamine in the culture medium.

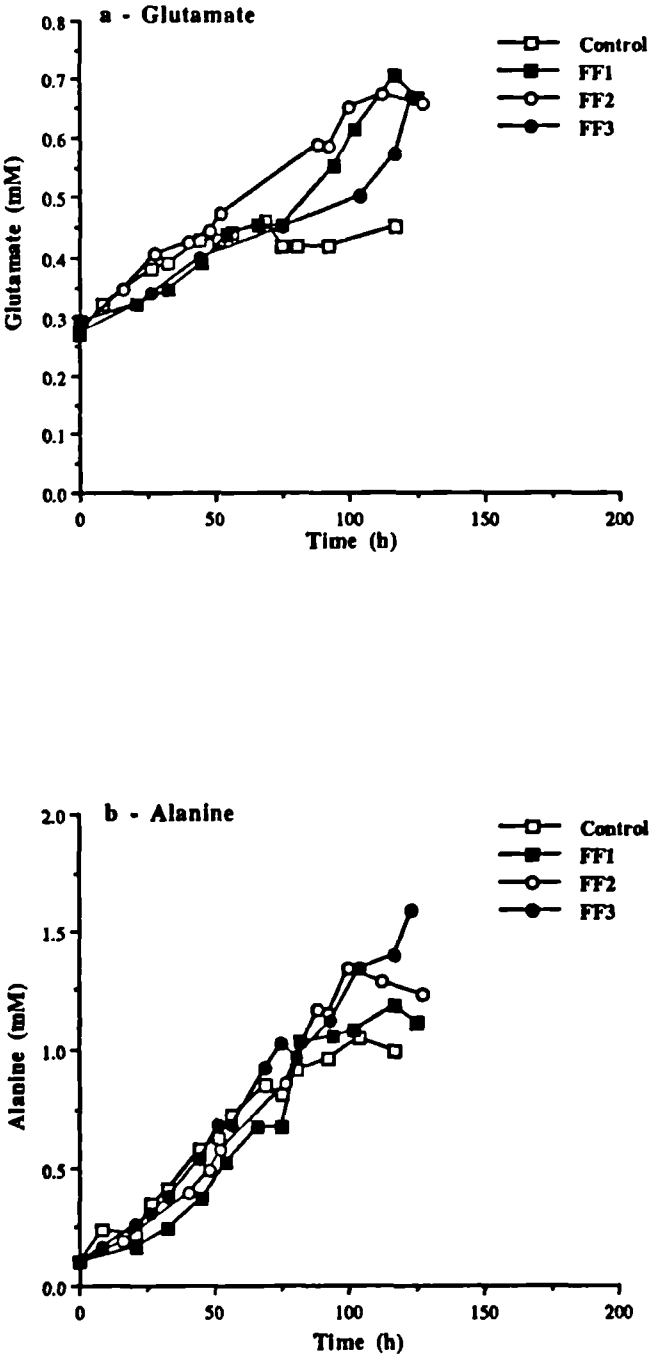


Figure 4.11- a) Glutamate and b) alanine production in fed-batch cultures.

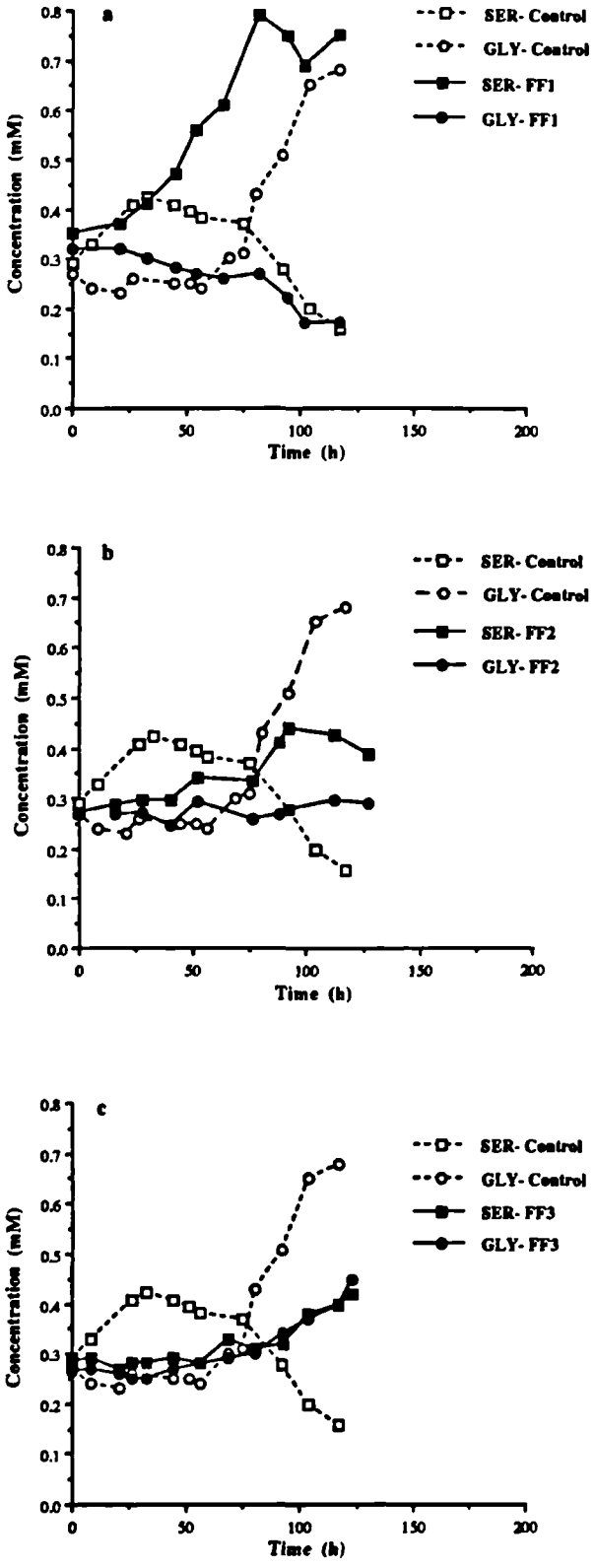


Figure 4.12- Metabolism of serine and glycine in fed-batch cultures a) FF1, b) FF2 and c) FF3.

5- THE EFFECTS OF BSA, LIPIDS AND PLURONIC F68 ON CHO CELL CULTURE

5.1- Introduction

In the first stage of this study the importance of low molecular weight nutrients, including glucose, amino acids and also protein supplements used in the serum-free medium was investigated. BSA was identified as an influential component for both cell growth and IFN-γ production. Subsequent experiments with different types of BSA indicated that the positive effect found for this medium component was probably related to differences in the source of BSA rather than to the actual amount present in the culture medium. Albumin has the ability to bind metals, lipids and hormones (Barnes and Sato, 1980; Iscove *et al.*, 1980). It is likely that its stimulating properties are due to the effects of unidentified molecules it carries or in the manner to which other components of the medium bind to it. BSA may also have a protective function because of its capacity to bind heavy metal ions, detergents and endotoxins. It can act as a detoxifying agent of the medium for some compounds, e.g., H_2O_2, and it has been reported to strongly inhibit copper-stimulated lipid peroxidation (Darfler and Insel, 1983; Gutteridge, 1986; Haliwell, 1988). There is also evidence that BSA may provide animal cells with protection from shear damage (Lambert and Birch, 1985; Hülscher and Onken, 1988; Papoutsakis, 1991a; Smith and Greenfield, 1992).

The trend in animal cell technology is to move towards more well-defined culture media. BSA is a component that introduces much variability into the culture medium. Its quality varies with the batch and source, and achievement of an absolute chemical definition is very difficult. Usually BSA is included in the culture media at relatively high concentrations, representing 80-90% of the total protein content of the culture medium (Graf *et al.*, 1991). This fact also makes downstream processing of the desirable product more difficult and costly.

Consequently, a study of possible BSA substitutes was undertaken. Considering the functions BSA may have in the culture medium, lipids and Pluronic F68 were candidates for this investigation.

Lipids have major functions in animal cells, both as structural components of membranes and as an energy source (King and Spector, 1981). The growth promoting effects of lipids have been referred to and they are included in most serum-free medium formulations (Barnes and Sato, 1980, Glassy *et al.*, 1988), but the lipid requirements of cells seem to vary considerably between cell lines. Lipids are usually added to the culture medium in the form of isolated plasma lipoprotein fractions, free fatty acids complexed to serum albumin, or fatty acid/phospholipid microemulsions.

The protection that BSA may provide to animal cells against mechanical stress caused by agitation and air bubbles may be substituted by polymeric surfactants. Pluronic F68 was chosen in this study. It is a block copolymer of polyoxyethylene and polyoxypropylene, 80% of which is polyoxyethylene, and has a relative molecular weight of approximately 8400. The protective effects of Pluronic F68 in adverse hydrodynamic environments has been often reported (Kilburn and Webb, 1968; Maiorella *et al.*, 1988; Murhammer and Goochee, 1988; Handa-Corrigan *et al.*, 1989; Michaels *et al.*, 1991; Smith and Greenfield, 1992, Zhang *et al.*, 1992a,b), but the mechanisms of protection are still unclear and opinions diverge. Pluronic F68 has been also reported to affect cell growth independently of shear damaging conditions caused by agitation or aeration (Bentley *et al.*, 1989; Mizrahi, 1975).

Reduction of the BSA content of the medium could affect IFN-γ glycosylation patterns. Glycosylation of epithelial basement membranes (EBM) has previously been reported to be influenced by the content of serum in serum-containing cultures (Megaw and Johnson, 1979). It has been shown recently that a different glycosylation of IgG is obtained if the antibody is

expressed in ascites, serum-containing media or serum-free media (Patel *et al.*, 1992). In this study, lipid supplementation was planned in conjunction with a reduction in BSA concentration, and this would alter the lipid composition of the medium. Since the cell fatty acid composition, either cellular storage lipids or membrane phospholipids, resembles that of lipids present in the culture medium, cell function could be affected. As referred in section 1.4.1, N-linked glycosylation starts with the synthesis of a lipid-linked oligossacharide moiety and its transfer *en bloc* to a nascent polypeptide chain in the ER. Whether the alteration of the lipid composition of the medium affects this process needed to be considered.

In this Chapter, the approach taken to investigate the possible reduction of BSA in the medium and its implications on cell growth and IFN-γ production are described. The effect of the various medium supplementations on the glycosylation profile of the protein is also addressed.

5.2- Definition of the culture conditions

As this Chapter involves the study of several combinations of medium components, a matrix with all the media used is specified in Table 5.1.

Table 5.1- Culture environments used in the present Chapter.

Medium	BSA* (mg/ml)	FAF-BSA* (mg/ml)	Lipid mixture (ml/l)	Linoleic (μg/ml)	Pluronic F68 (mg/ml)
A	5	-	-	-	-
B	5	-	1	-	-
C	5	-	-	-	1
D	1	-	-	-	-
E	1	-	1	-	1
F	1	-	1	-	2
G	1	-	1	-	4
H	1	-	2	-	2
I	1	-	2	-	4
J	1	-	4	-	1
K	1	-	-	-	1
L	1	0.8	-	8	1
M	1	0.4	-	4	1
N	1	1	-	-	-
O	1	1	-	-	1
P	1	1	-	8	1
Q	1	1	1	-	1
R	1	1	1	-	-
S	1	1	-	8	-

FAF-BSA= fatty acid-free BSA. Cultures having 1 mg/ml BSA in the base medium are designated as low-BSA cultures in the text. * Miles Pentex.

5.3- Effect of reducing the BSA concentration of the culture medium

The effect of reducing the BSA concentration of the culture medium was first investigated. Cell growth did not occur in cultures completely deprived of BSA. Growth in low BSA cultures (1 mg/ml) was also not achieved most of the time and when it occurred it was much poorer than a normal 5 mg/ml BSA culture (ca. 50% cell yield). A level of 1 mg/ml was set as the low limit for BSA concentration and so it was used as the basis for all the supplementation studies presented. A culture under such conditions was included as a comparative control for each set of experiments and results for this culture are not presented if there was no cell growth.

5.3.1- Possible substitutes for BSA- Lipids and Pluronic F68

Considering the nutritional role of BSA in culture media, its replacement by a commercial lipid mixture was investigated. The mixture chosen for this purpose is a water soluble media supplement containing lipoprotein, cholesterol, phospholipids and fatty acids, recommended for serum-free media (Sigma Catalogue). Its composition is as follows:

Total Cholesterol	11.4 ± 0.6 mg/ml
% HDL Cholesterol	96.8 ± 1.6
Total Phospholipids	11.3 ± 0.8 mg/ml
% Phosphatidyl choline	64.9 ± 15.2
% Lysophosphatidyl choline	9.7 ± 7.4
% Sphingomyelin	25.4 ± 7.9
Total Fatty Acids	12.5 ± 0.8 mg/ml
% Linoleic	51.0 ± 3.9
% Arachidonic	2.4 ± 0.9
% Polyunsaturated	59.4 ± 2.3
Total Protein	23.0 ± 1.9 mg/ml

The effect of adding this supplement to cultures containing 1 mg/ml BSA was initially studied and it was found that cell growth could not be maintained and cells died very quickly, in the same manner as with no supplementation. Simply supplying a lipid source to the culture was not enough to restore cell growth. BSA is also known to function as a protective protein for cultured cells. Reducing its concentration will affect the physical properties of the culture medium, such as viscosity and osmolarity, which will influence the cells susceptibilty to shear damage. Pluronic F68 was subsequently included in these cultures to provide the cells protection from possible shear damage.

Addition of different combinations of the lipid mixture and Pluronic F68 to the 1 mg/ml BSA cultures were investigated. The concentration of Pluronic F68 was varied between 0.1 and 0.4%, which is within the range usually reported by other researchers (Bentley *et al.*, 1989; Gardner *et al.*, 1990). The lipid mixture concentration was chosen in such a way as to keep the concentration of cholesterol and linoleic acid in the new medium within the range commonly used; usually these vary from 1 to 10 μg/ml for linoleic acid and from 1 to 20 μg/ml for cholesterol. Thus, the lipid mixture concentration varied from 1 to 4 ml/l although the manufacturer recommends its use between 1-10 ml/l. A summary of the major results obtained for the various combinations is presented in Table 5.2. Medium E, which contains the lowest combination of concentrations (0.1% Pluronic F68 and 1ml/l lipid mixture) improved growth significantly, when compared to cells grown with only BSA 1mg/ml (medium D), and cell titres similar to the control culture (medium A) were obtained. However, product formation was negatively affected, as indicated by an approximately 35% reduction in maximum titres obtained. Increasing Pluronic F68 concentration to 0.2% (medium F) caused a slight reduction in maximum cell titres, but product formation was unaffected. A further increase to 0.4% Pluronic F68 (medium G) had negative effects both on cell growth and product formation. Increasing lipid concentration to 2 ml/l, keeping Pluronic F68 at 0.2% (cf. medium F and medium H) had negative effects on cell growth and product formation; when Pluronic F68 was

kept at 0.4% (cf. medium G and medium I) cell growth was reduced but IFN-γ production was not affected. A more drastic increase of the lipid mixture level from 1 to 4 ml/l, with Pluronic F68 at 0.1% (cf. medium E and medium J) showed the inhibitory effects of higher doses of lipids on cell growth and IFN-γ production.

Table 5.2- Addition of different combinations of a lipid mixture and Pluronic F68 to 1 mg/ml BSA cultures.

Medium	μ (h^{-1})	Viable cell titre (cells/ml x 10^5)	IFN-γ production (IU/ml)
A	0.021 ± 0.001	9.6 ± 0.8	8423 ± 290
D	0.016 ± 0.001	5.8 ± 0.2	5330 ± 500
E	0.019 ± 0.001	8.8 ± 0.2	5633 ± 300
F	0.019 ± 0.001	7.3 ± 0.4	5725 ± 310
G	0.016 ± 0.002	6.0 ± 0.3	3765 ± 180
H	0.013 ± 0.001	5.4 ± 0.3	4641 ± 470
I	0.013 ± 0.001	4.5 ± 0.2	3985 ± 155
J	0.015 ± 0.001	5.9 ± 0.3	4067 ± 300

the concentrations of IFN-γ are the maximum obtained under each culture condition.

A- BSA 5 mg/ml; D- BSA 1 mg/ml

E- BSA 1 mg/ml + 1 ml/L Lipid mixture + 0.1% Pluronic F68

F- BSA 1 mg/ml + 1 ml/L Lipid mixture + 0.2% Pluronic F68

G- BSA 1 mg/ml + 1 ml/L Lipid mixture + 0.4% Pluronic F68

H- BSA 1 mg/ml + 2 ml/L Lipid mixture + 0.2% Pluronic F68

I- BSA 1 mg/ml + 2 ml/L Lipid mixture + 0.4% Pluronic F68

J- BSA 1 mg/ml + 4 ml/L Lipid mixture + 0.1% Pluronic F68

With specific supplementation of lipids and Pluronic F68 (media E and F), low BSA cultures could support cell growth to almost the same extent as control cultures (medium A). However, there was no improvement of product titre as compared to the low BSA non-supplemented cultures (medium D) despite the higher cell numbers. Furthermore, the IFN-γ titres in these experiments were always lower than the control culture.

Time course patterns for cultures grown in media A, D and E are shown in Figure 5.1a,b. The growth patterns of cultures supplemented with the lowest combinations and the control culture were very similar, but in the lipid supplemented cultures cells remained viable for a slightly longer period (Figure 5.1a). Product titres obtained in low BSA cultures were much lower than in the control culture. Initial IFN-γ production rates were also lower with lipid plus Pluronic F68 supplementation and similar to low BSA cultures (Table 5.3). In contrast to control cultures where IFN-γ was only associated with the growth phase, the accumulation of IFN-γ in supplemented cultures slowly increased throughout the duration of the culture (Figure 5.1b). This could suggest that some component in those additions maintained the production, or, it could be related to the physiological state of the cells.

Table 5.3- IFN-γ production rates in cultures A, D and E.

Medium	q_{IFN} (IU/10^6cells/h)
A- BSA 5 mg/ml	178
D- BSA 1 mg/ml	137
E- BSA 1 mg/ml + 1 ml/L Lipid mixture + 0.1% Pluronic F68	140

At this stage of the investigation it was possible to conclude that CHO cell growth occurred in low BSA cultures supplemented with a lipid mixture plus Pluronic F68, but IFN-γ production was always lower over the course of the culture. From this set of data it was not possible to identify the reason for the lower production of IFN-γ. Reduction of BSA concentration reduced the titres of IFN-γ achieved in culture; this was consistent with the lower cell numbers, although a reduction in the initial production rates was also noticed. Furthermore, the lower production observed in medium E as compared to the control culture could also be related to either of the supplements, lipids or Pluronic F68.

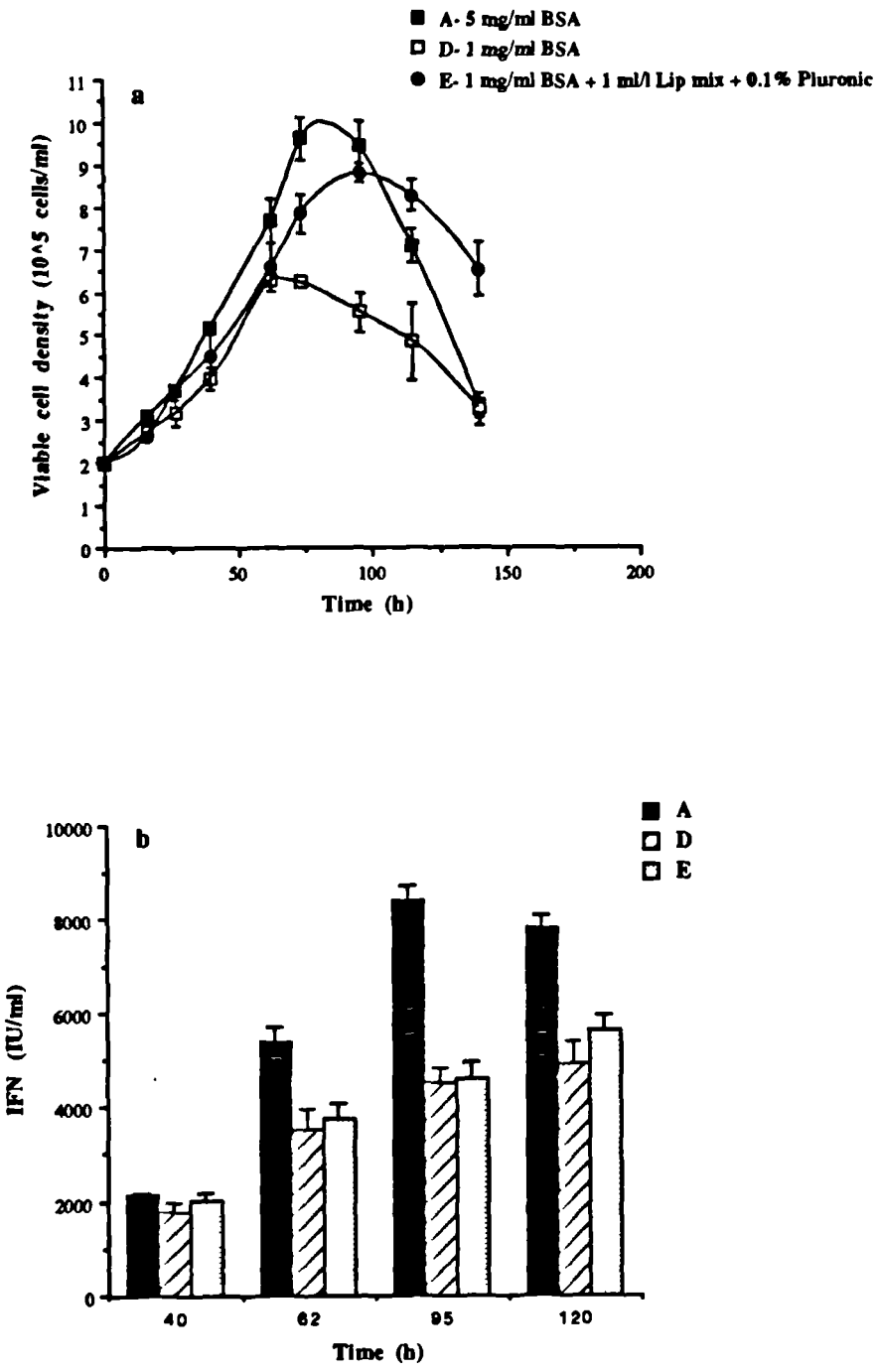


Figure 5.1- a) Cell growth and b) IFN-γ production in cultures A, D and E.

5.3.2- Influence of individual lipid components on the behaviour of low-BSA cultures: Plackett-Burman statistical analysis

One of the advantages of reducing the BSA concentration of the culture medium is to provide a more well defined medium that will be reproducible in terms of its components. The lipid mixture used was a multicomponent supplement containing lipoprotein, cholesterol, phospholipids and fatty acids. A search for possible individual lipid components responsible for the observed differential effects on cell growth and IFN-γ production was carried out. The effect of individual lipids on these parameters was investigated with the same Plackett-Burman statistical procedure used before (see section 3.3), however with a N=8 matrix (Table 5.4) to allow the study of 4 real variables against 3 dummy variables.

Table 5.4- Plackett-Burman matrix for the study of 7 variables.

		VARIABLES						
		A	B	C	D	E	F	G
F	1	+	+	+	-	+	-	-
L	2	-	+	+	+	-	+	-
A	3	-	-	+	+	+	-	+
S	4	+	-	-	+	+	+	-
K	5	-	+	-	-	+	+	+
	6	+	-	+	-	-	+	+
	7	+	+	-	+	-	-	+
	8	-	-	-	-	-	-	-

(+) high level; (-) low level.

Low BSA medium (1 mg/ml) supplemented with 0.1% Pluronic F68 was used as the basis of the matrix. Linoleic and oleic acids, cholesterol and choline plus ethanolamine were the components chosen for analysis, with corresponding levels shown in Table 5.5. Cholesterol and linoleic acid represent a major fraction of the lipid mixture used in the above section. Fatty acids and cholesterol constitute the basic lipid building blocks for the cells and growth promoting effects of these two components have been reported (Darfler and Insel, 1982;

Kovar and Franek, 1986; Sato *et al.*, 1987; Minamoto *et al.*, 1991). Lipid precursors, such as ethanolamine, were found to be essential for the growth of some hybridoma cell lines (Murakami *et al.*, 1982; Kovar, 1986) and ethanolamine is often found in serum-free media for hybridomas (Kovar, 1987; Schneider, 1989; Darfler, 1990; Fike, 1991). An increase in the medium concentration of choline and ethanolamine, to 75 and 20 μg/ml respectively, has been recently reported to substitute the requirement of lipoproteins by hybridoma cells (Maiorella, 1992).

Table 5.5- Components under study and corresponding concentrations.

Variable	Low level (μg/ml)	High level (μg/ml)
A- Oleic acid	0	5
B- Linoleic acid	0	5
C- Dummy		
D- Cholesterol	0	5
E- Dummy		
F- Dummy		
G- Choline/Ethanolamine	3/0	40/10

the lipid components were added as fatty acid-free BSA complexes, as indicated in section 2.1.2.2.

The individual results of each culture, as required for the following statistical analysis, are shown in Table 5.6 and some preliminary points can be made. This set of experiments revealed that Pluronic F68 by itself was capable of largely restoring the growth of CHO cells (culture 8 in the matrix), while growth with only 1 mg/ml BSA was unsuccessful.

Table 5.6- Cell growth and IFN-γ data for Plackett-Burman analysis [see Table 5.4].

EXP n°	μ (h^{-1})	Cell titre (10^5 cells/ml)	Interferon-γ titre (IU/ml)	
			50 h	116 h
1	0.025 ± 0.000	9.6 ± 0.4	2114 ± 196	4594 ± 196
2	0.022 ± 0.001	8.5 ± 0.3	1714 ± 245	4749 ± 158
3	0.017 ± 0.001	6.2 ± 0.5	1364 ± 84	3299 ± 605
4	0.015 ± 0.001	5.4 ± 0.2	1672 ± 112	4498 ± 429
5	0.022 ± 0.000	8.0 ± 0.5	2878 ± 78	5312 ± 746
6	0.023 ± 0.000	8.7 ± 0.1	2236 ± 113	4378 ± 157
7	0.024 ± 0.000	8.0 ± 0.3	1539 ± 61	3783 ± 552
8	0.018 ± 0.000	7.5 ± 0.2	3110 ± 450	6298 ± 640
Control	0.023 ± 0.001	8.5 ± 0.3	nd	9500 ± 320

nd= not determined.

A few considerations of this effect can be made. Pluronic polyols have been widely used in bioreactors as shear protective agents. There is evidence that the mechanism through which Pluronic F68 prevents cell damage in bioreactors is of a physical nature, by changing the properties of the gas-liquid interfaces (bubbles), e.g. stabilisation of foams, (Handa-Corrigan *et al.*, 1989; Michaels *et al.*, 1991; Zhang *et al.*, 1992b). However, the protective effect of Pluronic F68 has also been attributed to its interaction with cell membranes, increasing the cell membranes strength and resistance to shear damage (Murhammer and Goochee, 1988; Ramirez and Mutharasan, 1990; Zhang *et al.*, 1992a). It has also been reported that Pluronic F68 has a protective effect on the content of specific surface proteins of the cells during agitation (Lakhotia *et al.*, 1993) and it is possible that its protective effect is related to this function. On the other hand, Pluronic F68 has been shown to affect cell growth independently of shear damaging conditions. Mizhari (1975) suggested that low concentrations of Pluronic F68 (0.05 to 0.1%) could enhance the transport of metabolites into cells by lowering the surface tension of the medium, thus increasing cell growth rate. A concentration dependent effect in static

cultures has also been reported by Bentley *et al.* (1989), providing evidence that cell growth can be affected independent of agitation and aeration. In their study they also found that commercial Pluronic F68 or further purified Pluronic F68 exerted different effects on cell growth. The effect of the latter fraction varied with cell line, implying that the observed effect could in part be related to impurities present in commercial Pluronic F68 preparations. Since the present study was carried out in shake flasks the protective effect of Pluronic F68 against bubble damage is excluded. The stimulating effect may still be related to a protection of the cell integrity in lower protein content environment (1 mg/ml BSA), possibly by interaction with the cell membranes, and the restoration of the BSA protective effects. At the same time, it is also possible that any of the effects proposed by Mizrahi (1975) or Bentley *et al.* (1989) are reflected in this study.

A positive control (5 mg/ml BSA) was also included in this set of experiments, to allow a direct comparison of the results. Cell titres of approximately 8.5×10^5 cells/ml and maximum product concentrations of around 9500 IU/ml were obtained. Although some of the cultures inside the matrix were able to restore CHO cell growth, the resultant IFN-γ titres were always lower. Also, none of the lipid combinations used gave higher titres than cultures supplemented with only Pluronic F68 (culture 8), although cell titres were higher in some cases (cultures 1,2,6 and 7).

The results obtained from the statistical analysis are presented in Table 5.7. Only linoleic acid exhibited a significant positive effect for both cell growth rate and viable cell production (90% and 80% significance respectively) while cholesterol produced a significantly negative effect on both parameters (80% significance). Product titre at 50 h was used as an indication of the initial production rates. Cholesterol showed significantly negative effects (99%) for IFN-γ production at that stage of the culture. Product titre at 116 h was used as an indication of maximum titres obtained. Cholesterol, oleic acid and choline/ethanolamine all showed

significantly negative effects (80%, 70% and 80% respectively).

Table 5.7- Components and their effects on CHO cell growth and IFN-γ production.

Variable		Oleic acid	Linoleic acid	Cholesterol	Choline/Ethanolamine
Cell growth	a	0.002175	0.004625	-0.002625	0.001425
rate	b	1.44	3.06	-1.74	0.94
	c	**70%**	**90%**	*80%*	
		V= 0.00000228	SE= 0.0015		
Viable cell	a	0.375	1.575	-1.425	0.025
production	b	0.48	2.01	-1.82	0.03
	c		**80%**	*80%*	
		V= 0.616	SE= 0.785		
IFN-γ titre	a	-0.1225	0.0075	-0.3275	0.0525
(50 h)	b	-1.42	0.09	-3.78	0.61
	c	*70%*		*99%*	
		V= 0.00749	SE= 0.0865		
IFN-γ titre	a	-0.095	0	-0.170	-0.135
(116 h)	b	-1.22	0	-2.19	-1.74
	c	*70%*		*80%*	*80%*
		V= 0.00602	SE= 0.0775		

a- effect; b- t-value; c- significance level. Positive variables are shown in bold and negative variables in italics. V= variance; SE= standard error.

Two of the components under study must be pointed out, linoleic acid and cholesterol, the former for its growth positive effect and the latter for its negative effect on both cell growth and IFN-γ production. The fatty acid composition of membrane phospholipids is similar to that of the lipids present in the culture medium. Modification of the groups present in the membrane will be reflected in cell function, most likely affecting cell growth rates. The growth promoting effects of linoleic acid in serum-free cultures have been reported for hybridomas (Kovar and Franek, 1986; Schneider, 1989; Darfler, 1991) and it was part of the protein-free

medium developed for parental CHO cell lines, including the CHO-K1 (Hamilton and Ham, 1977). The maintenance of the bilayer fluidity seems to be essential for normal cell growth. Cholesterol is involved in the regulation of membrane fluidity and permeability (Phillips *et al.*, 1987) and an increase in cholesterol content tends to reduce the fluidity of the membrane. Ramirez and Mutharasan (1990) also found a decrease in the plasma membrane fluidity of hybridoma cells by cholesterol enrichment of the medium, which was claimed to result in a more robust cell, as measured by their survival to fluid mechanical stress. Doi *et al.* (1978) correlated the unsaturated fatty acid content of membrane phospholipids with cell growth and found severe growth inhibition if the content of unsaturated fatty acids in the membrane was reduced to less than 50%. The fact that this study indicated cholesterol as having negative effects on CHO cell growth and IFN-γ production may be related to this function. Low density lipoprotein, which contains cholesterol, has been shown to have either growth promoting or inhibiting effects, depending on the cell line (Kawamoto *et al.*, 1986). Albumin has been substituted by a complex composed of alpha-cyclodextrin with linoleic acid and cholesterol, at concentrations of 2 μg/ml and 0.1 μg/ml respectively, for the production of interferon by lymphoblastoid cells (Minamoto *et al.*, 1991). The concentration of cholesterol these authors used was much lower than that in the present investigation. Studies on the effects of lipid supplements on product formation are however relatively few. Schmid *et al.* (1991) found that supplementation of low serum medium with different combinations of fatty acids, namely oleic and linoleic acids, did not have any effect on BHK cell growth but that anti-thrombin production was a positive function of these supplements. Of the lipids used in the present study, there was no indication of any positive effect on IFN-γ production.

5.3.3- Linoleic acid substitution of the lipid mixture

The results described above showed that linoleic acid was a positive variable for cell growth, while no significant effect was detected for IFN-γ production, under the conditions of the study. As a consequence, the differential effect of linoleic acid on these parameters was then investigated. Low BSA cultures (1 mg/ml) containing 0.1% Pluronic F68 were supplemented with linoleic acid at two concentrations, 4 and 8 μg/ml, and culture performance was compared with the addition of the lipid mixture. Time courses for this set of experiments are shown in Figure 5.2a,b and specific growth and production rates are presented in Table 5.8. Linoleic acid was found to be a better substitute than the complex lipid mixture for cell growth (Figure 5.2a), as indicated by higher cell yields (ca. 35%) and higher specific growth rates (Table 5.8). Pluronic F68 supplemented cultures (medium K) showed better growth than cultures with further addition of the lipid mixture (medium E), stressing the growth inhibitory effects that some of its constituents, such as cholesterol, may have.

Table 5.8- Specific cell growth and IFN-γ production rates in low-BSA cultures.

Medium	μ (h^{-1})	q_{IFN} ($IU/10^6 cells/h$)
A- BSA 5 mg/ml	0.024 ± 0.002	235
E- BSA 1 mg/ml + 1 ml/l lipid mixture + 0.1% Pluronic F68	0.022 ± 0.001	106
K- BSA 1 mg/ml + 0.1% Pluronic F68	0.024 ± 0.001	169
M- BSA 1 mg/ml + 4 μg/ml linoleic acid + 0.1% Pluronic F68	0.026 ± 0.001	121
L- BSA 1 mg/ml + 8 μg/ml linoleic acid + 0.1% Pluronic F68	0.026 ± 0.001	89

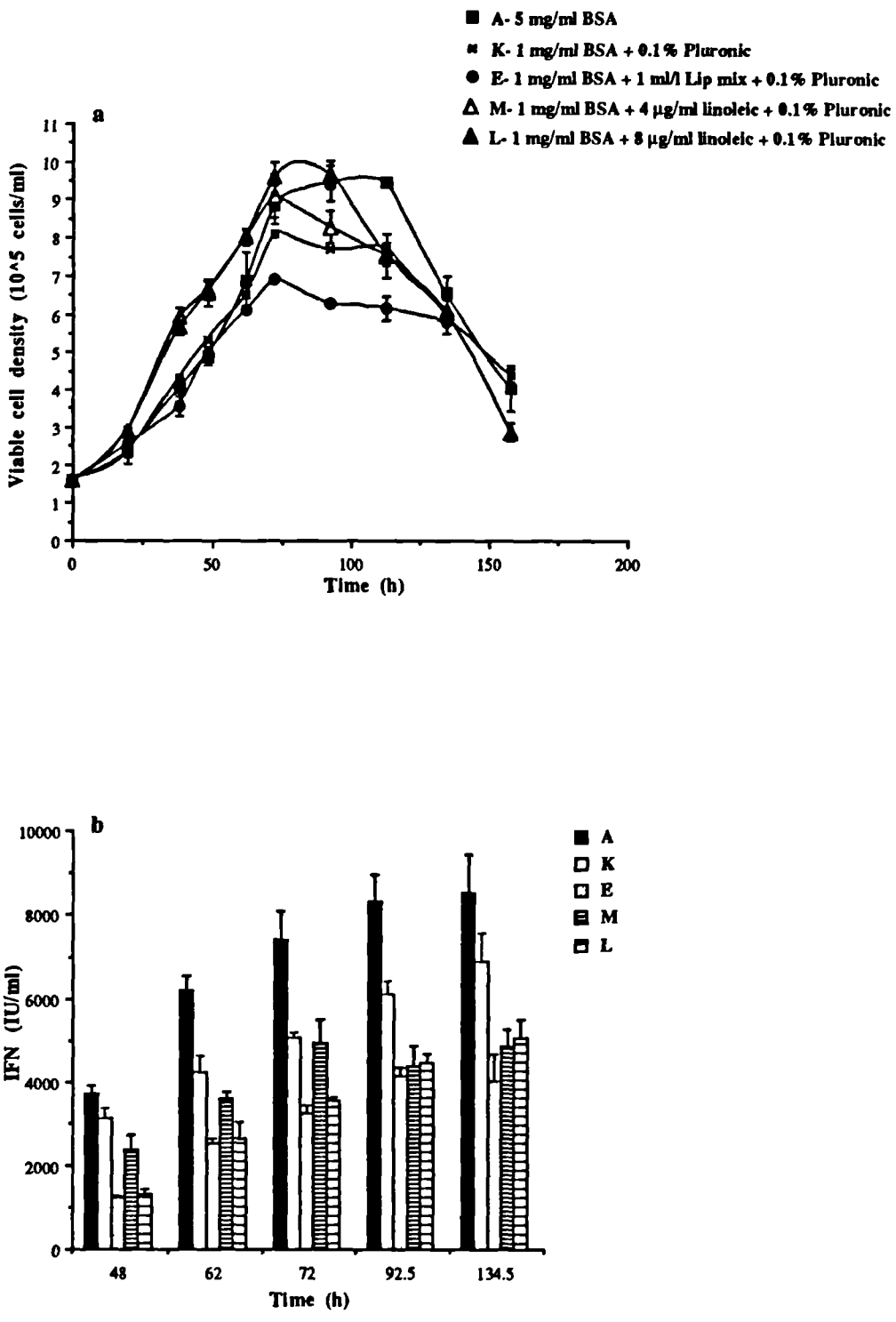


Figure 5.2- a) Cell growth and b) IFN-γ production in cultures A, K, E, M and L.

IFN-γ analysis showed a significant reduction in product titres in lipid/linoleic supplemented cultures (Figure 5.2b). Cultures with only Pluronic F68 supplementation showed higher IFN-γ titres than the latter cultures, indicating a detrimental effect of linoleic acid and lipid mixture on IFN-γ production. Although linoleic acid was identified by the Plackett-Burman procedure as having a positive effect on CHO cell growth, its negative effect on IFN-γ production, which was here determined, was not manifested. This could be a sign of interaction between variables but could also indicate that the magnitude of the effect of other variables, namely cholesterol, was high enough to mask the effect of linoleic acid.

Lipid mixture was less effective than linoleic acid itself, suggesting that something in addition to linoleic acid had a negative effect on IFN-γ production. Initially, cultures containing a lower concentration of linoleic acid (4 μg/ml; medium M), produced more IFN-γ than cultures with 8 μg/ml (medium L), but at ca. 90 h the production titres were the same. In the latter case, IFN-γ gradually increased in culture while in the former maximum titres were achieved earlier. A dose dependent effect of linoleic acid on IFN-γ production is suggested; while 8 μg/ml initially slowed down production, when compared to 4 μg/ml, it seemed to be more beneficial later in the culture. Initial production rates of IFN-γ are shown in Table 5.8. The lowest specific production was seen in cultures containing 8 μg/ml linoleic acid (medium L). The value found for the culture supplemented with the lipid mixture (medium E) was intermediate between the culture with 8 μg/ml (medium L) and the culture with 4 μg/ml (medium M) linoleic acid; it is interesting to note that the concentration of the lipid mixture used should provide the medium with ca. 6.5 μg/ml linoleic acid. It is possible that a cumulative negative effect of linoleic acid on productivity is reflected here. Although low BSA cultures all showed lower IFN-γ production rates, the decrease was much less pronounced if no lipid addition was made (cf. medium K and media E,M,L).

The possibility existed that these supplements (lipids, Pluronic F68) were affecting the

secretory machinery of the cell at some stage of the process and, as a consequence, the product was not being properly secreted by the cells. The determination of IFN-γ titres on cell lysates from these cultures indicated that this was probably not the case since the intracellular values were similar for all the cultures, and much lower than what was secreted (Table 5.9). It was interesting to note that the specific rate of IFN-γ secretion *per* 10^6cells/h (see Table 5.8) is not much different from the amount of IFN-γ/10^6cells kept intracellularly. As a percentage of the extracellular component, secretion appears to be affected in the early stages of the culture (media E,L) as compared with no addition (media A, K).

Table 5.9- Intracellular and extracellular IFN-γ accumulation in low-BSA cultures.

	IFN-γ (IU/10^6 viable cells)			
	62 h		*92 h*	
	Intracellular	Extracellular	Intracellular	Extracellular
Medium A	266	7645	242	8354
	3.48%		*2.90%*	
Medium K	173	5876	125	6242
	2.94%		*2.00%*	
Medium E	168	2493	110	4747
	6.74%		*2.32%*	
Medium L	167	2011	93	3717
	8.30%		*2.50%*	

The glycosylation profiles of the product obtained under each condition of medium supplementation were then determined. Analysis of the proportion of each of the glycoforms for all BSA-reduced cultures showed a different pattern from control cultures (Figure 5.3a-e). In Figure 5.4 is an example of the patterns obtained on a gel for a control culture and a low BSA culture, showing the stronger bands of the non-glycosylated form in the latter cultures.

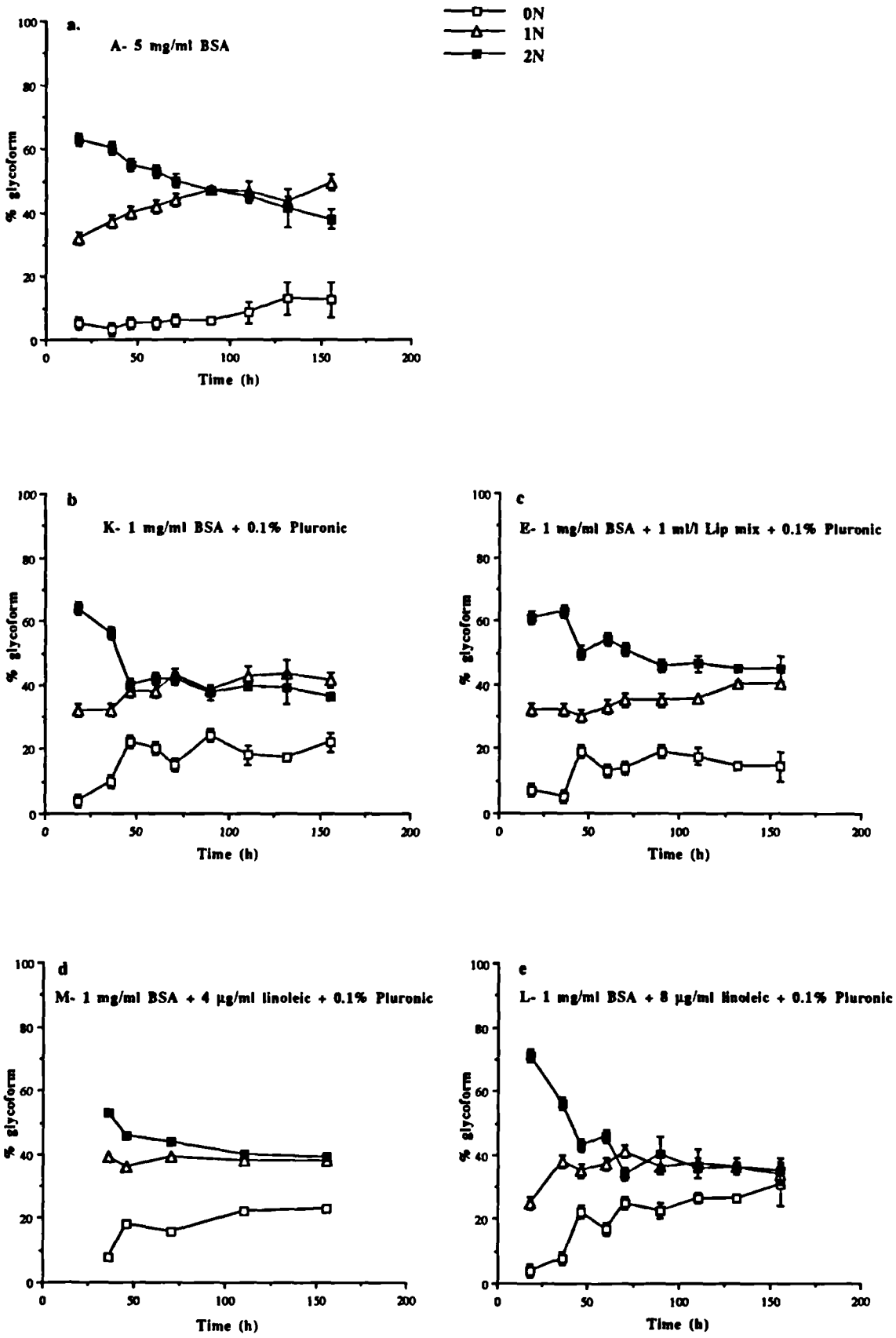


Figure 5.3- Glycosylation patterns of IFN-γ obtained in cultures A, K, E, M and L. [No error bars are given for culture M as only one set of results were obtained].

The control culture (5 mg/ml BSA) produced a relatively low level of non-glycosylated form, 5-7%, up to 100 h, while the 2N glycoform gradually decreased from ca. 65% at 20 h to ca. 45% after 100 h in culture, with a concomitant increase in the 1N glycoform (Figure 5.3a). A rapid increase in the proportion of the non-glycosylated form was noticed in all the low BSA cultures (Figure 5.3b-e), ca. 15-25% in 50 h, which for cultures with Pluronic F68 and Pluronic F68 plus linoleic acid was simultaneous with a rapid decrease in the proportion of the 2N glycoform to 40% and an increase in the 1N glycoform to the same value. This decrease was not so pronounced in cultures supplemented with the lipid mixture, in which the proportion of the 2N glycoform was maintained at higher levels for longer time (ca. 50% at 50 h). Since cells with only 1 mg/ml BSA did not grow, it could not be determined whether the addition of Pluronic F68 or the reduction of the BSA concentration itself was the cause for this change. As discussed earlier in this Chapter, Pluronic F68 has been shown to alter the cell membrane (Murhammer and Goochee, 1988; Ramirez and Mutharasan, 1990; Zhang *et al.*, 1992b), which may influence the latter's functionality. Reduction of BSA by itself may also affect the lipid composition of the membrane, which could also possibly affect the glycosylation process.

The distribution of the initial production rates of each glycoform was determined (Figure 5.5). Low BSA cultures supplemented with Pluronic F68 alone showed the highest initial rate of production of the non-glycosylated form. The control culture showed the highest production rates of IFN-γ glycosylated at 1 or 2 sites, while presenting the lowest rate for the non-glycosylated form. The titres of each of the glycoforms throughout the culture is shown in Figure 5.6. Lower titres of both glycosylated forms and relatively higher titres of non-glycosylated form were present at any time in low BSA cultures, as compared to the control culture (5 mg/ml). In terms of production of the glycosylated forms, none of the low BSA conditions presents a favourable alternative to the control situation, and among them supplementation with only Pluronic F68 yields the highest final titres of the 1N and 2N glycoforms.

Figure 5.4- Analysis of IFN-γ variants obtained in cultures A and L. Immunoprecipitated samples were resolved by SDS/PAGE on a 14% polyacrylamide gel and detected by silver stain, as described in section 2.2.2.2. The white arrow indicates the direction of migration. Lane 1- MW markers; lane 2 to 5- samples of culture A at 36, 60, 90 and 110 h, respectively; lane 6 to 9- samples of culture L at 36, 60, 90 and 110 h, respectively.

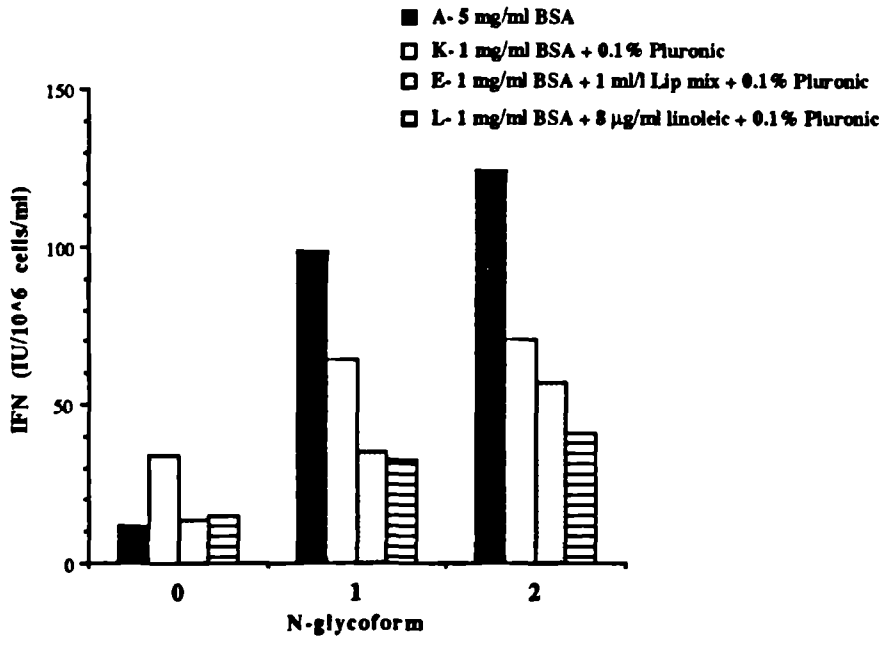


Figure 5.5- Initial production rates of the three IFN-γ glycoforms in cultures A, K, E and L.

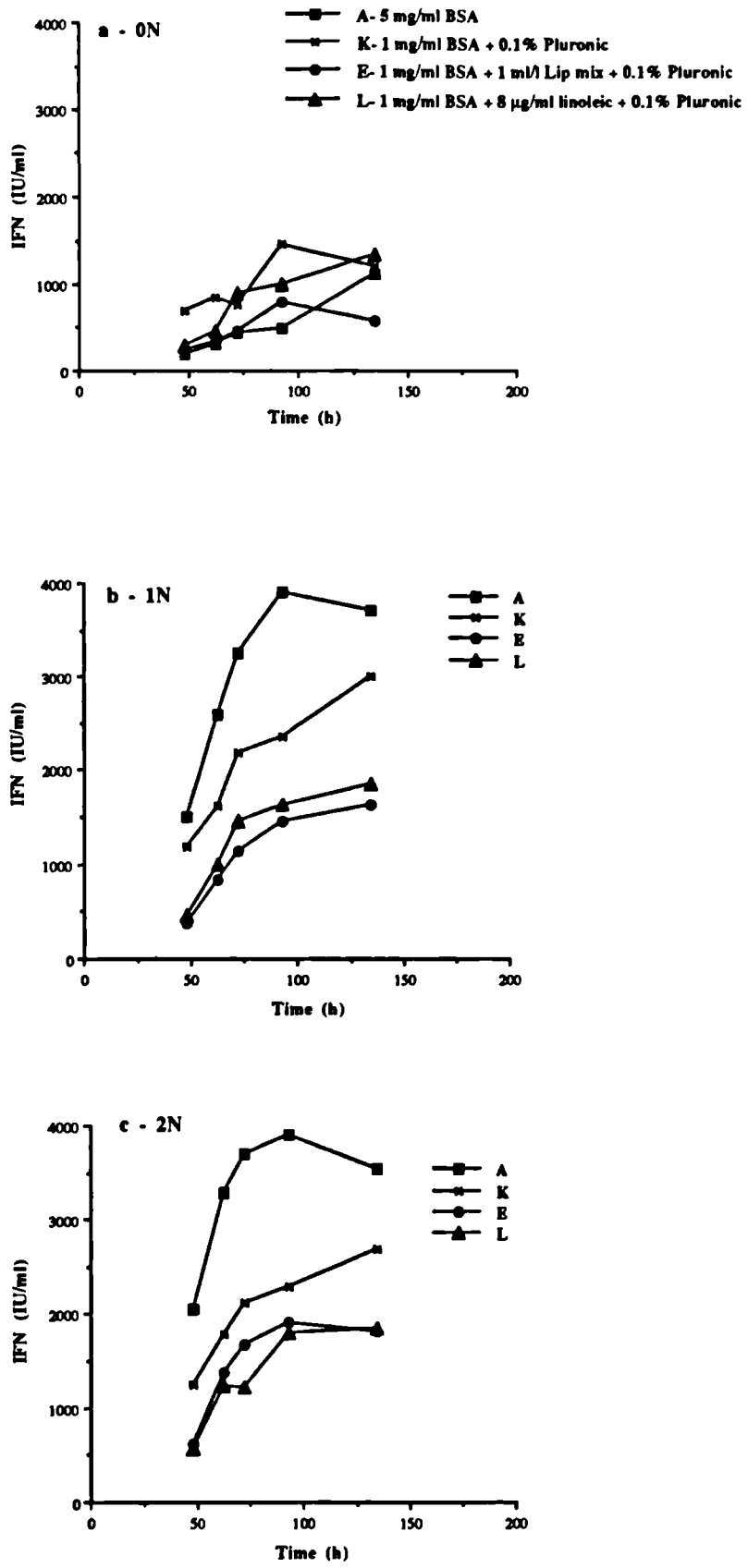


Figure 5.6- Accumulation of each IFN-γ glycoform during cultures A, K, E and L.

5.3.4- Effects of fatty acid-free BSA in culture

The results presented in the previous sections have consistently implied that BSA is a critical component in the culture medium with respect to IFN-γ production and glycosylation. One of the possible reasons for the shift in the proportions of IFN-γ glycoforms reported in the previous section was the decrease in the BSA concentration, which could be related to components it carries to the culture medium. The investigation of the effect of supplementing the medium with a more refined BSA, a fatty acid-free BSA (FAF-BSA), could provide more insight to the importance of the endogenous BSA lipid to the glycosylation of IFN-γ. Furthermore, linoleic acid itself is added to the medium as a FAF-BSA complex. The fact that the growth promoting effects observed for linoleic acid were not related to the additional FAF-BSA was also investigated. The effect of the FAF-BSA alone was studied by supplementing the same set of cultures as described in the previous section with FAF-BSA, in order to have a constant concentration 1 mg/ml. Time courses for those cultures are shown in Figure 5.7a. Addition of an extra 1 mg/ml FAF-BSA above the Pluronic F68 supplemented cultures did not have the same stimulating effect on cell growth as the addition of the linoleic acid complex (cf. medium O and medium P), attributing that effect to the linoleic acid itself. Again, Pluronic F68 showed its own stimulating effect on cell growth (cf. medium N and medium O). The extra FAF-BSA had a positive effect on Pluronic F68 plus lipid supplemented cultures (medium Q), which this time achieved the same growth as Pluronic F68 supplemented cultures (medium O), and this could be due to an increase in the total BSA itself or due to a protective effect of the FAF-BSA against any inhibitory lipid component. Media R and S, which corresponded to low-BSA cultures supplemented with lipids or linoleic acid but deprived of Pluronic F68 (specified in Table 5.1), were also studied in this set of experiments, but cell growth was unsuccessful, which once again reflects the importance of Pluronic F68 in low BSA cultures.

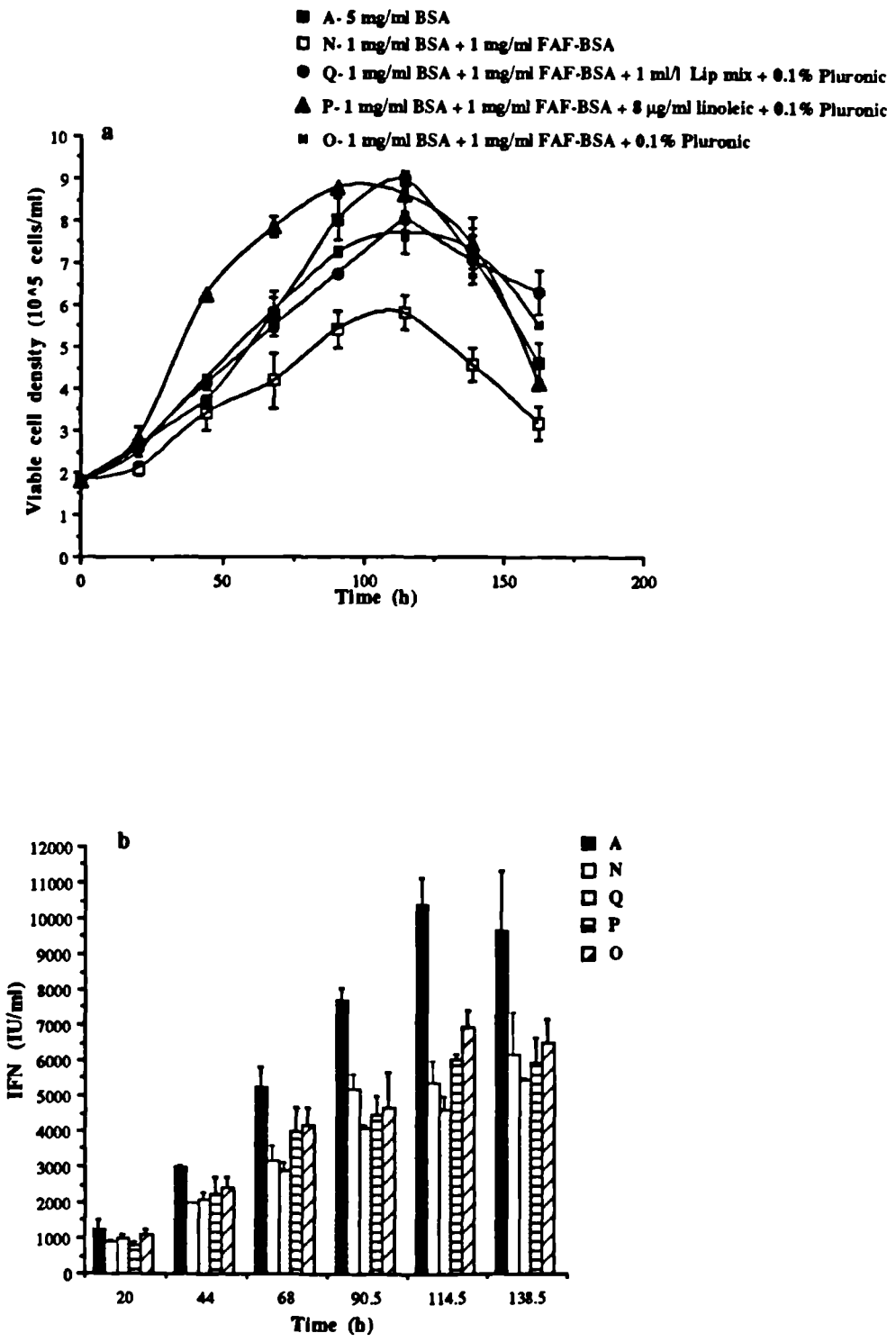


Figure 5.7- a) Cell growth and b) IFN-γ production in cultures A, N, O, P, and Q.

IFN-γ production for these cultures is shown in Figure 5.7b. Despite lower cell numbers, the IFN-γ titres obtained in cultures without lipids and Pluronic F68 supplements (medium N) were similar to the lipid supplemented cultures (media Q and P), reflecting a higher initial IFN-γ production rate in non-supplemented cultures (Table 5.10). Lipid addition alone was shown to have a negative effect on IFN-γ production, which can be seen by comparing medium O with medium Q, and is also indicated by lower rates of production observed in both media P and Q. None of the cultures achieved the same titres as the control culture, and no net positive effect on IFN-γ production was observed by adding the extra FAF-BSA.

Table 5.10- Initial IFN-γ production rates in low-BSA cultures.

Medium	q_{IFN} (IU/10^6cells/h)
A- BSA 5 mg/ml	203
N- BSA 1 mg/ml + 1 mg/ml FAF	156
O- BSA 1 mg/ml + 1 mg/ml FAF + 0.1% Pluronic F68	161
P- BSA 1 mg/ml + 1 mg/ml FAF + 8 µg/ml linoleic acid + 0.1% Pluronic F68	121
Q- BSA 1 mg/ml + 1 mg/ml FAF + 1 ml/L lipid mixture + 0.1% Pluronic F68	117

Examination of the different glycoforms revealed that the addition of extra FAF-BSA did affect the glycosylation process. A very low level of non-glycosylated form was observed in both high and low BSA control cultures (media A and N) while all lipid or Pluronic F68 supplemented cultures (media O,P and Q) showed a fairly constant level of about 9-12% (Figure 5.8 a-e). These data seem to indicate that it was not only the reduction in BSA concentration *per se* that caused the previous increase in the non-glycosylated form, but this may have also resulted from the addition of Pluronic F68 or lipids. It is interesting to note that the proportion of the 2N and 1N glycoforms were different in cultures containing FAF-BSA, as compared to the control culture suggesting that the addition of FAF-BSA alone may affect the glycosylation pattern of IFN-γ.

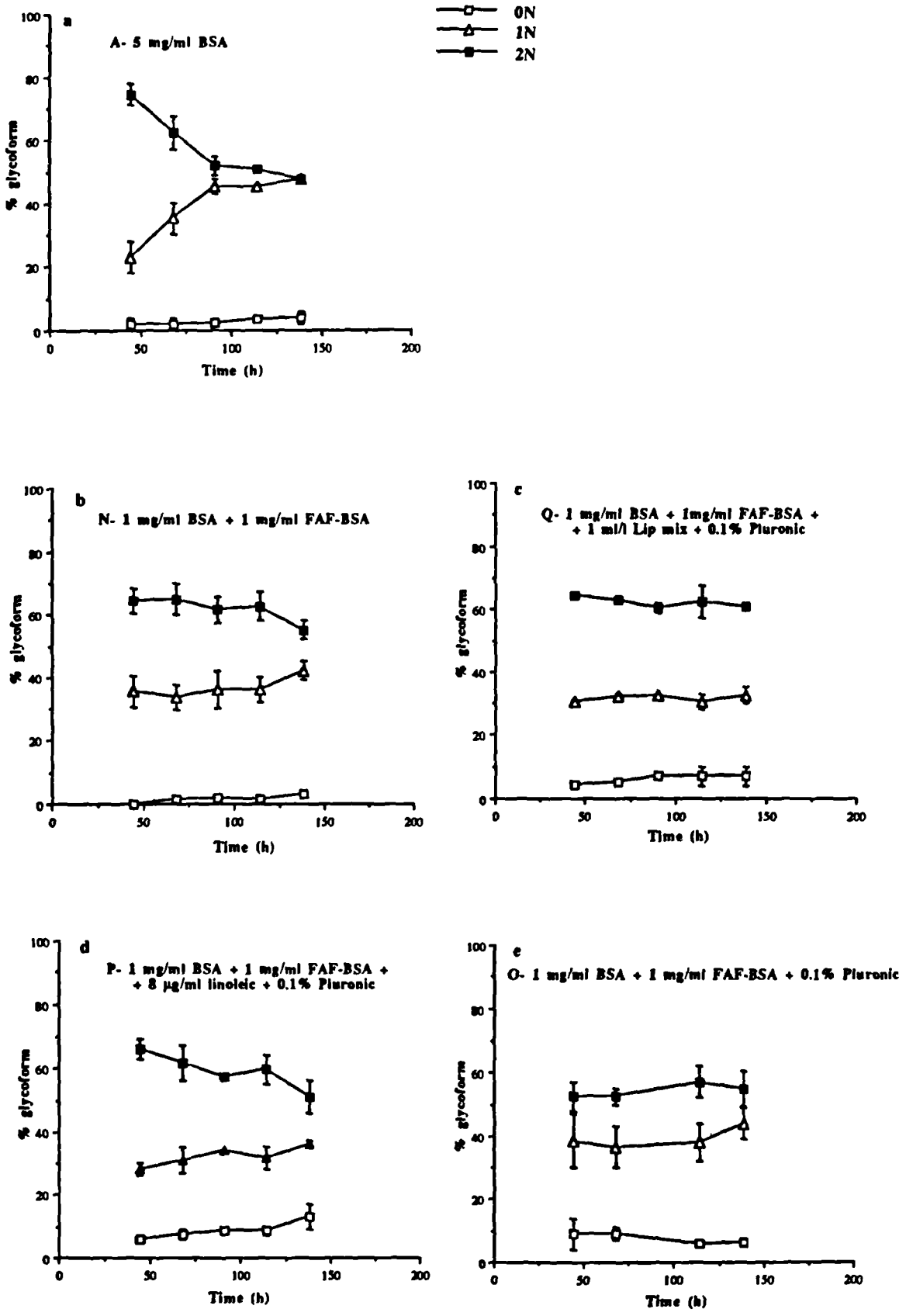


Figure 5.8- Glycosylation patterns of IFN-γ obtained in cultures A, N, O, P and Q.

Higher proportions of the 2N glycoform were found throughout the cultures containing FAF-BSA and the ratio of 2N glycoform to 1N glycoform seemed to be higher in those cultures supplemented with lipids, as compared to Pluronic F68 alone. The glycosylation could be affected by some component that is carried by FAF-BSA. However this is most unlikely, because the FAF-BSA used here comes from the same supplier as the medium BSA. It is in fact obtained by subsequent treatment (charcoal and solvent treatment) of the latter one, resulting in a more purified BSA (see section 3.4.3). Also, why that effect in the glycosylation of IFN-γ was not observed in culture L described in the previous section is not clear, as FAF-BSA was also used to prepare the linoleic acid complex, although at a lower concentration. A few considerations can be made. First, it is possible that the effect of FAF-BSA is only manifested above a certain concentration, below which other factors dominate the process. Also, it was noticed that IFN-γ obtained from cultures containing only 1 mg/ml BSA plus 1 mg/ml FAF-BSA (medium N) presented higher proportions of the double glycosylated form than control cultures with 5 mg/ml BSA (medium A). This can lead to the hypothesis that there is something present in BSA that inhibits glycosylation, for example the fatty acids it carries. Considering that linoleic acid itself can be one of these components, it is possible that its effect is only masked if a certain proportion of FAF-BSA is used.

Again, initial rates of production of the non-glycosylated form were higher in cultures supplemented with Pluronic F68 alone (Figure 5.9). The titre of each of the glycoforms in the different cultures is shown in Figure 5.10a-c. Despite the lower cell numbers obtained in non-supplemented low BSA cultures (medium N), the titres of glycosylated IFN-γ were either higher than or similar to those obtained in low supplemented ones (medium O,P,Q). In terms of production of the 2N glycoform, the level difference between the cultures was reduced, although the control culture still showed the highest titres. In this respect, among low BSA cultures a more favourable situation was found when supplementing low BSA cultures with Pluronic F68 alone.

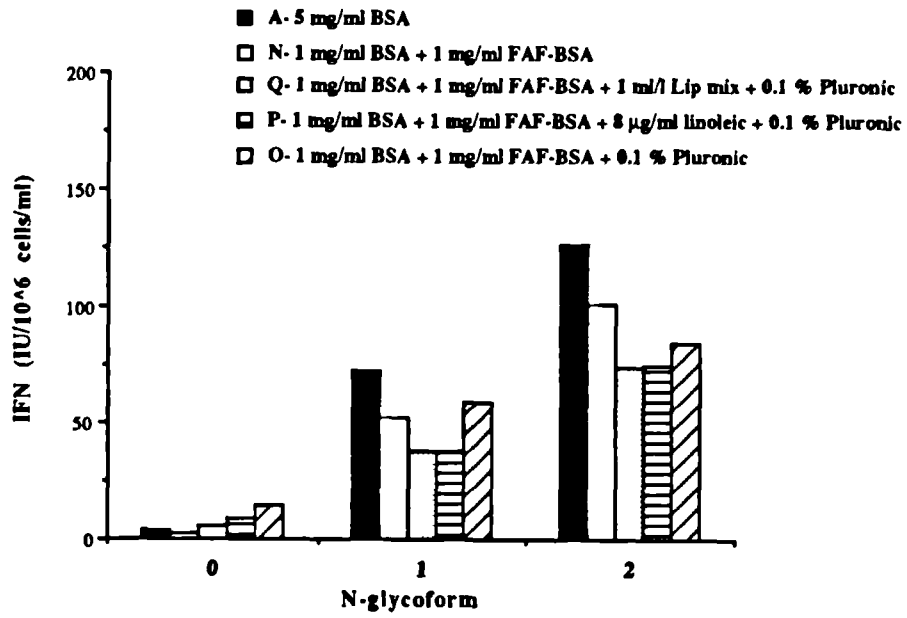


Figure 5.9- Initial production rates of the three IFN-γ glycoforms in cultures A, N, O, P and Q.

5.3.5- The independent effects of Pluronic F68 and fatty acid-free BSA

From the previous experiments, it seemed that Pluronic F68 and FAF-BSA *per se* could influence the glycosylation patterns of IFN-γ obtained from low BSA cultures. As a consequence, these effects were investigated by a simultaneous comparison of 1 mg/ml cultures supplemented either with Pluronic F68 or 1 mg/ml FAF-BSA or both. Cell growth and product titres for these cultures are shown in Figure 5.11a,b. Again it was observed that Pluronic F68 assisted cell growth, although its effect did not match that of the control culture (5 mg/ml). Addition of FAF-BSA alone supported cell growth, but to a lesser extent than supplementation with Pluronic F68 (cf. medium N and medium K). When Pluronic F68 and FAF-BSA were added together the effect of the former predominated. The typical reduction in IFN-γ titres was noticed in low BSA cultures (Figure 5.11b), but the final titres obtained in these cultures did not vary much, despite the lower cell numbers obtained in medium N.

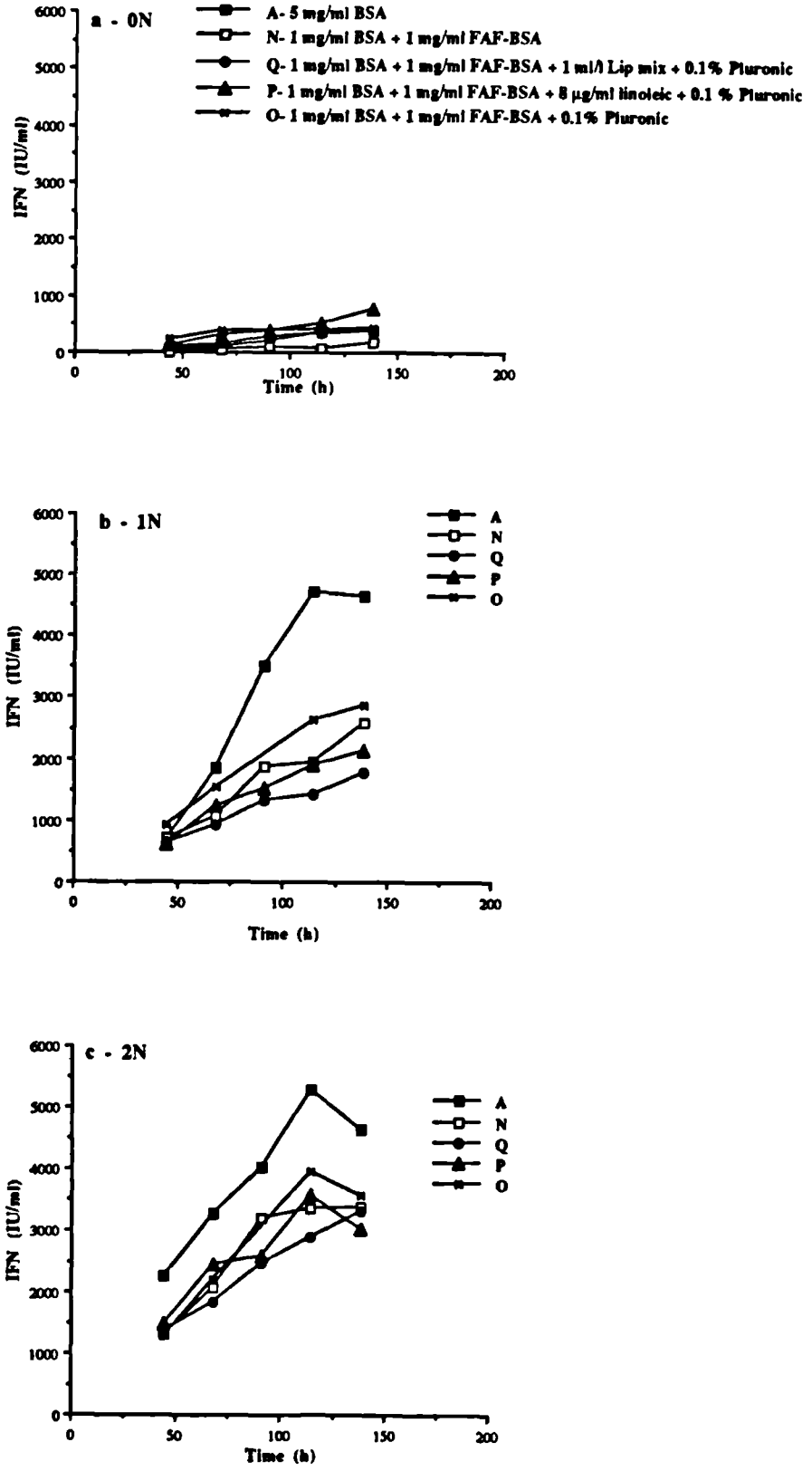


Figure 5.10- Accumulation of each IFN-γ glycoform during cultures A, N, O, P and Q.

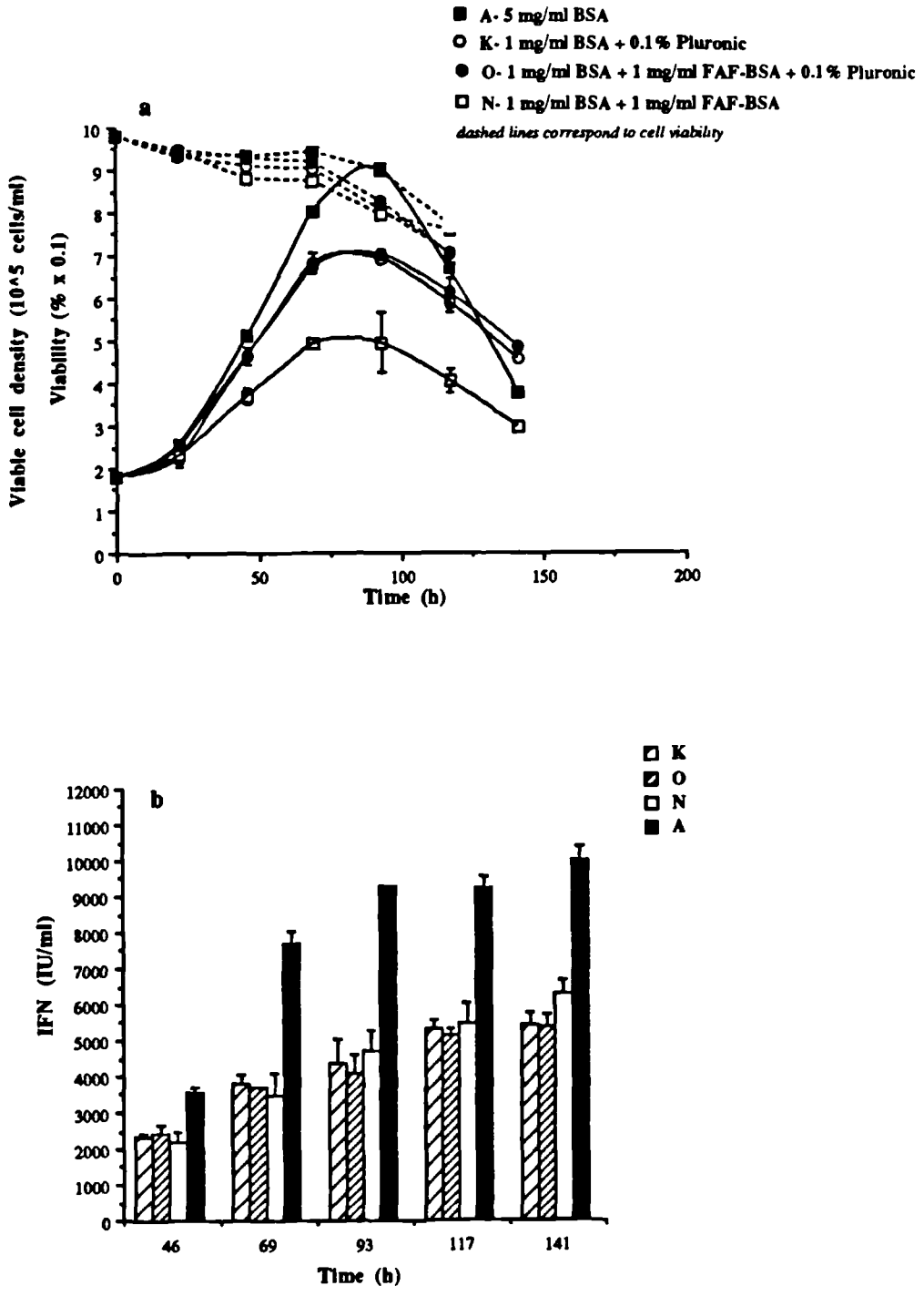


Figure 5.11- a) CHO cell growth and b) IFN-γ production in cultures A, K, N and O.

Considering that the only difference between medium N and O is the presence of Pluronic F68 in the latter case, it seems that Pluronic F68 exerts a negative effect on IFN-γ production since the product titres were similar on both cultures, despite the better growth achieved with medium O. The addition of FAF-BSA to Pluronic F68 supplemented cultures did not have any effect on IFN-γ production, as was also observed for cell growth.

Analysis of the glycosylation patterns of IFN-γ (Figure 5.12a-d) further demonstrated that in the presence of 1 mg/ml FAF-BSA a higher proportion of IFN-γ glycosylated at both sites is produced over the course of the culture (cf. medium N and medium O) while the addition of Pluronic F68 (cf. medium K and medium O) caused an increase in the proportion of non-glycosylated IFN-γ, although the latter was not very pronounced. Table 5.11 shows the proportions of non-glycosylated form and the 2N glycoform after ca. 95 h in culture. A lower proportion of the IFN-γ molecule is occupied at both sites in cultures supplemented with Pluronic F68, while further addition of FAF-BSA seems to increase this proportion and the highest proportion is obtained when Pluronic F68 is not present. This seems to reinforce the idea that the fatty acid associated with the BSA may decrease the glycosylation of IFN-γ, and the addition of FAF-BSA may counteract this effect.

Table 5.11- Proportions of the 0N and 2N IFN-γ glycoforms in low-BSA cultures.

Medium	**% glycoform**	
	0N	**2N**
K- BSA 1 mg/ml + 0.1% Pluronic F68	15	47
N- BSA 1 mg/ml + FAF-BSA 1 mg/ml	5	60
O- BSA 1 mg/ml + FAF-BSA 1 mg/ml + 0.1% Pluronic F68	10	55

values taken at ca. 90 h in culture.

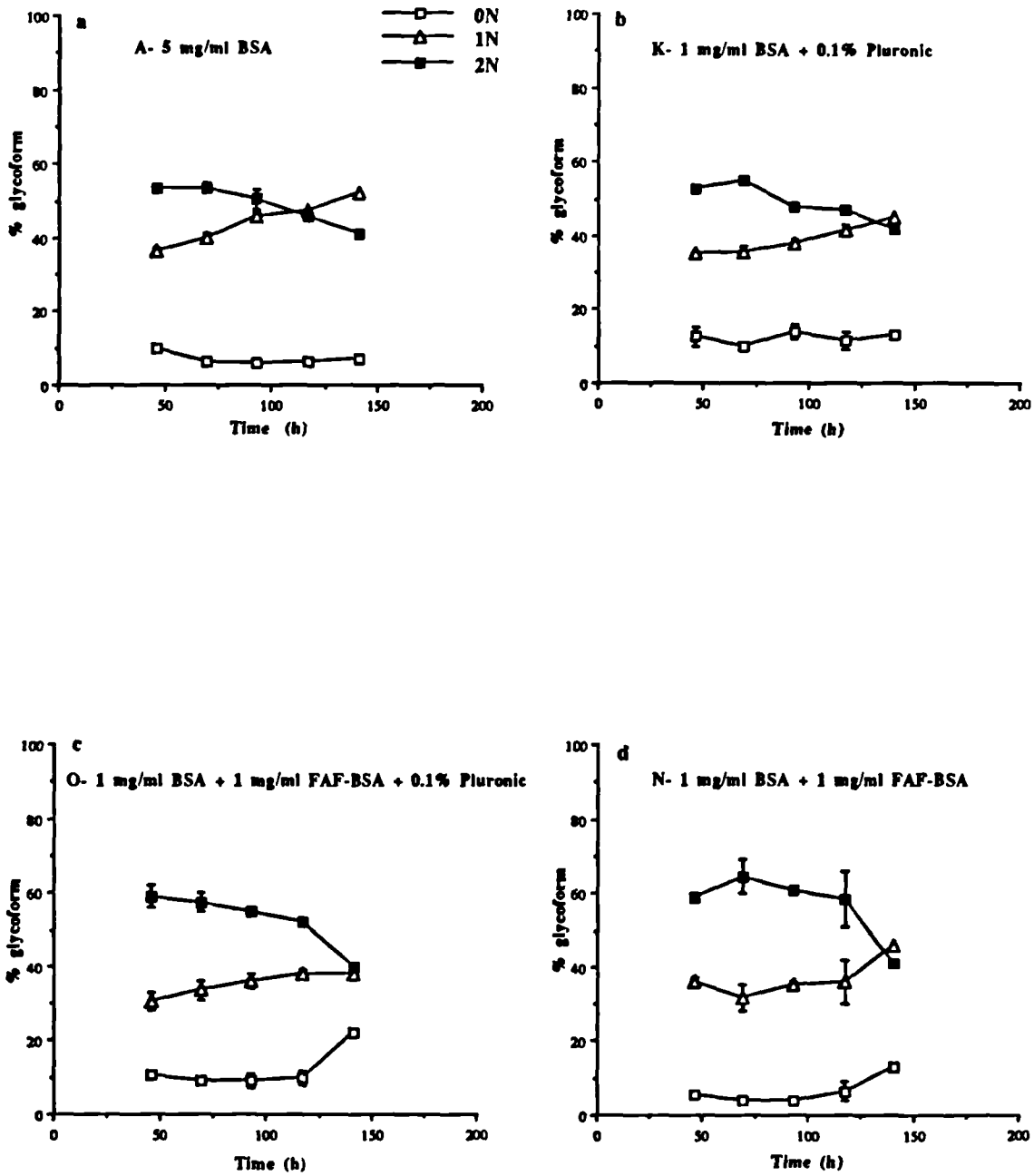


Figure 5.12- Glycosylation patterns of IFN-γ obtained in cultures A, K, N and O.

5.4- Supplementation of control cultures with lipids and Pluronic F68

The hypothesis put forward until now were based mostly on low BSA cultures, and it was not clear how much the reduction in BSA itself could account for the results obtained. Analysis of the effect of lipid and Pluronic F68 over control cultures (5 mg/ml BSA) on cell growth and IFN-γ production was important to determine their independent effects on these parameters.

5.4.1- Addition of lipids to control cultures

Addition of the previously used lipid mixture (medium B) to high BSA cultures (medium A) improved cell growth, with approximately 25% increase in cell titres and a slight extension of the stationary phase (Figure 5.13a), reinforcing the idea that the positive effect of BSA observed before was of a nutritional nature. The fact that the same lipid supplementation did not have the same effect on cell growth over 1 mg/ml BSA plus 0.1% Pluronic F68 cultures (cf. medium E and medium K, section 5.33) further suggests that the net effect may be dependent on the overall lipid balance of the medium. However, despite the higher cell numbers achieved with this formulation, IFN-γ titres were reduced and this was more predominant as the time course of the culture increased (Figure 5.13b), with approximately 35% reduction on initial production rates. This result supports the finding that lipid supplementation has negative effects on IFN-γ production.

Analysis of the proportions of the three glycoforms throughout the cultures showed little difference in the glycosylation patterns of the product (Figure 5.14a,b). Addition of the lipid mixture over 5 mg/ml BSA cultures did not seem to affect the glycosylation pattern of the product. This is an indication that the glycosylation process is not directly linked with the rate of product formation, since cells with lipid supplementation secreted lower amounts of product. This is consistent with the findings of Bulleid *et al.* (1992), who reported that while

the extent of N-glycosylation of t-PA was dependent on the state of folding of the polypeptide chain, the variation in glycosylation was independent of the rate of protein synthesis. Lin *et al.* (1993) has also found that while the production rate of t-PA decreased by 80% under anoxic conditions, the quality of the product was not affected. However, there are also reports suggesting that the variation in the extent of N-linked glycosylation may be dependent on the rate of synthesis of these proteins (Miletich and Broze, 1990).

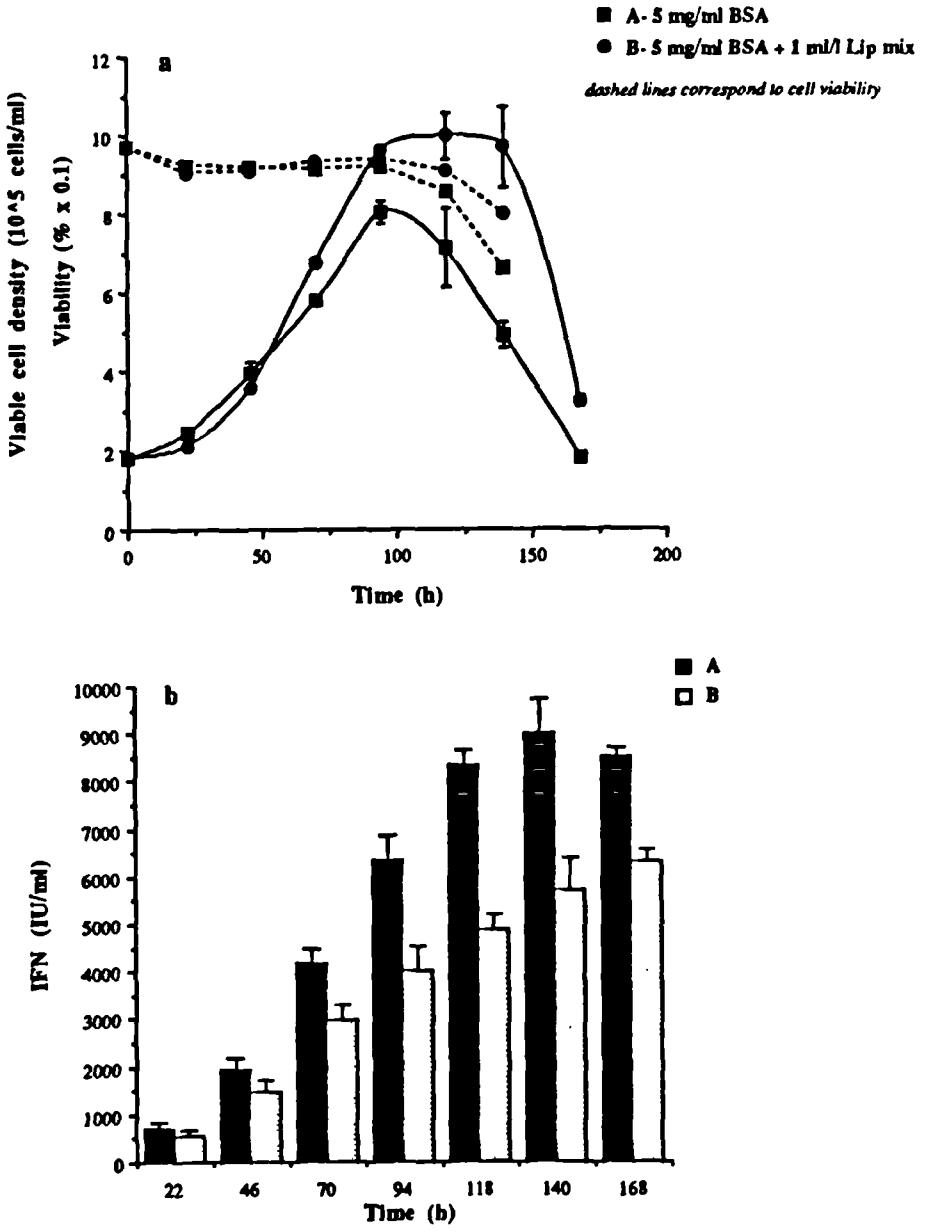


Figure 5.13- a) CHO cell growth and b) IFN-γ production in 5 mg/ml BSA cultures supplemented with 1 ml/l lipid mixture.

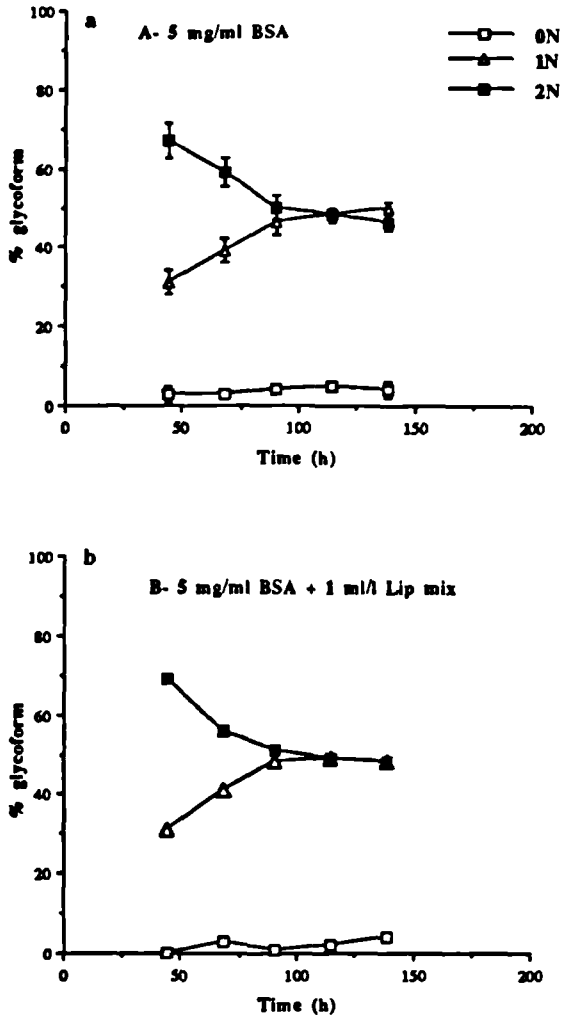


Figure 5.14- Glycosylation patterns of IFN-γ obtained in 5 mg/ml BSA cultures supplemented with 1 ml/l lipid mixture. [No error bars are given for culture B as only one set of results were obtained].

5.4.2- Addition of Pluronic F68 to control cultures

No significant effect on cell growth was observable with 0.1% Pluronic F68 supplementation (medium C) of high BSA cultures (Figure 5.15a). This seems to support the idea that the positive effect observed in low BSA cultures is due to a replacement of the protective effects of BSA. A decrease in initial production rate of IFN-γ was detected, 186 against 235 IU/10^6cells/ml for the control culture, but final product titres were similar (Figure 5.15b).

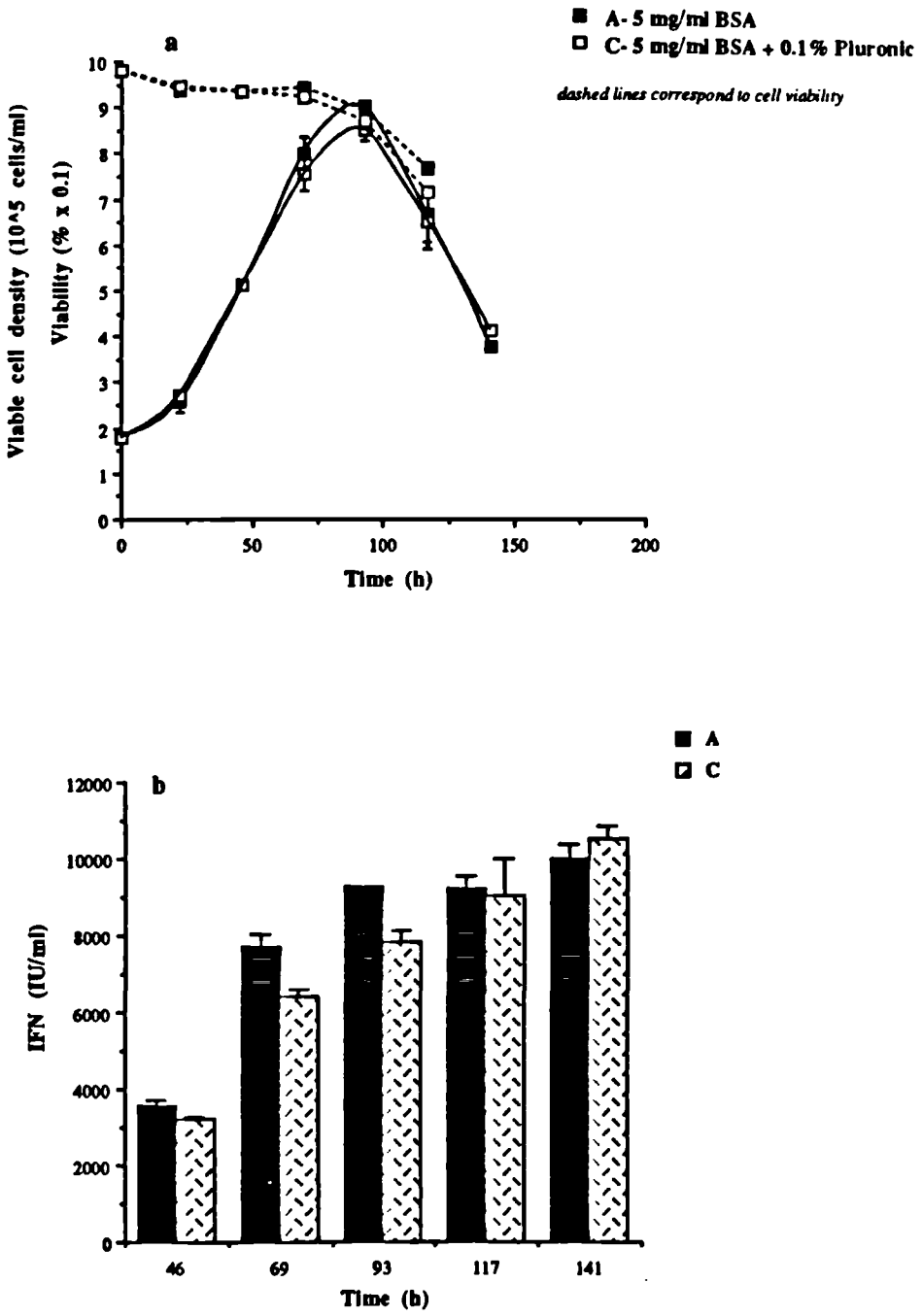


Figure 5.15- a) CHO cell growth and b) IFN-γ production in 5 mg/ml BSA cultures supplemented with 0.1% Pluronic F68.

A slight increase in the proportion of the non-glycosylated form of IFN-γ was noticed in Pluronic F68 supplemented cultures (Figure 5.16). After 90 h in culture ca. 5% the non-glycosylated form was found in control cultures against ca. 13% in the supplemented ones. This seems to indicate that the increase in non-glycosylated IFN-γ observed in the low BSA cultures described before could be partially due to the presence of Pluronic F68, but there is no substantial evidence for this. The level difference in 2N glycoform was even less pronounced, ca. 45% against 50% respectively. Considering that at that time titres of total IFN-γ between both cultures were not much different, the net effect of Pluronic F68 over high BSA cultures probably is not significant.

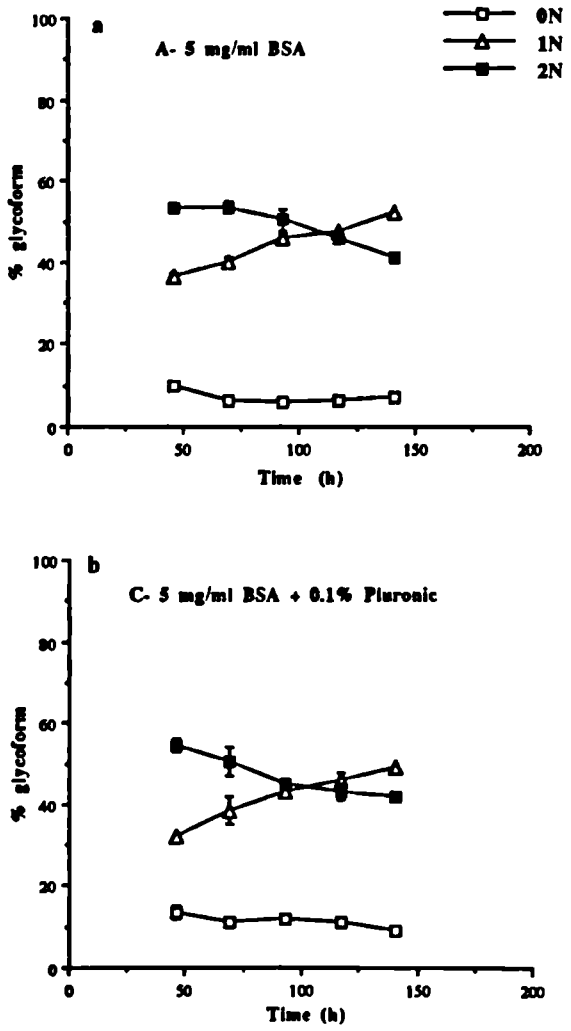


Figure 5.16- Glycosylation patterns of IFN-γ obtained in 5 mg/ml BSA cultures supplemented with 0.1% Pluronic F68.

5.5- Effect of serum in culture

The process of developing a more defined medium did not seem to be straightforward and just looking at cell growth was not enough. All along, BSA seemed to play a major role, both in the level of IFN-γ production and the authenticity of the molecule itself. It seemed sensible to question cell behaviour and product characteristics in the presence of serum, by making the medium even less defined. Protein and trace elements supplements were omitted in this experiment and foetal calf serum was added at a concentration of 8%. Growth was improved to a large extent, with higher specific growth rates and an increase of ca. 90% in viable cell titre (Figure 5.17a). Cell viability was maintained for a longer period. However, there was a great reduction in product titres (Figure 5.17b), in a similar way to that previously observed with lipid supplementation. The negative effect of serum may be related to the endogenous lipid fraction that it carries. Serum also carries growth factors, such as EGF and FGF. They have both been shown to stimulate the growth of these cells, while reducing the specific rate of production of IFN-γ and in the latter case also reducing the titres achieved in culture (Tomlinson, 1991).The results obtained here reinforce the idea that an optimal medium for cell growth is not necessarily the best for IFN-γ production.

IFN-γ glycosylation analysis showed a different distribution pattern of the several variants within the same major molecular weight glycoform (Figure 5.18), with stronger bands in the regions of lower range of molecular sizes. In terms of proportion of each of the glycoforms, no major differences were detected (Figure 5.19). The typical initial decrease of the 2N glycoform was noticed, and after 70 h the proportions remained the same throughout the culture, with ca. 50% of the 2N glycoform, 40% of the 1N glycoform and 10% of the non-glycosylated form.

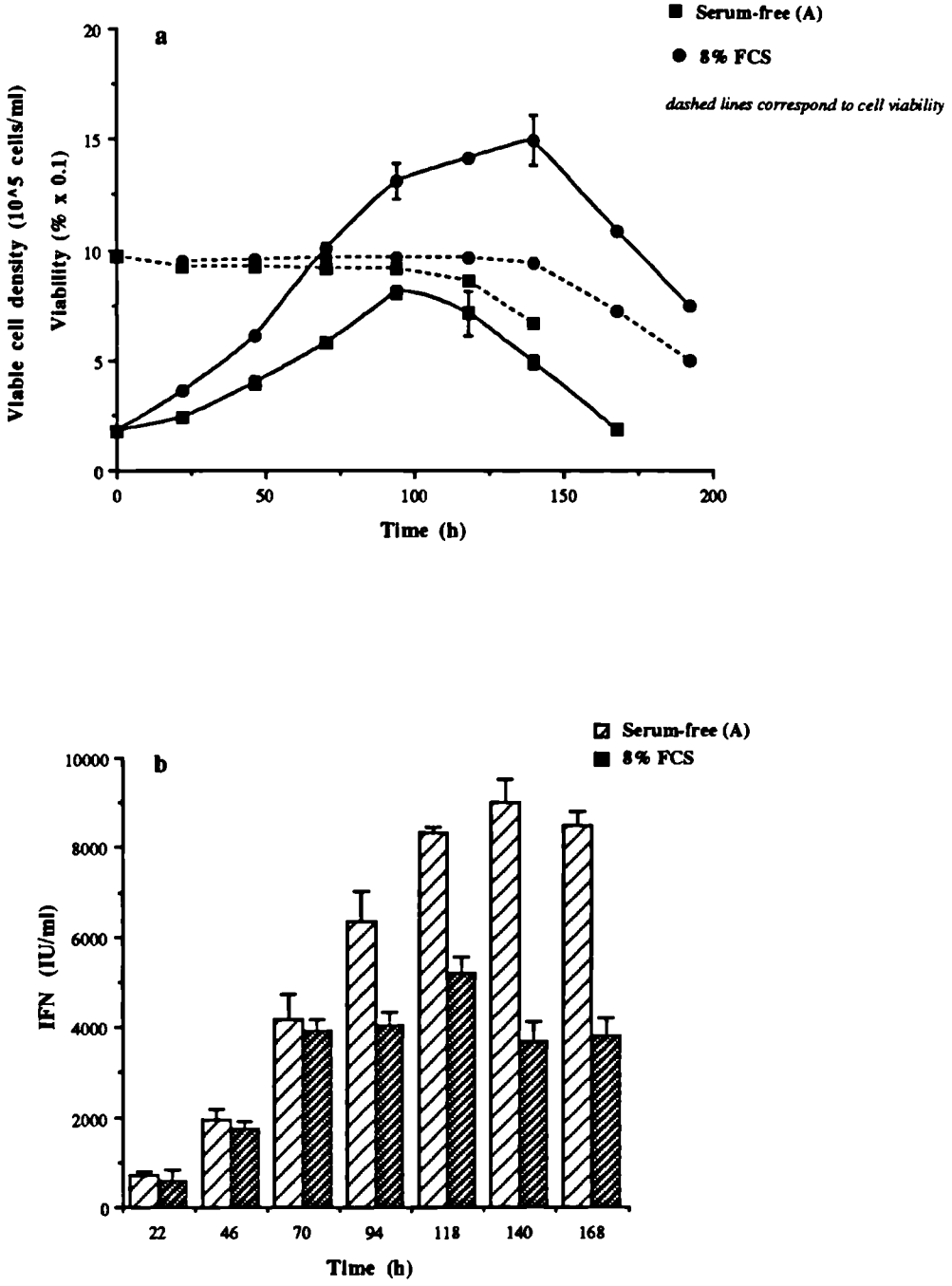


Figure 5.17- a) CHO cell growth and b) IFN-γ production in serum supplemented cultures.

It is possible that the differences observed in the patterns were due to proteolytic cleavage of IFN-γ by proteases carried by serum, as it has been reported for t-PA (Lin *et al.*, 1993). In other reports, there was evidence that serum also affected the sugar content of the oligosaccharide chains attached to the protein, e.g., changes in the sialic acid content (Megaw and Jonhson, 1979; Patel *et al.*, 1992; Maiorella *et al.*, 1993). However, in the present study no studies were conducted to elucidate any of these hypotheses.

5.6- Concluding remarks

The results of studies reported in this chapter have shown clearly that cell growth and production require different nutritional stimuli, giving support to the proposition made in Chapter 3. The role of the culture composition was highlighted by the different responses that were achieved for CHO cell growth, IFN-γ production and IFN-γ glycosylation towards several culture environments. This has striking implications on the design of a medium culture for biological production.

CHO cell growth was successfully achieved in low-BSA (1 mg/ml) cultures, but there was always a reduction in IFN-γ production. The importance of BSA in the culture medium was once more evidenced. Furthermore, not only cell growth and/or IFN-γ titres were altered in low-BSA cultures, but also different IFN-γ glycosylation patterns were obtained from different environments. Both concentration and type of BSA seemed to be important factors for this process, although the glycosylation analysis was rather puzzling.

Among others, to obtain a more reproducible culture environment while reducing the fermentation costs, were the main reasons for reducing the BSA concentration of the medium. The fact that different types of BSA gave different responses in terms of authenticity of the

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Among others, to obtain a more reproducible culture environment while reducing the fermentation costs, were the main reasons for reducing the BSA concentration of the medium. The fact that different types of BSA gave different responses in terms of authenticity of the

product obtained put emphasis on the reproducibility issue and the choice of the starting material for a production process was given primary attention. This consideration is particularly important for components which are obtained from fairly undefined sources, such as BSA.

An estimate of the unit cost of IFN-γ obtained from each low-BSA culture in relation to the cost of the culture medium and compared with the control culture, is shown in Table 5.12. The culture with serum supplementation is also shown to give evidence of the large contribution that serum has on the cost of animal cell culture fermentation. These figures do not reflect commercial production costs because of the differential costs of small and large scale purchasing and possibly the grades of chemicals used. However, the figures do reveal valid comparative costs.

No improvements were obtained in terms of cost of production; at least a 50% increase in the unit cost of IFN-γ was obtained in the low-BSA cultures, reflecting the lower titres achieved. However, the various conditions which were used led to the production of IFN-γ with different glycosylation patterns in terms of macroheterogeneity (proportion of oligosaccharide attached) in the case of low-BSA cultures, and in relation to the distribution of the molecular weight variants among the three major forms (proteolytic cleavage and/or type of sugars attached) in the case of serum supplemented cultures. How much this would affect the biological properties of the product, such as pharmokinetics and immunotolerance, would be the kind of analysis required for the evaluation of each production system.

Table 5.12- Relative unit cost of IFN-γ produced using different media.

	Relative Unit Cost (per IU IFN-γ per volume)
Control- BSA 5 mg/ml	1.00
Pluronic F68	
+ BSA 1	1.50
+ BSA 1 + FAF-BSA 1	1.65
Lipid mixture	
+ BSA 1 + Pluronic F68	2.31
+ BSA 1 + Pluronic F68 + FAF-BSA 1	2.20
Linoleic acid*	
+ BSA 1+ Pluronic F68	1.73
+ BSA 1 + Pluronic F68 + FAF-BSA 1	1.63
FAF-BSA 1	
+ BSA 1	1.60
8% Serum	28.20

*added as a FAF-BSA complex.

6- GENERAL DISCUSSION

6.1- The Plackett-Burman statistical design applied to the optimisation of the cell culture composition

The benefits arising from using statistically designed experiments have been illustrated in this study. Clearly, the Plackett-Burman procedure is a very valuable tool to process optimisation. The identification of important variables to the process could be made using a fairly small number of experiments and an improvement of both CHO cell growth and IFN-γ production was achieved based on those variables (Chapter 3). In fact, when all the nutrients identified as positive for both parameters were added simultaneously to the culture, improvements in excess of 40% were obtained in CHO cell growth and IFN-γ production. The effect of individual lipid components on CHO cell growth and IFN-γ production in low-BSA cultures (1 mg/ml) was also determined with this technique (Chapter 5). A few studies on parameters affecting cell culture performance have already emphasised the benefits of including such methods in a rational optimisation procedure (Ganne and Mignot, 1990; Gebert and Gray, 1990; Gaertner and Dhurjati, 1993). There are limitations to these techniques but a cautious analysis of the results can identify key factors for further investigation.

The possible interactions between nutrients that may take place in the cell culture medium were not accounted for in this study. The Plackett-Burman experimental design does have its limitations, arising from the fact that it is a highly fractionated procedure. Interactive effects among variables can not be identified, unless the number of experiments N is an integral power of 2. This is a weak aspect of the technique and must be considered when evaluating the results. It is possible that an assigned main effect to an independent variable is in fact the result of two-factor or even higher order interactions between other variables, i.e., the effect of main variables and interactions between variables are "confounded". However, high-order

interactions usually are not as important as main effects. In this study, a more efficient utilisation of glucose and glutamine was seen when increasing the concentration of the positive variables in the culture (Chapter 3), possibly due to interactive effects between variables. In Chapter 5, linoleic acid was identified as having a positive effect on CHO cell growth, but its negative effect on IFN-γ production, which was later determined, was not manifested in the Plackett-Burman analysis. It is possible that the magnitude of the effect of other variables, namely cholesterol, was high enough to mask the effect of linoleic acid. In fact, other restrictions of the technique are associated with the choice of the nutrient concentration to include in the experimental matrix. The difference between the high and low level should be large enough to allow a measurable effect but there is the danger of masking the results of other variables if that differential is too high.

Other statistical procedures, also based on fractional factorial studies, allow the quantification of interactive effects. The effect of the main independent variables may be separated from the effect of the appointed interactive variables (Gebert and Gray, 1990). However, if the total number of variables, including main plus interactive variables, exceed the number of experiments, then not all the effects can be isolated, leading again to the occurrence of "confounding" among the determined effects (Gaertner and Dhurjati, 1993).

6.2- The cell culture environment

The present study gave clear indication that CHO cell growth and IFN-γ production exhibit different dependencies on the cell culture environment. Furthermore, the glycosylation profile of IFN-γ also varied with some of those changes. It is not easy to assign specific roles to each of the investigated components, and the net effect obtained on either CHO cell growth or IFN-γ production is probably related to interactive effects, e.g., synergism and/or competition,

between the various components.

The major effects found for the environmental conditions which were investigated are summarised in Table 6.1. This overall view is an attempt to provide a better understanding of the whole process and to illustrate the different behaviour of CHO cell growth and IFN-γ production under some of the culture conditions. The analysis is separated into high-BSA cultures (5 mg/ml) and low-BSA cultures (1 mg/ml), and a comparison in relation to the control culture is always provided. This Table will be used as a guideline for the following discussion. The same numbering system used in the Table for each particular condition is also used in the text.

6.2.1- CHO cell growth and IFN-γ production enhanced by different stimuli

The production kinetics of IFN-γ in a control batch culture showed an apparent relationship between CHO cell growth and IFN-γ production, as IFN-γ accumulated during the cell growth phase (section 3.2). This is supported by earlier observations in batch culture (Hayter *et al.*, 1991a) and also by studies performed in chemostat cultures (Hayter *et al.*, 1993). However, IFN-γ production at a relatively high rate has also been achieved when cells were grown under glutamine starvation (Hayter *et al.*, 1991a), thus cell growth association kinetics can not be firmly established.

Table 6.1- Effect of several components on CHO cell growth and IFN-γ titre and glycosylation.

		Cell titre	IFN-γ production	
			Titre	%Glycosylation
[1] Control- BSA M 5				
[2]	+ PB positive group A + BSA S 2.5	+	+	ns
[3]	+ PB positive group A	+	+	ns
[4]	+ BSA S 2.5	+	+	nd
[5]	+ BSA M 2.5	ns	ns	nd
[6]	+ Lipid mixture	+	-	ns
[7]	+ 0.1% Pluronic F68	ns	ns	ns
[8] BSA M 5 + positive group A		*a*	*a*	*s*
[9]	+ Glucose & Glutamine feeding	ns *a*	ns *a*	ns *s*
[10] BSA M 1 + 0.1% Pluronic F68		*b*	*b*	*b*
[11]	+ Lipid mixture	- *b*	- *b*	ns *b*
[12]	+ Linoleic acid*	+ *s*	- *b*	- *b*
[13] BSA M 1 + FAF-BSA 1		*b*	*b*	*a*
[14] BSA M 1 + FAF-BSA 1 + 0.1% Pluronic F68		*b*	*b*	*a*
[15]	+ Lipid mixture	ns *s*	- *b*	ns *a*
[16]	+ Linoleic acid	+ *s*	- *b*	ns *a*
[17] 8% Foetal calf serum		*a*	*b*	ns §

The effect of each component(s) is related to the correspondent base culture: (+) positive; (-) negative; (ns) not significant; (nd) not determined. A comparison between each culture and the original control culture [1] is also provided: (*a*) above [1] (*b*) below [1]; (*s*) similar to [1]; (§) differences observed in relation to the micro-heterogeneity of IFN-γ. M- Miles Pentex; S- Sigma; Positive group A = amino acids and sodium pyruvate as identified by the Plackett-Burman analysis. *- added as a FAF-BSA complex. The numbers following the type of BSA correspond to its concentration in the culture. Cell and IFN-γ titres are related to the maximum achieved in culture; % glycosylation is related to ca. 100 h in culture.

Evidence that CHO cell growth and IFN-γ production may not be enhanced by the same stimuli was found throughout this investigation. In Chapter 3, an improvement in IFN-γ production was obtained simultaneously with an improvement in cell growth when the concentration of all the positive variables was increased in the beginning of the culture [2]. However, the individual analysis of the Plackett-Burman experimental data has also shown that a positive response for CHO cell growth did not always imply a positive response in IFN-γ production. Furthermore, BSA seemed to be one of the major components responsible for the improvements in IFN-γ production, as revealed by the analysis of the separate effects of BSA. The results obtained in Chapter 5 strongly supported the latter view. When CHO cell growth in non supplemented 1 mg/ml BSA cultures was achieved, IFN-γ was produced slowly, up to the end of the culture, although cell decline started prematurely (ca. 60 h). In addition, whereas CHO cell growth in low-BSA cultures could be totally restored by adding a mixture of Pluronic F68 and linoleic acid [(12), (16)- see Table 6.1], IFN-γ production was still much less in those cultures. The more drastic situations were found when clear improvements in cell growth above control cultures were achieved by lipid or serum supplementation and IFN-γ production was strongly suppressed [(6), (17)].

The apparent relationship between cell growth and IFN-γ production may thus not be directly related to the growth rate *per se*, but may be a reflection of other cellular physiological characteristics, such as the activity of certain metabolic pathways and/or the integrity of the cell membrane, which are related to the environment that surrounds the cell. As a result, cell function may be ultimately affected.

6.2.2- Influence of BSA on CHO cell growth and IFN-γ production

Throughout this investigation there was clear evidence of the influence that BSA exerted on CHO cell growth and IFN-γ production. Although its relevance was manifested early in the first Plackett-Burman analysis (Chapter 3), the studies reported in Chapter 5, attempting to establish the role of BSA in culture, helped to clarify some of the questions associated with its function. BSA seemed to be the major component for IFN-γ production within the positive group of variables identified by the Plackett-Burman procedure, but different types of BSA had distinct effects on CHO cell growth and IFN-γ production [(4), (5)]. Furthermore, none of the low-BSA cultures [10-16] produced the same levels of IFN-γ as the control culture [1], although CHO cell growth could be totally restored by adding Pluronic F68 and linoleic acid [(12), (16)].

BSA is a very poorly defined component in the culture and most of the serum-free media that have been published in the literature include BSA in their formulation (Barnes and Sato, 1980; Iscove *et al*, 1980; Glassy *et al*, 1988; Büntemeyer *et al*, 1991). However, the mechanism behind its stimulating effect is the subject of some debate and it has been mostly related to the following properties: (1) BSA functions as a carrier for several components, including lipids (Barnes and Sato, 1980; Iscove *et al*, 1980) and its fatty acid binding properties may prevent the toxicity of free fatty acids in culture (Darfler and Insel, 1983); (2) BSA provides cells with protection from shear damage in adverse environments (Hülscher and Onken, 1988; Papoutsakis, 1991a; Smith and Greenfield, 1992) and, (3) the ability of BSA to inhibit copper-stimulated peroxidation and the generation of free hydroxyl radicals from H_2O_2 prevents toxicity by oxidation of unsaturated fatty acids in the membranes or oxidation of cellular proteins (Darfler and Insel, 1983; Gutteridge, 1986; Halliwell, 1988). The data obtained in the present study seem to provide evidence for both former mechanisms while not excluding the latter one, and the effects observed on CHO cell growth and IFN-γ production will be discussed in this light.

6.2.2.1- Nutritional effect

In this study, a nutritional role for BSA was indicated by the following results: an increased concentration of one type of BSA exerted a positive response on CHO cell growth and IFN-γ production [4], whereas increasing the concentration of the type of BSA already present in the culture did not have any effect [5]. The growth stimulating effect of BSA was thus dependent on the source used (BSA Sigma or BSA Miles). This suggests that some undefined component of BSA may be important for CHO cell growth and IFN-γ production. Sigma and Miles do use different purification strategies to obtain BSA Fraction V, which may be the reason for the different responses obtained. The work of Jayme (1991) with hybridomas gives support to this view. The latter study has shown that different sources of BSA have distinct effects on cell growth, and the authors related that effect to the distinct lipid profile of each preparation, as determined by HPLC separation. The fact that in the present study the addition of a lipid mixture to cultures containing 5 mg/ml BSA promoted an increase in cell numbers further supported the possible nutritional role of BSA. However, there was a decrease in the production of IFN-γ under those conditions, which leads also to the possibility that the positive effect of BSA Sigma on CHO cell growth and IFN-γ production may result from components other than the lipid fraction.

6.2.2.2- "Protective" effect

The role of BSA as a protective agent against adverse culture conditions seemed to be emphasised in this study. An alteration of physical parameters of the culture, such as viscosity, when reducing the BSA content of the culture was expected. Considering that the cells were grown in suspension culture, the susceptibility of the cells to mechanical forces, particularly agitation, could be affected. The results obtained by supplementing the cultures with Pluronic F68 supported the protective role of BSA. In fact, although Pluronic F68 did not have any

significant effect over 5 mg/ml BSA cultures [7], it was able to substantially restore cell growth in 1 mg/ml BSA cultures [10]. Its effect may be related to its postulated interaction with the cell membrane, as it was reported for insect cells (Murhammer and Goochee, 1988), affording cells shear protection. This view is supported by the studies of Ramirez and Mutharasan (1990) with hybridomas, which assigned the Pluronic F68 protective properties to its ability to form a semi-stagnant film at the cell surface, leading to a decrease in the apparent fluidity of the cell membrane and increased cell resistance to shear. Later studies (Zhang *et al.*, 1992a) have also shown that Pluronic F68 had a strengthening effect on the cell membrane of hybridoma cells. The interaction of Pluronic F68 with the cell membrane is further supported by the work of Lakhotia *et al.* (1993), which has shown that Pluronic F68 has a protective effect on the content of a specific surface protein of hybridoma membranes, independently of shear damaging conditions. It is possible that BSA acts by a similar protection mechanism to that ascribed to Pluronic F68, and the effect of the latter observed in this study in low-BSA cultures is related to a replacement of this function. However, the protection afforded by BSA seems to be dependent on cell line and culture conditions. Hülscher and Onken (1988) did not find any influence of BSA on hybridoma cell growth and monoclonal antibody production in static and spinner suspension cultures in the range of 0 to 1 mg/ml BSA, but the presence of BSA was absolutely required for growth to occur in an airlift bioreactor. This seemed to be due to the action of BSA at the gas liquid-interface, as proposed by Handa-Corrigan *et al.* (1989) for Pluronic F68. Given that the present studies were carried out in shake-flasks, this mechanism is not suitable to explain the observed action of Pluronic F68. Smith and Greenfield (1992) also reported a protective effect of BSA on hybridoma growth in bioreactors at high agitation rates (600 rpm) but the presence of BSA was also essential for growth to occur in static cultures. Thus, while BSA provides protection to the cells in adverse environmental conditions, which may result from agitation and aeration, there are other factors which support its importance in culture.

6.2.2.3- Antioxidant effect

The effect of the lipid mixture on IFN-γ production was negative both in low-BSA [(11), (15)] and high-BSA cultures [6], but the cell growth response towards the same component was dependent on the BSA content of the culture. The growth stimulating effect observed in cultures containing 5 mg/ml BSA [6] was not manifested in low-BSA cultures [(11), (15)]. In fact, the effect was negative in cultures containing the lowest BSA content, 1mg/ml [11], while no effect was observed if an extra 1 mg/ml FAF-BSA was included in the culture [15]. It is feasible that a more extensive oxidation of unsaturated fatty acids in the membranes may occur at lower BSA concentrations, and this is possibly avoided when extra FAF-BSA is included in the culture. The following mechanisms could contribute to this protection: (1) inhibition of peroxidation by an increase in total BSA content and (2) enhanced binding of free fatty acids by the FAF-BSA, preventing their possible toxic effects as free components in culture. This could in some way justify the variable effects on cell growth obtained with the lipid mixture in high and low-BSA cultures, strengthening the role of BSA as an antioxidant agent in culture. The fact that IFN-γ production is lower when the BSA content of the culture is reduced and when lipids are added to the culture, may indicate that cells respond to these new environments by increasing the production of antioxidant molecules, as it has been reported for CHO cells under stressful conditions, e.g. anoxia and hypoxia (Lin and Miller, 1992), and it is possible that it leads to competition with other cellular activities, such as product formation.

6.2.2.4- Cell structural integrity

The distinct responses obtained for CHO cell growth and IFN-γ production obtained under different culture environments may be related to the structural "integrity" of the cell, namely the cell membrane. This may be once again reflect the BSA content of the culture, as suggested by the effect of Pluronic F68 in low-BSA cultures. The lipid composition of the

culture is another aspect to consider. Due to the different lipid content expected in each BSA preparation, the culture lipid composition is expected to be determined by the type and concentration of BSA used, together with each specific lipid supplementation. This in turn affects the lipid composition of the cell membrane. It has been long reported for mouse LM cells that the unsaturated fatty acid content of the membrane strongly affected cell growth, and less than 50% unsaturated fatty acids on the acyl groups of the membrane phospholipids led to severe growth inhibition (Doi *et al.*, 1978). Phospholipids are one of the major components of the membranes and are critical in maintaining the integrity and regulation of various membrane functions. Indeed, several components added to the cell culture, including cholesterol and Pluronic F68, have been shown to affect the fluidity of the membrane (Ramirez and Mutharasan, 1990). In this study, cholesterol exhibited negative effects on CHO cell growth while linoleic acid (C18 with two double-bonds) was shown to have a stimulating effect. Whether the latter effect is related to a re-establishment of such membrane lipid equilibrium is a possibility to consider. Also, although cell growth in 1 mg/ml BSA cultures was restored, cell function in terms of IFN-γ production was affected differently. The improvement in cell growth, concurrent with a decrease in IFN-γ production obtained by supplementing 5 mg/ml BSA cultures with a lipid mixture, may be also a reflection of the disparate effects in cell function. This is supported by the work of Schmid *et al.* (1991), who reported different effects of linoleic and oleic acids on BHK cell growth and anti-thrombin production. In their study, an increase in product titre concurrent with no changes in cell growth was obtained by supplementing batch cultures with those fatty acids. A certain lipid composition of the membrane may in fact be essential for the cells to achieve an equilibrium appropriate for both growth and production and this is determined by the cell culture environment.

6.2.3- Glycosylation of IFN-γ

In the initial stages of a control batch culture, approximately 70 % of IFN-γ is glycosylated at 2 sites, 25 % is glycosylated at only 1 site and 5% is not glycosylated. A continuous shift throughout the duration of the culture leads to the following profile after approximately 100 h: ca. 6% remains non glycosylated whereas only ca. 48% is glycosylated at 2 sites and ca. 46% is glycosylated at 1 site. There is some evidence that this change in glycosylation profile is not due to instability of the carbohydrate residues, as the incubation of exogenous ^{35}S-labelled IFN-γ for up to 96 h in cell-free conditioned medium does not affect the proportion of IFN-γ glycoforms (Curling *et al.*, 1990). It is also important to note that the shift does not reflect the net production pattern of each of the forms. IFN-γ is produced during the growth phase of the cells, reaching its maximum titre at ca. 100 h, and that is followed by an increase in the amount of the three forms (see Chapter 5, Figures 5.6 and 5.10). As such, although there is a decline in the proportion of the 2N glycoform during the culture, its maximum titre is also reached at ca. 100h. In some cultures IFN-γ concentration declined slightly at the end of the culture (after 100 h); the liberation of intracellular proteases by non-viable cells may lead to proteolysis of IFN-γ and there is evidence that glycosylated forms are more resistant to proteolysis (Curling *et al.*, 1990). The data obtained in this study are not sufficient to provide any further understanding of the dynamics of IFN-γ synthesis and degradation.

The changes of the culture environment with the age of the culture, e.g., nutrient depletion, may be responsible for the shift in the proportions of each IFN-γ form observed in batch cultures. It was also found that the glycosylation pattern of IFN-γ was affected by some of the alterations of the cell culture environment, reinforcing the importance of the cell culture environment on the glycosylation process. Table 6.1 shows that in the present study although distinct glycosylation responses were obtained under some of the culture conditions, the effect was dependent on the type of modification. Indeed, in Chapter 3, similar glycosylation patterns

of IFN-γ were obtained under different culture environments [(1), (2), (6), (7)]. It is worth pointing out that all these cultures had the same concentration of BSA, 5 mg/ml. However, in Chapter 5, changes occurred when modifying the concentration [(10), (11), (12)] and type of BSA [(13), (14), (15), (16)] used in the culture and when supplementing the cultures with serum [17]. With serum, it was the micro-heterogeneity of IFN-γ which was affected. Also, in Chapter 4, a change in the initial glycosylation pattern of the produced IFN-γ was seen when the initial glutamine concentration in the culture was reduced by 75% [8], although the patterns were similar to the control [1] after 100 h. This behaviour will be discussed in the following sections, in light of the existing knowledge of glycosylation and cell functionality.

6.2.3.1- Glycosylation of IFN-γ is not dependent on the rate of IFN-γ production

Under the conditions of this study, there was evidence that the efficiency of IFN-γ glycosylation was not directly related to its production rate or cell growth rate. For example, the lower production of IFN-γ obtained with lipid supplementation over 5 mg/ml BSA cultures [6] did not have any significant effect on the extent of glycosylation. Furthermore, higher proportions of the 2N glycoform were achieved in some of the low BSA cultures [(13), (14), (15) and (16)], where a reduced IFN-γ production was seen. This is consistent with the findings for glucose-limited chemostat cultures of the same CHO cells (Hayter *et al.*, 1993). In the latter case, similar IFN-γ glycosylation patterns were obtained at growth rates varying from 0.014 and 0.021 h^{-1}, for which increases in IFN-γ production rate were also noticed. In the case of t-PA there is also evidence that the extent of N-linked glycosylation is not dependent on the rate of protein synthesis (Bulleid *et al.*, 1992; Lin *et al.*, 1993), while for protein C it has been suggested that glycosylation may be partially dependent on this rate (Miletich and Broze, 1990). In the latter case the glycosylation site which presented variable glycosylation has a Asn-X-Cys sequence, unlike the more common Asn-X-Ser/Thr sequence, and the postulated relation may well be dependent on each specific type of protein.

6.2.3.2- The effect of glucose and glutamine on the glycosylation of IFN-γ

Glutamine was one of the components that seemed to influence the efficiency of IFN-γ glycosylation. In fermenter fed-batch cultures containing lower initial concentrations of glutamine, 0.5 mM, a lower initial proportion of the 2N glycoform (45-50% as compared to a typical 70%) and higher initial proportion of the 0N glycoform (ca. 15% as compared to a typical 5%) was obtained. Thus, the availability of glutamine may be an important limiting factor in the glycosylation pathway. A certain driving force may be required for the glycosylation process, and this is probably derived from glutamine. Glutamine is a nitrogen donor for the synthesis of purines and pyrimidines and is also involved in the biosynthesis of amino sugars. In its initial steps, the glycosylation process requires the nucleotide sugars, UDP-glucose, GDP-mannose and UDP-N-acetylglucosamine. A limited supply of any of these precursors may impair the glycosylation process, which may be the reason behind the lower glycosylation of IFN-γ at lower availability of glutamine in the beginning of the culture. A similar comparison with the reported findings for the effect of the glucose supply on the glycosylation process may also be established. Glucose is required for the assembly of nucleotide sugars and there is some evidence that a limited supply of glycosylation precursors derived from glycolytic intermediates may be in the origin of a decrease in the glycosylation of IFN-γ obtained from glucose-limited chemostat cultures showing low glucose uptake rates (Hayter *et al.*, 1993). Furthermore, it has also been shown that the glycosylation pathway is impaired by glucose starvation (Rearick *et al.*, 1981).

In this study the presence of excess glucose throughout fed-batch cultures containing high initial glutamine concentrations (2 mM) did not prevent the typical shift in the glycosylation pattern of IFN-γ, suggesting that it is not only the availability of glucose which affects the glycosylation process. In fact, increasing the initial glucose concentration in batch cultures has proven to be ineffective in maintaining the initial IFN-γ glycosylation pattern (Hayter *et al.*,

1991b). However, glucose pulses in glucose-limited chemostat cultures were able to temporarily increase the proportion of the 2N glycoform (Hayter *et al.*, 1992), which was simultaneous with a marked increase in the uptake rate of glucose. It was postulated then that the transient increase of the 2N glycoform could be due to changes in cell physiology following the addition of the growth-limiting nutrient and not to an increase in glucose concentration.

Goochee *et al.* (1992) proposed that an alteration in the cellular energy state, which is dependent on glucose and glutamine metabolism, may be one of the factors limiting the extent of the glycosylation process. In this study, the lower extent of IFN-γ glycosylation obtained in cultures with low initial glutamine concentration is consistent with such mechanism. A change in the metabolism of certain amino acids was in fact detected in those fed-batch cultures. In particular, a lower initial uptake of glutamine and an alteration in the metabolism of glycine and serine metabolism, components closely associated with glucose and glutamine, was observed. It is feasible that changes in glycosylation are associated with changes in the activity of key metabolic pathways.

6.2.3.3- The extent of IFN-γ glycosylation varies with the concentration and type of BSA in culture

Different patterns of N-glycosylation of IFN-γ were observed in cultures containing high BSA or low BSA concentrations. Not only the concentration but also the type of BSA affected the glycosylation process. In order to obtain the maximum possible information from the different data sets presented in Chapter 5, the results for the various cultures (sections 5.3 and 5.4) were amalgamated for comparison in Table 6.2, which will be used as a guide in this section. The values for IFN-γ were calculated from the maximum yields achieved in the various cultures. The same Table provides data for cell productivity (IU/10^6cells) and volumetric productivity

(IU/ml), strenghtening the argument for uncoupled growth and production achieved when lipids and/or Pluronic F68 were added to the cultures, and the apparently independent effect on IFN-γ glycosylation.

Table 6.2- Effect of BSA and medium supplements on interferon-γ production.

	Interferon-γ production				
	Relative proportion		Percentage of glycoform		
	IU/10^6 cells	IU/ml	0N	1N	2N
Control- BSA 5 mg/ml (A) [a]	1	1	6 ± 1	46 ± 0.5	48 ± 1.5
+ P F68 (C) [c]	0.99	1	11 ± 1.5	43 ± 2	45 ± 2
+ Lipid mixture (B)	0.53	0.70	4	47	49
P F68					
+ BSA 1 (K) [b]	0.78	0.63	17.5 ± 3	40 ± 0.5	42.5 ± 2.5
+ BSA 1 + F-BSA 1 (O)[b]	0.76	0.61	9 ± 1	37 ± 0.5	54 ± 1.5
Lipid mixture					
+ BSA 1 + P F68 (E)[c]	0.62	0.47	19 ± 2.5	35 ± 0.5	46 ± 2.5
+ BSA 1 + P F68 + F-BSA 1 (Q)[c]	0.62	0.52	7 ± 2	31.5 ± 1.5	61.5 ± 1
Linoleic acid*					
+ BSA 1+ P F68 (L)[c]	0.58	0.58	24 ± 1.5	38 ± 4	38 ± 3
+ BSA 1 + P F68 + F-BSA 1 (P)[c]	0.60	0.60	8.5 ± 1	33 ± 1.5	57.5 ± 2.5
F-BSA 1					
+ BSA 1 (N)[b]	1.04	0.59	3 ± 0.5	36 ± 3	61 ± 2.5

* added as a FAF-BSA complex. P F68= Pluronic F68; F-BSA= fatty-acid free BSA. 1= 1 mg/ml. The letters in brackets correspond to the medium code. Glycosylation values taken at ca. 95-110 h in culture; [a] - 6 observations, [b] - 4 observations, [c] - 2 observations.

The various media supplementations utilised in Chapter 5 reflect different lipid compositions of the medium, due to different type/concentration of BSA and/or addition of lipids/linoleic acid. As discussed before, the lipid composition of the cell membrane reflects that of the growth environment and exposing the cells to different medium constituents can alter appreciably the lipid composition of both surface and intracellular membranes, due to a rapid phospholipid turnover (King and Spector, 1981). Membrane lipid composition, namely the equilibrium between saturated fatty acids and unsaturated fatty acids, is known to affect cell growth (Doy *et al.*, 1978); cell membrane fluidity is affected by cholesterol and Pluronic F68 (Ramirez and Mutharasan, 1990) affecting cell sensitivity to the culture conditions. It is feasible that the alterations made in the present study (Chapter 5) may be translated into an altered cell function, e.g., post-translational processing.

The suggestion that the types of lipid, e.g. extent of unsaturation, incorporated by the membrane may affect the glycosylation process can be partially supported in this study. The efficiency of N-glycosylation decreased in 1 mg/ml BSA cultures [media K, E and L]. Among those, cultures supplemented with linoleic acid [medium L] showed the highest proportion of the 0N form and lowest proportion of the 2N glycoform, suggesting a contribution by linoleic acid to the change in the glycosylation patterns. Also, the ratio of the 2N to the 1N IFN-γ glycoform was higher when cultures were supplemented with the lipid mixture. Common features of such cultures, in comparison to the control 5 mg/ml BSA cultures, were the presence of Pluronic and the lower BSA concentration. Pluronic itself was also a likely candidate for the cause of the change in the glycosylation pattern of IFN-γ observed in the low-BSA cultures. Addition of Pluronic to 5 mg/ml BSA cultures [medium C] did not give very strong support to this view, but whether Pluronic would have the same global effect in 1 mg/ml BSA cultures and 5 mg/ml BSA cultures cannot be assured. Also, in the set of 1 mg/ml BSA cultures supplemented further with 1 mg/ml FAF-BSA [media O, P Q and M], those containing Pluronic gave slightly higher levels of 0N IFN-γ, again suggesting its involvement

in the glycosylation process. This can be a reflection of its postulated interaction with the plasma membrane (Murhammer and Goochee, 1988; Ramirez and Mutharasan, 1990; Zhang *et al.*, 1992a; Lakhotia *et al.*,1993). The fact that cell growth was not successful in cultures containing 1 mg/ml BSA, with no other supplementation, prevented the determination of whether the changes in glycosylation profile were mainly due to the reduction in BSA concentration.

The possibility that the lower extent of N-glycosylation was due to the deficiency of some important component carried by BSA lost its relevance when glycosylation efficiency increased in 1 mg/ml BSA cultures supplemented with a further 1 mg/ml FAF-BSA [media O, Q, P and N], and higher proportions of the 2N glycoform were seen throughout the cultures as compared to the control 5 mg/ml BSA. Low-BSA cultures supplemented solely with FAF-BSA (total 2 mg/ml BSA) showed very low proportions of the 0N form and higher proportions of the 2N glycoform [medium N]. The reduction in BSA concentration did not seem to be the only cause for the previous shift observed in the patterns of IFN-γ. There was, however, possible evidence that the fatty acid-free BSA plays a major role in the glycosylation process. As the FAF-BSA itself is obtained from the original BSA by subjecting it to further treatment to remove lipid components (see Chapter 3), it is unlikely that the positive effect was due to any component carried by the FAF-BSA. The role of FAF-BSA may derive from the fact that it can complex certain components present in the medium, which might have been carried by the lipid containing BSA. The fact that the latter is obtained by extensive heat treatment may result in oxidation of some of its components, e.g., fatty acids, which may be toxic to cultured cells. This last observation can lead to the hypothesis that lipid components, probably linoleic acid, carried by the more crude BSA impairs the glycosylation process. The fatty acid may become complexed by the FAF-BSA and above a certain ratio of fatty-acid to BSA their potential inhibitory effects may not be manifested. A ratio of 1:100 has been reported as required to eliminate the toxic effects on hybridoma growth (Kovar and Franek, 1986), but the

situation may be different for production purposes. At the same time, the FAF-BSA may have modified the lipid composition of the culture to something which is more favourable to cell function, and this may reflect changes in the state of the membrane, particularly membrane fluidity. Post-translational processing may be affected by these alterations, as was previously discussed for IFN-γ production.

IFN-γ obtained from the 5 mg/ml BSA cultures presented similar extent of N-glycosylation independently of the additions made to those cultures, such as lipids or Pluronic F68 [media B and C], or even BSA Sigma [Chapter 3]. This seems to reflect the protective effect that BSA exerts in the culture medium, which may derive from its binding and antioxidant capacities (see properties described in section 6.2.2). Components which affect the glycosylation process in low-BSA cultures, e.g., lipids and Pluronic, may not have the same effect in cultures containing higher concentrations of BSA, where the effect of BSA probably predominates.

There is also the possibility that the supply of components taking part in the glycosylation pathway are affected by alterations in the culture environment. The glycosylation process is initiated by the synthesis of a lipid-linked oligosaccharide moiety, the $Glc_3Man_9GlcNAc_2$-P-P-Dolichol. When lipids are available in the culture *de novo* synthesis of fatty acids and cholesterol is suppressed and the regulation of their biosynthesis occurs through inhibition of rate-limiting enzymes. The synthesis of dolichol-P-P happens along the same pathway of cholesterol biosynthesis, and its formation occurs after the rate-limiting enzyme step (Lehninger, 1975). It is thus possible that inhibition of cholesterol synthesis, which will be mainly dictated by the lipid content of the culture, may also limit the supply of dolichol-P-P, impairing the glycosylation process.

The state of the cell membrane (lipid composition, fluidity), which is largely affected by the medium composition, does seem to be a critical parameter for cell function and may well be

one of the mechanisms controlling the glycosylation process. This kind of rationale seems more reasonable than attributing the cause of the reduction in IFN-γ glycosylation seen early in the low BSA cultures to the lack of any component carried by BSA. Furthermore, it could also explain the higher proportion of the 2N glycoform in 1 mg/ml BSA cultures further supplemented with FAF-BSA, even if the total concentration of BSA is lower.

6.2.3.4- Serum affects the pattern of IFN-γ

Glycosylation analysis showed that the heterogeneity of IFN-γ was different in serum-free and serum-containing cultures. In this case, it was the distribution of the molecular weight species within each major variant (0N, 1N and 2N) which was affected. Serum has been reported by others to influence the glycosylation pattern of glycoproteins. Megaw and Johnson (1979) have reported major differences in the glycosylation of a glycoprotein, epithelial basement membranes, in media containing different levels of foetal calf serum; a decrease in sugar content with a decrease in serum concentration was noticed. Patel *et al.* (1992) have shown that differences in the glycosylation profile of a murine IgG monoclonal antibody ocurred depending on whether it was produced in ascites, serum-free or serum-supplemented cultures. These differences were found to be related to the sialic acid content and incidence of outer-arm galactosylated oligosaccharides, and not to the number of attached N-glycan chains. Also, studies conducted on the production of IgM have shown that the monoclonal produced in ascites had a lower average degree of sialylation than what was obtained *in vitro* (Maiorella *et al.*, 1993). In the latter study, the pharmokinetics of the product, namely plasma clearance rate and binding activity, were clearly affected by the glycosylation changes. Product derived from ascites had variable plasma clearance rate from batch to batch, as it was previously reported (Anderson *et al.*, 1985), while consistent results were obtained for the serum-free cultures. These data reinforce the importance of evaluating the culture method for product authenticity and pharmokinetics of the product. Furthermore, the advantage of using serum-free medium in

terms of consistency of the product from batch to batch is well illustrated.

In the present study, sialic acid or sugar content was not analysed. Stronger bands were found for the lower molecular weight variants for the three major IFN-γ forms. As such, the third variant of the non-glycosylated form, which is rarely detected on the gel, was the most abundant of the 3 non-glycosylated variants. Since this occurred with all the forms, it is possible that there was proteolytic cleavage of the molecule, which may be due to proteases present in the serum. Lin *et al.* (1993) reported differences between t-PA produced by CHO-K1 in serum-free and serum-containing cultures. In the latter conditions, the authors found degradation of the product, which was attributed to proteases present in serum. However, in the present investigation, no studies were conducted to elucidate this hypothesis, so it was not determined whether the observed effect is a consequence of protein degradation in the medium or due to intracellular differences in the post-translational process, as was observed in studies referred above.

6.2.3.5- The impact of the media composition on the production of glycoproteins

The mechanisms underlying the changes in protein glycosylation patterns under different culture conditions are not yet clarified and are a subject of major interest because the implications for the production of recombinant proteins that have therapeutic applications are great. The pharmacokinetic behaviour of the product *in vivo* can be affected by the glycosylation profile of the product (Grinnel *et al.*, 1991; Delorme *et al.*, 1992; Maiorella *et al.*, 1993) and if such structural changes occur when modifying a production method then any proposed change must be evaluated before the product goes into clinical trials. The studies of Grinnel *et al.* (1991) and Delorme *et al.* (1992) focused on the removal of any of the potential glycosylation sites of Protein C and EPO, respectively. Glycosylation of Protein C at different sites had distinct effects on its functional anticoagulant activity; also, one of its carbohydrate

chains was found to be essential for efficient protein secretion. Glycosylation of EPO was found to be essential for its *in vivo* activity, with each of the carbohydrate chains providing a distinct contribution. The work of Maiorella *et al.* (1993) has shown that variations in the sugar content of the carbohydrate chains affected biological properties of monoclonal antibodies, such as plasma clearance rate and binding activity. In the present study, large variations in IFN-γ glycosylation profile were observed when the medium composition was modified (Table 6.2). In light of the studies reported so far on the effect of glycosylation on the pharmacokinetic properties of certain recombinant proteins, it is feasible that changes in glycosylation profile as those reported here for IFN-γ also affect the therapeutic *value* of such proteins. Product consistency is another factor to reinforce the importance of characterising the factors and mechanisms affecting the glycosylation process.

The work described in this study reinforced the importance of the medium composition on the post-translational processing of glycoproteins. The influence of the extracellular environment on the glycosylation of other proteins now has been recognised by other researchers (see Chapter 1, Table 1.5), although such studies are still scarce and the glycosylation pattern of the product throughout the production process is rarely reported (Curling *et al.*, 1990; Robinson *et al.*, 1993). Most of the studies reported allude to the effect of serum on the glycosylation patterns, mainly sugar composition (Megaw and Johnson, 1979; Patel *et al.*, 1992; Maiorella *et al.*, 1993; Lin *et al.*, 1993). The influence of serum on the pattern of IFN-γ also was manifest in this study, although it was not determined if that was due to changes in sugar content and/or to proteolytic degradation. Glucose supply does affect the glycosylation process (Rearick *et al.*, 1981; Hayter *et al.*, 1992); a similar result was found in this study for glutamine. In both glycosylation may be related to the supply of important precursors for the glycosylation pathway.

In the present study, the protein (BSA) content of the medium was also found to be of great

significance for the glycosylation profile of IFN-γ. The effect was related to both its concentration and degree of purification. Other components [Pluronic, linoleic acid, lipids] also affected the efficiency of glycosylation, but the magnitude of their effect was dependent on the BSA content of the medium. The present trend in culture media design is the removal of proteins, mainly due to the risk of contaminants, however findings of the type reported here may have a strong impact on such developments.

No direct relationship between IFN-γ production and IFN-γ glycosylation was found in this study, which emphasizes the necessity of considering the effects of culture media on product quality as well as on product quantity during process optimisation.

6.3- Nature of CHO cell growth limitation

Providing the cells with glucose and glutamine, the two nutrients most likely to become limiting, failed to prevent CHO cell growth and IFN-γ production from ceasing [9]. Ammonia accumulation, which could become a limiting factor, was controlled by adding those nutrients in a fed-batch mode, using low initial concentrations of glutamine and low feeding rates, as it has been previously reported (e.g., Glacken *et al.*, 1986; Ljunggren and Häggström, 1990). Net improvements in CHO cell growth and IFN-γ production were not achieved, and other factors may be involved in the limitation process.

Another possibility that may also be considered for growth inhibition of CHO cells is self inhibition by IFN-γ. There is some evidence of specific binding of IFN-γ (provided by Wellcome Biotech) to CHO 320 cells (N.J. Tomlinson and N. Jenkins, unpublished results), by a mechanism still unknown. Evidence that intracellular IFN-γ may affect cell growth in the absence of specific receptors was also found (Sanceau *et al.*, 1987). However, it was postulated in the former study that IFN-γ obtained from Wellcome might have been contaminated with

some toxic agent that also showed the protein inhibitory effect. More recent studies with the same cell line have shown that the levels of IFN-γ usually obtained in batch culture do not cause any growth inhibition, when added in the beginning of batch cultures (J.M. Tong and A.T. Bull, unpublished results) but a different source of purified IFN-γ was used (purification carried out at the University of Kent). The specific activity of IFN-γ used in the two studies may have not been the same, and further work would be necessary to elucidate this possibility.

The hypothesis of an optimal range of concentrations necessary to sustain cell growth may be a reasonable one. Factors such as competition between amino acids for transport, feed-back effects on metabolic pathways by higher concentrations of particular metabolites, may reflect the postulated range.

7- CONCLUSIONS

The influence of the culture environment on CHO cell growth, IFN-γ production and IFN-γ authenticity was well illustrated in this study. These parameters were not always enhanced by the same stimuli, a conclusion which strongly supports the view of optimising the cell culture system for the desired biological response.

A Plackett-Burman statistical design proved to be a very useful tool for identifying key factors in the optimisation procedure. Increasing the initial concentration of a group of growth medium components, identified as positive by the latter technique, in batch cultures led to improvements of approximately 40% in CHO cell growth and IFN-γ production. These effects were not due to nutrient depletion, showing that the initial composition of the culture medium is as important as the feeding of depleted nutrients.

Bovine serum albumin was found to be a very important component in culture and its multiple roles were manifested in this study. Nutritional, protective and possibly antioxidant functions were apparent. Different types of BSA produced different responses on CHO cell growth and IFN-γ production and this seems to reflect the purification procedures to which BSA is subjected. BSA introduces much variability to the culture, but it is essential for cell growth to occur. Pluronic F68, alone or in combination with a lipid mixture or linoleic acid, was able to restore cell growth in low-BSA cultures (1 mg/ml), to different extents. However, the levels of IFN-γ production were always reduced in these cultures, especially in the presence of lipids. In fact, the lipid composition of the culture was important for cell function, and CHO cell growth and IFN-γ production were differently affected by both type and concentration of these components. Furthermore, the extent of IFN-γ glycosylation was affected by (1) the concentration of BSA, (2) the type of BSA, e.g., fatty acid-free BSA, and (3) the lipid composition of the culture. The latter is affected by both (1) and (2) and all of them, together with Pluronic F68, contribute to the structural integrity of the cell membrane, which is

reflected in cell function. It was also found that the glycosylation of IFN-γ is not dependent on its production rate.

The inclusion of serum in the culture medium stimulated cell growth, but had adverse effects on IFN-γ titres, stressing that an optimum environment for CHO cell growth may not be the same for IFN-γ production. The predominant molecular weight species within each IFN-γ major variant was altered in the presence of serum, indicating cleavage of the core polypeptide.

Glucose and glutamine were depleted early in batch cultures and were *prima facie* candidates for growth and/or production limitation. However, the results of fed-batch fermenter studies with supplementation of glucose and glutamine did not support this hypothesis, as no net improvements in growth or productivity were obtained when both nutrients were available throughout the cultures. The glycosylation pattern of IFN-γ was, however, affected by the initial glutamine level of the culture.

The caution that should be taken when dealing with the optimisation of recombinant glycoprotein production by animal cells was highlighted in this study. The rather undefined nature of BSA is a major concern when considering the batch consistency of the product and the characterisation and choice of the starting material is an element that must be addressed when appraising the design of a reproducible culture system. The development of protein-free media is an area which deserves more attention. Successful cultures under such conditions have already been achieved, but there are no reports on the influence of those changes on the quality of recombinant proteins. Moving towards a more defined culture is most desirable, however the repercussions for the authenticity of the product need to be assessed. This study has shown that product consistency would benefit enormously from a clearly defined medium. The development of this medium would elucidate many of the complex steps involved in the growth of mammalian cells and their ability to produce authentic recombinant proteins.

8- SUGGESTIONS FOR FUTURE WORK

The work described here has stressed the importance of the cell culture environment for the production of recombinant IFN-γ by CHO cells. The study focused on CHO cell growth, IFN-γ production and also IFN-γ glycosylation. Some of the points raised by this study deserve further investigations.

The influence of the type and concentration of BSA seen on cell growth and IFN-γ production (titre and glycosylation) and the undefined nature of that component calls for more detailed investigations on the protein content of the culture medium. Moving towards a more defined culture medium is of primary importance in terms of reproducibility of culture performance. There is a need to better define the multiple roles of BSA in culture, such as serving as a carrier of vitamins, hormones and growth factors (Barnes, 1987). Based on the information already obtained in this study with Pluronic F68 and some lipids, these components could be analysed with a further statistical analysis, attempting to eliminate the BSA from the culture. Considering the antioxidant role of BSA, components with such function, e.g., catalase, glutathione or vitamin E (Darfler and Insel, 1983; Darfler, 1990), should be included in that study. The original control cultures without BSA and supplemented with Pluronic F68 could become the basal culture composition. The analysis of second-order interactions between components should be also included, if the statistical design selected allows such evaluations (Gaertner and Dhurjati, 1993).

Studies on specific supplementation strategies of amino acids which have high utilisation rates, while increasing the initial concentration of the components already identified as important by the Plackett-Burman design (excluding BSA), could follow.

Glycosylation of IFN-γ seemed to be, in some way, a function of glutamine concentration at the beginning of the culture. It is suggested that the main function of glutamine could be the provision of nucleotide precursors, which are required for the glycosylation process. The effect of supplementing the cell culture with specific nucleotides on the glycosylation patterns of the molecule could give some more insight to the understanding of this process.

The microheterogeneity of IFN-γ, as a result of differences in carbohydrates, was not analysed in this study. Analysis of IFN-γ glycoforms could be accomplished by a variety of techniques (reviewed by Dwek *et al.*, 1993), which can provide more detailed information about the molecule. Techniques being developed in this laboratory are Capillary Zone Electrophoresis, which can be used to rapidly profile IFN-γ glycoforms, and Laser Desorption-Mass Spectrometry, which can provide detailed site-specific mass structural information.

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