AMMONIA EFFECTS ON CHO CELL GROWTH, METABOLISM, ERYTHROPOIETIN PRODUCTION AND GLYCOSYLATION

Ming Yang

A THESIS

Submitted to the Faculty of Graduate Studies
In Partial in Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology
University of Manitoba

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FACULTY OF GRADUATE STUDIES

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ABSTRACT

The effects of ammonium on cell growth, metabolism, protein production and glycosylation were investigated for Chinese hamster ovary (CHO) cells transfected with the human erythropoietin (EPO) gene and grown in serum-free medium during batch culture. Cell growth was inhibited above a culture concentration of 5 mM NH_4Cl. The amount of EPO produced decreased in the presence of ammonium. The specific production of EPO increased with the addition of NH_4Cl above 10 mM. The metabolic effects of added NH_4Cl included higher specific consumption rates of glucose and glutamine and an increased rate of production of alanine and glutamate.

The EPO analyzed from control cultures had a molecular weight range of 33-39 kDa and an isoelectric point range of 4.06-4.67. Seven distinct isoforms of the molecule were identified by two dimensional electrophoresis. Complete enzymatic de-glycosylation resulted in a single molecular form with a molecular weight of 18 kDa. Addition of NH_4Cl to the cultures caused a significant increase in the heterogeneity of the glycoforms as shown by an increased range of molecular weight and pI. The increased pI indicated that the effect of ammonia was in the reduction of terminal sialylation of the glycan structures. The addition of a sialidase inhibitor to the cultures had no effect on the ammonia-induced increase in EPO heterogeneity. Also, the effect of ammonia on glycosylation could not be mimicked using the weak base chloroquine in our system.

The FACE N-linked oligosaccharide profile showed that the density of the major band was greatly diminished and the width was significantly increased in cultures containing added ammonia. The proportion of tetra-antennary structures was reduced by 60%, while the tri- and bi-antennary structures were increased proportionally in the presence of ammonia. Glycan analysis by HPLC using a weak anion exchange column showed that the most significant characteristic effect of ammonia was a reduction of the proportion of glycans with four sialic acids from 46% in control cultures to 29% in ammonia-treated cultures. Analysis by normal phase chromatography indicated a distribution of tetra-, tri-

and bi-antennary structures similar to that shown by FACE. The N-linked glycan sequence determination by FACE indicated that EPO contained a typical N-linked complex oligosaccharide structure. Glycans from ammonia-containing cultures showed the same sequence pattern.

The molecular heterogeneity of EPO increased during the course of a batch culture. Analysis of the secreted EPO indicated a time-dependent increase in the molecular weight band width of the peptide consistent with degradation rather than changes in the glycan structure. A high glutamine concentration (16-20 mM) in the culture decreased the apparent degradation of the EPO.

Glucosamine induced EPO heterogeneity which was significantly different from ammonia. The pI range was increased and extended from 3.5 to 7.5 in 10 mM glucosamine supplemented culture . The number of protein spots from seven in a control culture increased to more than twenty by 2-D electrophoresis. Carbohydrate analysis indicated that both ammonia and glucosamine inhibited the oligosaccharide sialylation and complexity but glucosamine had less effects. The addition of ammonia and glucosamine resulted in a significant increase of the intracellular nucleotide sugar pool. The UDP-GNAc pool of ammonium and glucosamine treated cultures accounted for 60% of total nucleotides compared to 9.2% for the control culture.

In conclusion, ammonia in the culture medium affected EPO glycosylation which was observed as a reduction of the tetra-antennary and tetra-sialylated oligosaccharide structures. The added ammonia and glucosamine to CHO cell culture had different effects on EPO heterogeneity although both significantly increased intracellular UDP-sugar pool concentrations. There may not be a direct relationship between intracellular nucleotide sugar pool and glycan structures from this study.

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LIST OF ABBREVIATIONS

2-AB	2-aminobenzamide
4-AB	4-aminobenzonitrile
ANTS	8-aminonaphthalene-1,3,6-trisulfonic acid
AP	alkaline phosphatase
BHK	baby hamster kidney
BSA	bovine serum albumin
CHO	Chinese hamster ovary
CV	coefficient of variation
DHFR	dihydrofolate reductase
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
rDNA	recombinant DNA
EDTA	ethylene dinitrilo-tetraacetic acid
EPO	erythropoietin
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FACE	fluorophore-assisted carbohydrate electrophoresis
GDH	glutamate dehydrogenase
G-CSF	granulocyte colony-stimulating factor
GnT	N-acetylglucosamine transferase
GPI	glycophosphatidylinositol
GU	glucose unit
GuHCl	guanidine hydrochloride
HPLC	high performance liquid chromatography
IC-50	50% inhibitory concentration
IEF	isoelectric focusing
IFN	interferon
Ig	immunoglobulin
IL	interleukin

kDa	kilodalton
KSCN	potassium thiocyanate
LDH	lactate dehydrogenase
Mab	monoclonal antibody
2-ME	2-mercapto-ethanol
mPL-I	mouse placental lactogen-I
mRNA	messenger ribonucleic acid
MS	Mass spectrometry
MW	molecular weight
$NaBH_3CN$	sodium cyanoborohydride
NC	nitrocellulose
NCAM	neural cell adhesion molecule
NP-40	NONIDET P-40
OD	optical density
PAS	periodic acid-Schiff
PBS	phosphate-buffered saline
pI	isoelectric point
pKa	the negative logarithm of a dissociation constant
PNGase F	peptide-N-glycosidase F
pNPP	p-nitrophenyl phosphate
q	specific consumption or production
RIA	radioimmunoassay
rpm	revolutions per minute
SAR	scaffold attachment region
SCLC	small cell lung cancer
SEM	standard error of the mean
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TNFR	tumor necrosis factor
tPA	tissue plasminogen activator
U	unit
UV	ultraviolet

WHO	World Health Organization

Amino acids

ala	alanine
asn	asparagine
gln	glutamine
glu	glutamate
gly	glycine
ser	serine
thr	threonine

Carbohydrates

Frc	fructose
Fuc	fucose
Gal	galactose
Glc	glucose
Man	mannose
GlcN	glucosamine
GalNAc	N-acetylgalactosamine
GlcNAc	N-acetylglucosamine
NeuNAc	N-acetylneuraminic acid (sialic acid)

Nucleotides and nucleotide sugars

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
CTP	cytidine diphosphate
GDP	guanosine diphosphate
GTP	guanosine triphosphate
NAD	nicotinamide adenine dinucleotide
UTP	uridine triphosphate
UDP-Glc	uridine diphosphate-glucose
UDP-GalNAc	uridine diphosphate-N-acetylgalactosamine
UDP-GlcNAc	uridine diphosphate-N-acetylglucosamine
UDP-GNAc	UDP-N-acetylhexosamines (sum of UDP-GalNAc and UDP-GlcNAc)

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CHAPTER 1

Introduction

1.1 Genetic engineering in mammalian cells

Synthesis of significant quantities of human therapeutic proteins intended for clinical use to correct deficiencies of endogenous biomolecules or to augment their existing level is a great challenge. Since there is no good natural source for these proteins, the only possible way to obtain the necessary quantities of human bioactive proteins is recombinant DNA technology.

Simple polypeptides can be produced with pharmacological activity when produced in *E coli* as a non-glycosylated forms e.g. insulin, human growth hormone. However, the majority of extracellular proteins require sugars for biological activity. Sugars are usually efficiently transferred to proteins in heterologous eukaryotic cells. However, the resulting sugar chain structure may be different from the native structure. Such a difference in structure might result in inactivity or antigenicity. To avoid these problems, some therapeutic glycoproteins were originally produced in their native host cells without gene manipulation, e.g. interferon-β (IFN) produced in fibroblasts (Utsumi et al., 1984) and tissue-type plasminogen activator (tPA) produced in Bowes human melanoma cells (Collen et al., 1982). However, in the case of HuEPO, no native host was available. The use of recombinant mammalian cells remained the only option for the mass production of HuEPO.

The production of monoclonal antibodies and recombinant proteins are the two major developments in new biotechnology. The major events in the commercialization of genetic engineering are shown in Table 1.1. Genetic engineering can be defined as the ability to transfer genes from one organism to another, to cross barriers which would not be possible by conventional genetics. The aim of genetic engineering in mammalian cells

is to achieve high-level production of a particular protein in a stable cell line that can be cultivated in bulk cell culture. When the goal is to reproduce the complex molecules found in animals, cultured animal cells offer unique qualities to produce new natural products. This approach requires high expression levels and stable production capacities. The products should have a precisely defined structure to maintain safety and efficacy.

Recombinant DNA techniques depended upon three independent discoveries. The first was the discovery of restriction enzymes, which are capable to cutting DNA at specific sites to give discrete fragments. The second discovery was the presence of independently replicating circular DNA in bacteria (plasmids), which could be cut by restriction enzymes and repaired to include other DNA fragments. The third discovery was the method of treating bacteria so that they were capable of taking up plasmid DNA, and hence contain new genes (Scragg, 1988). These three discoveries have allowed the development of genetic engineering which has formed the basis for many new products. The general approach taken by genetic engineering is outlined in (Fig. 1.1):

Table 1.1 Major events in commercialization of new biotechnology (adopted from Scragg, 1988)

1973	First gene cloned
1974	First expression of a gene cloned in a different species of bacteria
1975	Monoclonal antibodies
1976	First firm (Genentech) to exploit recombinant DNA technology
1981	First monoclonal antibody diagnostic kit approved in US
1982	First rDNA animal vaccine (colibacillosis) approved in Europe
	First rDNA pharmaceutical product, human insulin

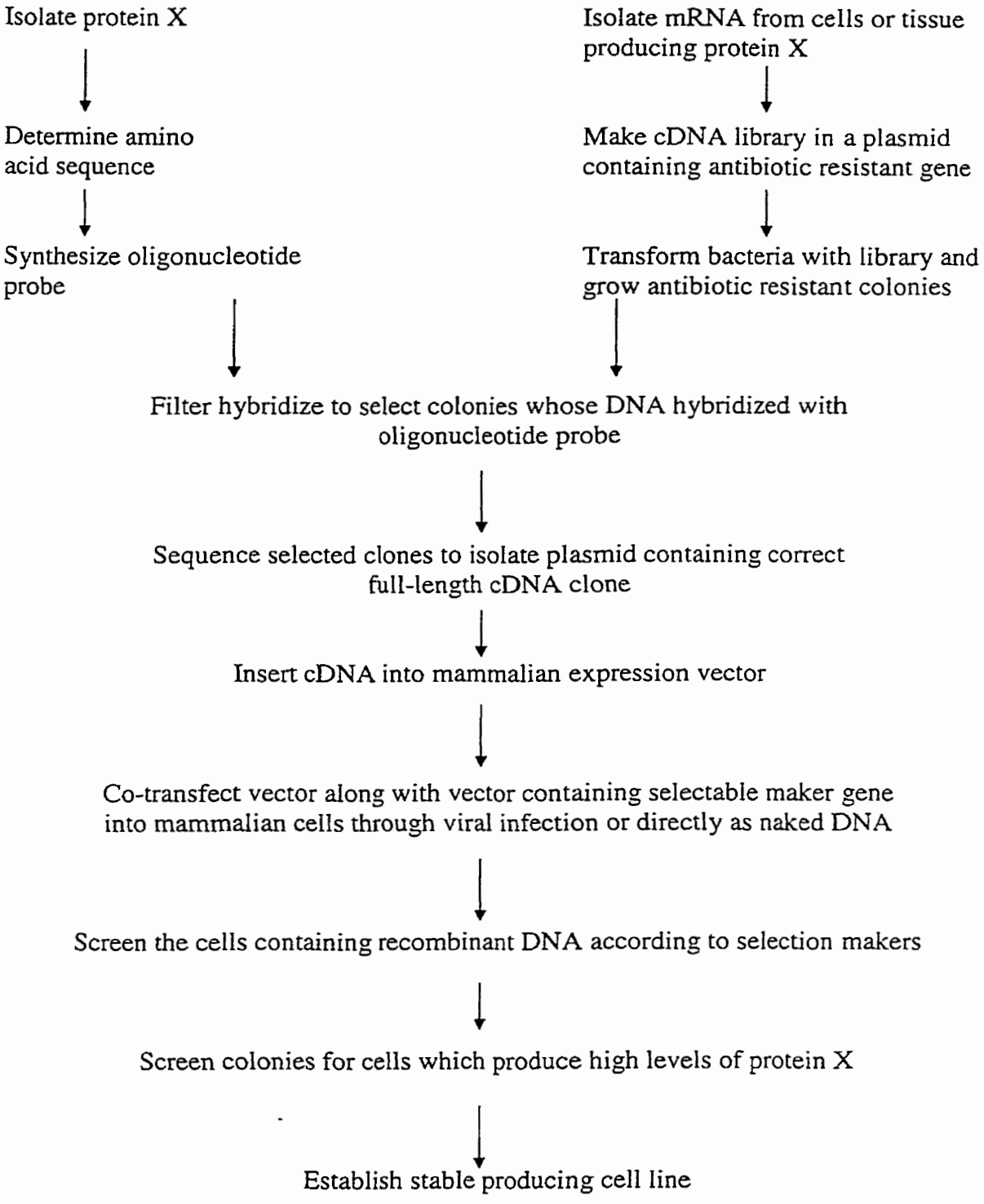


Fig. 1.1 Generation a genetically engineered mammalian cell line (Adapted from Weymouth and Barsoum, 1986).

1.2 Expression system

A basic understanding of the composition of the wild-type protein conformation and the functional role of each component in the protein's structure is necessary before recombinant protein production. The initial choice of expression system is crucial important (Jenkins et al., 1996). To choose either an eukaryotic or a prokaryotic host, we should have a complete understanding of the differences in protein expression in these two systems.

1.2.1 Prokaryotic

Bacteria. The expression of proteins in bacteria is the widely used approach for the production of cloned gene products. Common bacterial expression systems such as *E. coli*, with its rapid growth rate and high-density fermentation, can result in high product expression levels. However, significant disadvantages exist in using *E. coli*. These include the high level of endotoxin present, the insoluble and misfolded nature of the recombinant protein (Williams et al., 1982). The effort required to refold proteins obtained from *E. coli* may result in difficult and labor intensive recovery steps. Most important is that common bacterial expression system has no capacity to glycosylate proteins in either N- or O-linked positions. Therefore, for many molecules, the high-level *E. coli* expression system may not be appropriate.

1.2.2 Eukaryotic

For eukaryotic hosts, protein expression involves a series of complex processing steps. Eukaryotic hosts have cellular machinery that is dramatically different from prokaryotic.

(a) Yeast. Heterologous protein production in yeast has many commercial, medical, and basic research applications. As an organism easily manipulatable by genetics, plasmid introduction, and growth conditions, yeast are also ideal eukaryotic hosts for performing many of the post-translational modifications required for the large scale production of bioactive proteins. Yeast expression systems have been used to produce correctly

assembled proteins such as the hepatitis B surface antigen (Valenzuela et al., 1982) and secreted glycosylated proteins such as insulin-like growth factor 1 (Elliott et al., 1990).

However, yeast glycosylation patterns do not resemble glycosylation forms found in higher organisms. Yeast cells do not add complex oligosaccharides and are limited to the high-mannose-type carbohydrates (Marino, 1989). These high-mannose, hyperglycosylated structures are potentially immunogenic, can be rapidly cleared from serum in therapeutic targets, and can interfere with the biological activity of the protein (Rademacher et al., 1988). With these difficulties, yeast expression systems are not generally suitable for production of glycosylated recombinant proteins.

(b) Plants. Transgenic plants are emerging as an important system for the expression of many recombinant proteins, Especially those intended for therapeutic purposes (Moffat, 1995). One of their major attractions is the potential for protein production on an agricultural scale at an extremely competitive cost. Most plant transformation techniques result in the stable integration of the foreign DNA into the plant genome, so genetic recombination by crossing of transgenic plants is a simple method for introducing new genes and accumulating multiple genes into plants. Furthermore, the transformed plant line can easily be stored as seeds almost indefinitely under ambient conditions (Ma and Hein, 1995). Several studies reporting the production of human proteins in plants have suggested that simple N-glycan structures that lack sialic acids are added. For example, EPO produced in tobacco cells has no biological activity *in vivo* (Matsumoto et al., 1995). The other obstacle is the presence of potentially allergenic residues such as xylose β1,2 mannose (Ma and Hein, 1995) or core α1,3 linked fucose (Tretter et al., 1993).

(c) Insects. Baculovirus-infected insect cells perform many of the post-translational modifications observed in other eukaryotic systems. These include glycosylation, phosphorylation as well as many others (Luckow, 1996). The baculovirus-infected insect cell expression system has become a popular route for recombinant protein synthesis because of its short process development time and potentially high yields. However, the N-glycosylation capabilities of this system are limited to producing only simple

mannose-type oligosaccharides (James et al., 1995; Jarvis and Finn, 1995). In contrast, a few studies have demonstrated the production of complex N-linked oligosaccharides (Davidson et al., 1990).

(d) Mammals. Mammalian cells have the exquisite ability to produce properly folded and biologically active molecules at reasonable yields. Mammals that are physio-genetically closer to human have more elements of the glycosylation machinery in common. For production of these complex molecules, mammalian cell cultures offer an advantage over other organisms such as *E. coli.* and yeast.

1.3 Advantages and disadvantages of mammalian cell for recombinant protein production

1.3.1 Advantages

Mammalian cell culture offers the advantage that complex post-translational events are an inherent property of the secretion and processing system. The main reason why prokaryotic and lower eukaryotic systems have not proved satisfactory for the production of all proteins is their incapacity to reproduce mammalian proteins with complete fidelity. This is because, even after accurate translation of the relevant mRNA, many proteins produced in animal cells undergo a number of post-translational chemical modifications or processing steps before the mature protein is secreted. These modifications can't all be accurately reproduced by microbial systems. A considerable range of these so-called post-translational modifications are encountered in different proteins. These are summarized in Table 1.2.

In addition, engineered proteins from mammalian expression systems are secreted into the cell culture medium. The recombinant expression unit includes an N-terminal signal peptide which initiates the insertion of the nascent peptide into endoplasmic reticulum (ER). This signal peptide is removed during the secretion process, generating a molecule with the correct N-terminal amino acid. The secretion events from the ER to Golgi to the

extracellular space result in glycosylation and protect the protein from intracellular proteases (Gething and Sambrook, 1992). The secretion process leads to the additional advantage that products accumulate in the culture medium. Because the product is not associated with the intracellular proteins, proteolysis is limited and the purification process is simplified. The use of serum-free media has simplified the recovery process by removing the potentially contaminating serum proteins.

Most cells that are available for recombinant gene expression are immobilized cell lines. Commonly used cell lines are listed in Table 1.3. These cell lines have gone through a transformation process and can be propagated indefinitely. With this property of immobilization, cells divide rapidly to high cell densities and have fewer nutritional requirements. Immobilization allows for easy handling and a consistency between experiments (Etcheverry, 1996).

Table 1.2 The post-translational modifications most frequently encountered in mammalian proteins (Cartwright, 1994a).

Post-Translational Modifications
Glycosylation
γ-carboxylation of glutamic acid
β-hydroxylation of aspartic acid
Phosphorylation and sulphatations
Proteolytic processing
Amidation

Table 1.3 Common cell lines for biotechnology usage (Primrose, 1994; Etcheverry, 1996; Butler, 1996)

Cell line	Specific origin	Cell type	Characteristic
BHK-21	Baby hamster kidney	Fibroblast	Cells are anchorage dependent but can be induced into suspension; used for vaccine production; high yield and stable expression
CHO	Chinese hamster ovary	Epithelial	Cells will attach to a surface if available but can be adapted in suspension; used for genetic engineering; high yield and stable expression
COS	African green monkey kidney	Fibroblast	Cells contain a mutant of SV40 virus. Used for transient expression of recombinant genes
293-HEK	Human embryonic kidney	Fibroblast	Anchorage-dependent; efficient transient expression
Hela	Human cervical carcinoma	Epithelial	Fast-growing human cancer cell isolated in the 1950s
Hep-2	Human carcinoma of larynx	Epithelial	
L	Mouse connective tissue	Fibroblast	Many culture techniques developed from the 1950s were based on this cell line
MDCK	Canine kidney (Madin Darby)	Epithelial	Anchorage-dependent cells with good growth characteristics; used used for veterinary vaccine production
MRC-5	Human embryonic lung	Fibroblast	Finate life-span, "normal" cells used for human vaccine production
MPC-11	Mouse myeloma	lymphoblast	Derived from a mouse tumour; secretes immunoglobulin
Namalwa	Human lymphoma	Lymphoblast	Derived from cells from a human suffering from Burkitt's lymphoma; used for α-interferon production
NB41A3	Mouse connective tissue	Neuronal	Tumor cells with good growth rate. Cells have nerve cell characteristics including a response to nerve growth factor
NIH-3T3	Mouse connective tissue	Fibroblast	Vigorous growth in suspension; cells used widely in the development of cell culture techniques and for viral replication
Wi-38	Human embryonic lung	Fibroblast	Finate life-span, 'normal' cells; used for human vaccine production
WISH	Human SMNION	Epithelial	
Vero	African green monkey kidney	Fibroblast	An established cell line capable of continuous growth but with many 'normal' diploid characteristics; used for human vaccine production

1.3.2 Disadvantages

The difficulties of using mammalian cells for making recombinant protein involve the following: (1) Animal cells generally growth slower and produce lower quantities of recombinant protein per cell than microbial systems. (2) The inherent fragility of the cells means that they can be damaged by shear forces during fermentation. Fermentation equipment required is complicated and costly. (3) The effective control of culture parameters is essential in animal cell culture if reproducible results are to be achieved. (4) The medium used for animal cell growth is expensive and may be variable, poorly defined and a serious potential source of contamination. The design of effective, chemically defined culture medium can avoid these technical difficulties. (5) Purification may be difficult due to high levels of exogenous protein in the medium used (Cartwright, 1994a).

1.4 Erythropoietin

1.4.1 History of EPO

"Hemopoietine" was used to describe the first hypothetical humoral factor in erythropoiesis (Carnot and Deflandre, 1906). In 1948, Bonsdorff and Jalavisto, introduced the term, erythropoietin. More convincing evidence of the existence of EPO was seen in experiments where injection of large quantities of plasma from anemic rabbits into normal rabbits stimulated erythropoiesis and maintained high red blood cell number when stimulation was maintained for a longer period (Erslev, 1953).

Approximately 90% of adult EPO is produced in the kidneys. The remaining 10% is produced in the liver and bone marrow macrophages (Fried, 1973). The tubular epithelium, might be the oxygen-sensitive cells, which, when stimulated by reduced 0_2 tension, could induce the synthesis of EPO mRNA in the contiguous peritubular tissue.

In 1977, EPO was purified from the urine of patients with aplastic anemia (Miyake et al., 1977). To obtain EPO in quantity, human EPO gene was cloned and sequenced in several laboratories (Lin et al., 1985; Jacobs et al., 1985). The expression of EPO cDNA clones has also been achieved by transfecting mammalian cells, Chinese hamster ovary (CHO) and baby hamster kidney (BHK) cell lines (Goto et al., 1988). Since no native host for EPO is available, the above process is the only way to produce HuEPO in quantity.

1.4.2 Function and application

The process that regulates the production of erythrocytes depends on the hormone, EPO, is called erythropoiesis (Jacobson et al., 1957). Erythropoiesis is the only possible way of making more erythrocytes by means of stem cells. Because of the absence of nuclei, endoplasmic reticulum, mitochondria and ribosome, erythrocytes can't grow or divide in an adult mammal (Peschele and Condoerlli, 1975). EPO is active in almost all stages of red blood cell differentiation, except in the very early step (Koury et al., 1987). The capacity of cells to respond to EPO stimulation is linked to a receptor. The specific receptor in humans is located on erythroid cells (Eridani, 1990).

The main stimulus for EPO production is a low concentration of oxygen as detected by renal sensor cells. Tissue hypoxia stimulates cells in the kidneys to synthesize and secrete 1000-fold increased amounts of EPO into the blood stream. EPO, in turn, enhances the production of more erythrocytes until the level of tissue oxygenation reaches normal levels.

Recombinant human EPO (rHuEPO) has been approved by the U.S. Food and Drug Administration for the treatment of the anaemia of chronic renal failure since June 1989. The main clinical use of EPO is the treatment of the anaemia induced by chronic renal failure. Besides chronic renal failure, rHuEPO has been extensively used for therapy in non-renal associated anaemias, e.g. rheumatoid arthritis patients suffer from anemia due to a defective marrow response (Foa, 1991); cancer patients suffer anemia from either chemotherapy or as an effect of the cancer itself (Foa, 1991); transfusion-dependent

patients; AIDS patients and pretreatment of surgery candidates (Ridley et al., 1994). Treatment with rHuEPO can restore a normal hematocrit, eliminate the need for transfusion and improve the quality of life of its recipients. Thus, rHuEPO is a safe drug with a high benefit-risk ratio.

1.4.3 Molecular structure

The gene for human EPO is located on human chromosome 7 (Powell et al., 1986). The human EPO gene encodes a protein of 193 amino acids and the last 166 residues correspond to the mature protein (Fig. 1.2). The first 27 amino acids predicted by the DNA coding sequence are presumed to be the hydrophobic leader sequence that is cleaved during secretion of the protein (Lin et al., 1985; Egrie, 1990). Both rHuEPO and urinary HuEPO (uHuEPO) are proteolytically processed at their C terminus and lack the amino acid arginine at position 166 by posttranslational modification (Jelkmann, 1992). The mature hormone is composed of 165 amino acids, has two disulfide bonds (between 7 and 161, between 29 and 33) and four oligosaccharide chains (three N-liked at Asn24 Asn 38, Asn 83, and one O-liked at Ser126) as shown in Fig. 1.3 (Lai et al., 1986; Recny et al., 1987). The molecular mass of glycosylated EPO is 34-38.5 kDa, but the molecular mass of the peptide chain is only about 18 kDa (Goldwasser and Kung, 1972; Sasaki et al., 1987).

The sugar chains compose 40% of the molecular weight (M.W.) of HuEPO and probably cover most of its molecular surface. N-linked carbohydrate units present in EPO are very diverse. There are several characteristic structures in the Asn-linked sugar chains of uHuEPO. The major structure is a tetra-antennary complex type. This structure is very large and is relatively rare among serum glycoproteins (Yoshima et al., 1981). Less branched structures (tri- and bi-antennary) also exist. The ratio of the di-, tri- and tetra-antennary moieties is 1.4:13.5:85.1, respectively (Sasaki et al., 1987; 1988). The degree of sialylation is about 80-97% (Rice et al., 1992). About 80% of the sugar chains have fucose linked to GlcNAc at the reducing terminal (Takeuchi et al., 1987; Ohashi et al., 1989).

The uHuEPO has one O-linked sugar chain in addition to the three Asn-linked chains (Fukuda et al., 1986). Lai et al., (1986), who sequenced uHuEPO purified by Miyake et al., (1977), determined that Ser 126 was glycosylated with a branched oligosaccharide (Sasaki et al., 1987).

1.4.4 Comparison of natural EPO and rHuEPO

Natural EPO and rHuEPO both consist of one polypeptide chain (Jacobs et al., 1985; Recny et al., 1987). The amino acid sequence of natural EPO and rHuEPO are homologous. However, the carbohydrate moiety may be different. uHuEPO migrates as a diffuse band at 34-38.5 kDa and rHuEPO migrates at 32-38 kDa by SDS-PAGE (Sasaki et al., 1987). By comparing the characteristic structure of N-linked saccharides obtained from the bioanalytical method, a few differences have been elucidated between uHuEPO and rHuEPO (Recny et al., 1987; Takeuchi et al., 1988; Tsuda et al., 1988). However, the glycosylation pattern of rHuEPO obtained in one cell type will differ from that obtained after expression of the same gene in another cell type.

First, both uEPO and rHuEPO have a significant amount of tetra-antennary oligosaccharides containing Galβ1,3GlcNAc. Secondly, uEPO apparently has a lower portion of bi-antennary saccharides, but rHuEPO contains heterogeneous bi-antennary saccharides depending upon the batches. Thirdly, uEPO lacks the tetra-antennary structure with N-acetyllactosaminyl repeats that can be found in rHuEPO.

Differences in sialylation have been observed between uHuEPO and rHuEPO/CHO. Two distinct sialic acid linkages, NeuAcα2,3Gal and NeuAcα2,6Gal, are found in N-linked glycoproteins isolated from human urine. For example, EPO isolated from human urine contains about 60% of its sialylated structures with the NeuAcα2,3Gal linkage and 40% with the NeuAcα2,6Gal linkage (Takeuchi et al., 1988).

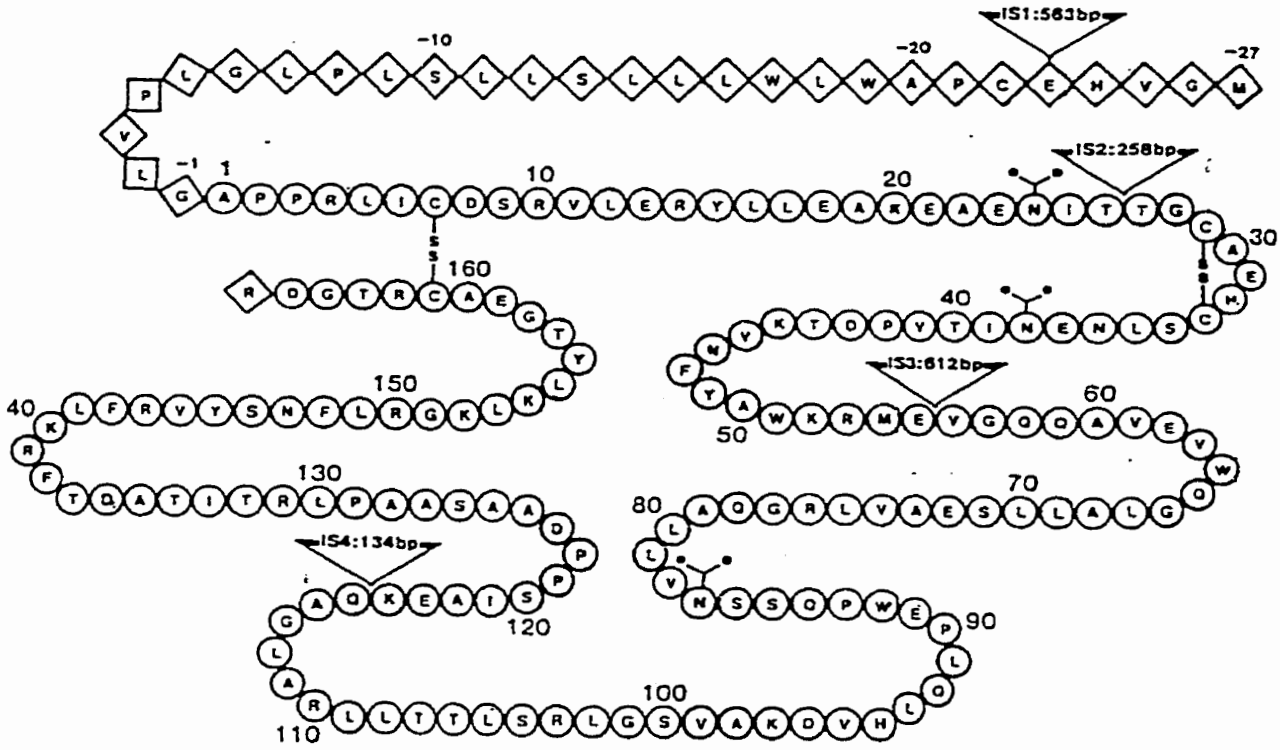


Fig. 1.2 Amino acid sequence of the human EPO gene. The 27 amino-terminal amino acids of the putative signal peptide, and the carboxyl-terminal arginine are boxed, indicating posttranslational cleavage. 165 amino acids of the mature hormone are included in circles. Positions of the two disulfide bonds (S-S), and three N-linked glycosylation sites are indicated (Browner et al., 1986).

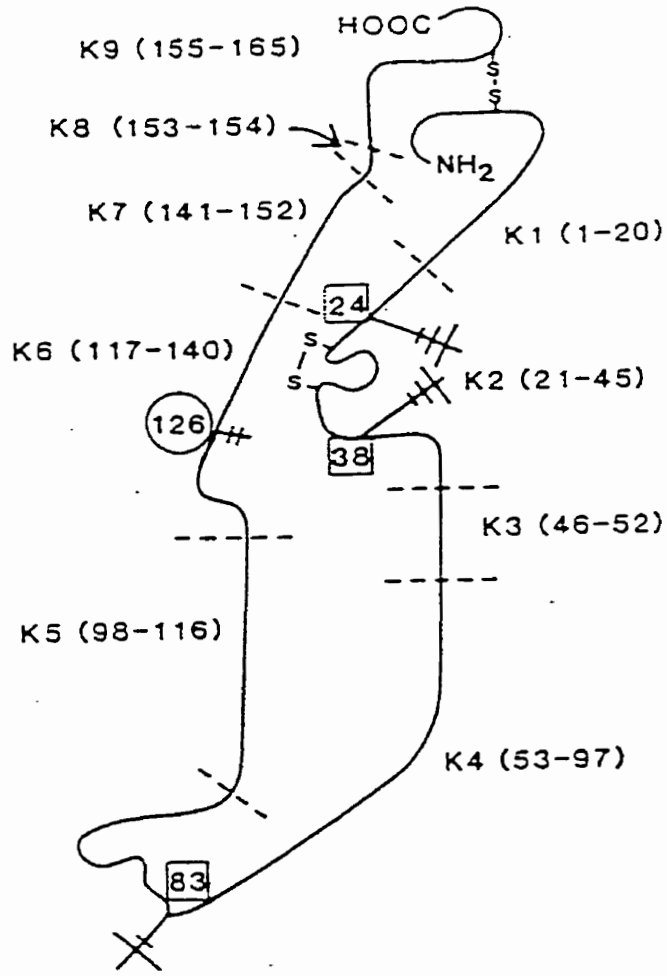


Fig. 1.3 Predicted structure of human EPO. K1-K9 are peptides generated by endoLys-C, and the amino acid residue numbers are indicated in parentheses. The N-linked oligosaccharides are located at the amino acid residue 24, 38, and 83, whereas O-linked oligosaccharide is located at the residue 126 (Sasaki et al., 1988).

1.5 Effects of oligosaccharides on EPO properties

The majority of bioactive proteins are made not only of amino acids, but also of sugars which are important for biological activity. Studies of glycoprotein hormones have demonstrated widely varying functions for the carbohydrate chains. The carbohydrate chains of a glycoprotein may affect its conformation leading to changes in intracellular transport and secretion, solubility, susceptibility to proteases, *in vivo* half life by altering routes of clearance, and receptor binding properties.

1.5.1 Effects on biosynthesis, secretion and stability

The effects of removing glycosylation sites on the biosynthesis and secretion of rHuEPO have been tested. Dubé *et. al.* (1988) genetically changed the asparagine (Asn) and serine (Ser) at the glycosylation sites of HuEPO into glutamine (Gln) and glycine (Gly), respectively, and produced the mutants in BHK cells. They reported that only the Gln 24 mutant was synthesized and secreted into the media as efficiently as native EPO. Less than 10% of other mutant proteins were processed properly and secreted from the cells.

Narhi et al., (1991) determined the role of carbohydrate in maintaining the conformational stability of the protein. The protein expressed in CHO cells (fully glycosylated) and *E.coli* (no carbohydrate) were compared for their stability to guanidine HCl, pH, and temperature. From their experiments, the carbohydrate played a critical role in stabilizing the EPO molecule to denaturing conditions. Goldwasser *et. al.* (1974) reported asialo-erythropoietin is more sensitive to heat denaturation and trypsin action than the native hormone. Recently, Uchida et al., (1997) reported that the deglycosylation of EPO caused an increase of susceptibility to oxygen radicals compared to intact EPO. They indicated that the role of oligosaccharides in EPO might be to protect the protein structure from active oxygen radicals.

Although EPO has only one O-linked oligosaccharide, prevention of this glycosylation inhibits secretion and results in rapid degradation of the mutant EPO (Dubé et al., 1988).

All data demonstrated that carbohydrates on EPO are required for chemical and biological stability.

1.5.2 Effects on *in vitro* and *in vivo* biological activities

Oligosaccharides play a significant role in defining the *in vivo* glycoprotein clearance rate, a critical property in determining the efficacy of an injected therapeutic protein. Since Lowy et al., (1960); Winkert and Gordon, (1960) found that sialidase treatment inactivates the *in vivo* biological activity of uHuEPO, the role of sialic acid of EPO in its biological activity has been studied extensively. Dordal *et. al.* (1985) reported that desialylated rHuEPO lost its *in vivo* activity, while a increase in *in vitro* activity was observed. Sialidase treated rHuEPO (Takeuchi et al, 1990; Tsuda et al., 1990) showed no *in vivo* activity in spite of a 2-to 3-fold increase in *in vitro* activity. Fukuda et al.,(1989); Spivak and Hogans, (1989) examined the organ distribution of asialo- and intact HuEPO injected into rat, and found that asialo-EPO rapidly accumulated in the liver with a $t_{1/2}$ =2.0 min, while intact HuEPO circulated with a $t_{1/2}$ =180 min. Recently Morimoto et. al. (1996) noted a linear relationship between the *in vivo* bioactivity and the sialic acid content in the range of 9.5-12.1 mol. of sialic acid per mol. of EPO. Obviously, the role of sialic acid at the terminal of oligosacchride could be to protect EPO from the trapping of hepatic asialo-glycoprotein binding lectin *in vivo,* which induce the rapid clearance from the body.

Glycoprotein oligosaccharides can also affect clearance rates by mechanisms which do not involve high-affinity receptors. To study the roles of the remaining sugar moieties of HuEPO, Takeuchi et al., (1989) found a unique cell line which produced an unusual form of HuEPO (EPO-bi-antennary) in addition to the usual form (EPO-tetra). In spite of a 3-fold higher *in vitro* activity than standard HuEPO, EPO-bi showed very little *in vivo* activity, even though it had sufficient sialic acid to cover most of its galactose residues. This finding suggested that sugar moieties other than sialic acid residues might be important for the expression of *in vivo* EPO activity. A good positive correlation between the *in vivo* activity of HuEPO and the ratio of tetra-antennary to bi-antennary oligosaccharides was found. From these results, it was suggested that the branching

structure of sugar chains of HuEPO have a role in decreasing a non-specific clearance mechanism, such as filtration by the kidney, or in enhancing homing to bone marrow, which is the target organ of EPO (Takeuchi et al., 1989).

Takeuchi et al., (1990) found that the *in vitro* bioactivity of HuEPO was increased by the action of sialidase, galactosidase and N-acetylhexosaminidase, but further removal of sugar chains by the action of α- and β-mannosidases decreased the *in vitro* and *in vivo* activities. Finally, the removal of all N-linked oligosaccharides chains of HuEPO by extensive PNGase F digestion resulted in almost complete loss of activity. These investigators concluded that the core portions of the N-linked oligosaccharide chains are necessary for HuEPO to retain its full biological activity and that removal of the core portion of the sugar chains destroys the active conformation of HuEPO.

All investigators found that the activity was almost the same before and after the removal of the O-linked sugar chain indicating that the O-linked carbohydrate chain did not contribute much to bioactivity of HuEPO at all.

In summary, the sugar moieties of HuEPO are not just decoration, but have physiological functions. The Asn-linked sugar chains can be divided into three functional units: core, branching and terminal parts. The core part appears to maintain the bioactive conformation of the polypeptide. The branching of glycans on rHuEPO affects its filtration rate in the kidney and allows a longer lifespan in the blood stream. The terminal part appears to act as a signal for recognition by the hepatic asialoglycoprotein binding lectin and determines the lifespan of glycoproteins in the blood stream through lectin-mediated clearance systems (Takeuchi and Kobata, 1991).

1.6 Glycobiology

1.6.1 Glycoconjugates

(a) Attachment of oligosaccharides to glycoprotein

The glycans of glycoproteins can be classified into two groups:

(i) Those that are called Asn-linked or N-linked glycans contain an N-acetylglucosamine (GlcNAc) residue at their reducing termini and are linked to the amide group of an Asn residue within the consensus sequence Asn-X-Ser/Thr of a polypeptide (Fig. 1.4).

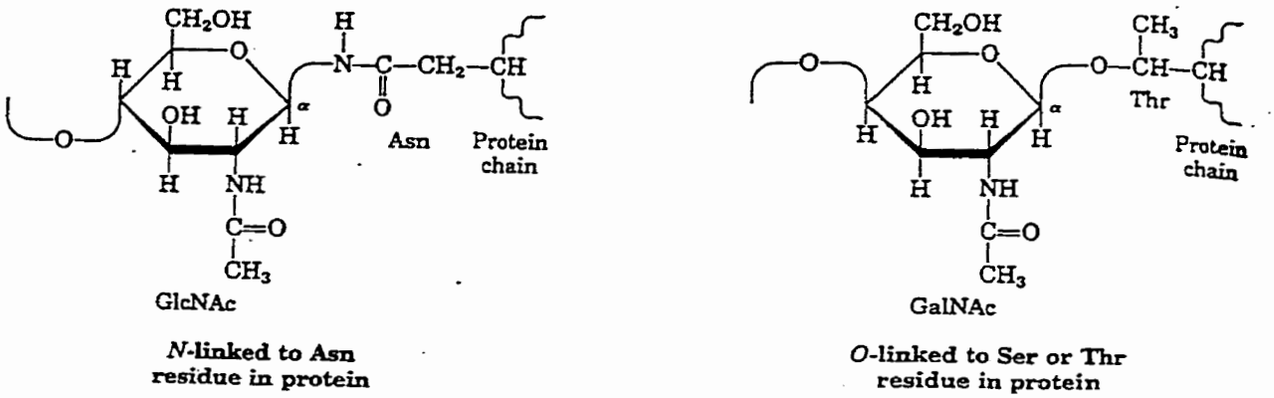
(ii) The glycans that belong to another group called mucin type or O-linked glycans generally contain an N-acetylgalactosamine (GalNAc) residue at their reducing termini. This GalNAc is linked to the hydroxyl group of either a serine (Ser) or a threonine (Thr) residue of a polypeptide. Although the glycans are often linked to Ser or Thr residues through GalNAc, the linkage may occasionally be through other residues e.g. fucose. It is also recognized that single glycans such as GlcNAc or fucose may be O-linked to the peptide.

(b) GPI membrane anchors

As shown in Fig. 1.5, glycophosphatidylinositol (GPI) membrane anchors are complex glycophospholipids found covalently attached to a wide variety of externally disposed plasma membrane proteins in eukaryotes (Cross, 1990; Ferguson and Williams, 1988). The primary function of GPI anchors is to afford a stable association of proteins with membrane lipid bilayers.

(c) Other glycoconjugates

In addition to glycoproteins and GPI membrane anchors, there are a number of other types of glycoconjugates. The oligosaccharide moieties present on polypeptides and proteins are discrete, specific and conserved structures, but this is not always the case on these other glycoconjugates, e.g. glycolipids and proteoglycans.



(a) (b)

Fig. 1.4 Two types of glycosidic linkage between protein and oligosaccharide. (a) The N-glycosidic bond to the nitrogen of the Asn side chain. (b) The O-glycosidic bond to the hydroxyl group of Ser or Thr side chains.

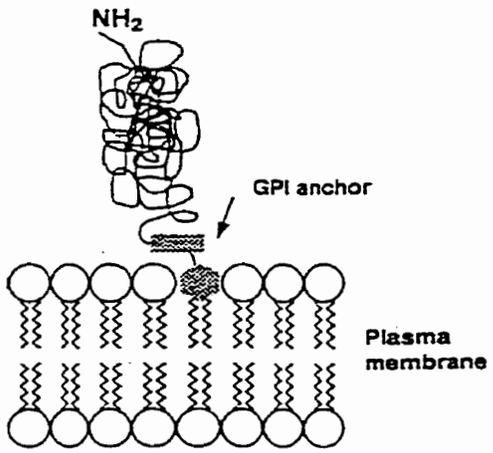


Fig 1.5 GPI-anchored protein. A GPI-anchored protein has an N-terminal domain and is embedded in the outer of the lipid bilayer via the lipid portion of the GPI anchor.

1.6.2 Oligosaccharide structures found on glycoproteins

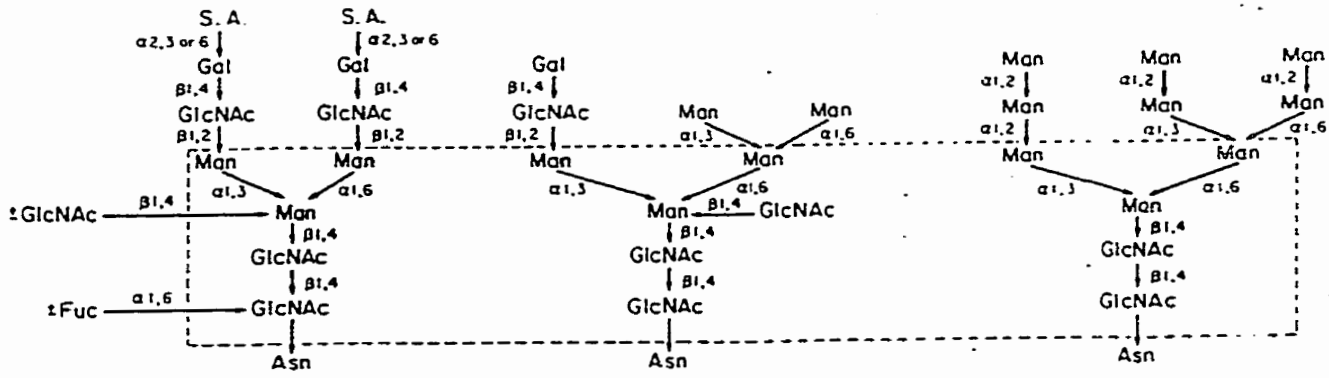
(a) N-linked

Asn-linked oligosaccharides fall into three main categories termed high mannose, hybrid, and complex (Fig.1.6). They all share the common core structure $Man_3GlcNAc_2$-Asn, but differ in their outer branches.

The high mannose-type oligosaccharides typically have two to six additional mannose residues linked to the pentasaccharide core. High-mannose types of oligosaccharides are most commonly found associated with 'immature' glycoproteins (i.e. glycoproteins still in the Golgi during the process of biosynthesis), but they can also be found associated with mature glycoproteins of yeast, fungi and higher plants and on some viral envelope glycoproteins (Elbein, 1991).

The hybrid molecules contain both high-mannose and complex-type oligosaccharides. Most hybrid molecules contain a "bisecting" N-acetylglucosamine.

The complex-type structure contains two to four outer branches with the typical sialyllactosamine sequence. The complex-type structure may be modified both by the addition of extra branches on the α mannose residues or by the addition of extra sugar residues that elongate the outer chains (Kornfeld and Kornfeld, 1985). In the biantennary complex chain the core region is lengthened by the trisaccharide sequences composed of NeuAc-Gal-GlcNAc. However, other complex structures may have three (triantennary) or four (tetraantennary) of these trisaccharide units. In addition, some of them may have an α-linked fucosyl unit attached to the innermost GlcNAc, or they may have other sugars in place of the sialic acids such as polylactosamine chains. These complex structures are generally found on plasma membrane receptors, as well as on secreted proteins.



1 2 3

Fig. 1.6 Three subgroups of N-linked sugar chains. (1) complex type sugar chain; (2) hybrid type sugar chain; (3) high mannose type sugar chain. The structure within the shaded box is core structure common to all N-linked glycans. Structures outsides these lines can vary by glycans.

(b) O-linked

In contrast to N-linked glycans, O-linked have fewer structural rules. They do not share a common core structure. The O-linked glycan is simpler with a structure of -Gal-GalNAc but variability exists with the attachment of one or two NeuAc residues. A common mammalian O-linked oligosaccharide structure is shown below:

NeuAcα2-3Glaβ1-3GlaNAcα1-Ser/Thr
|
NeuAcα2-6

1.6.3 Glycosylation processing

(a) N-linked glycosylation

The biosynthesis of the oligosaccharide portion of the Asn-linked glycoproteins involves three stages that can be described as follows:

(i) Assembly of the lipid-linked oligosaccharide

A series of lipid-mediated reactions in which the sugars GlcNAc, mannose, and glucose are transferred from sugar nucleotides to dolichyl-linked oligosaccharides to form a large lipid-linked oligosaccharide, $Glc_3Man_9GlcNAc_2$-pyrophosphoryl-dolichol (Dol-P) (Li et al., 1978; Hubbard and Ivatt, 1981) (Fig. 1.7). The oligosaccharide is assembled in the ER on the lipid carrier dolichol phosphate. The sugars are added in a stepwise fashion with the first seven sugars derived from the nucleotide sugars, UDP-GlcNac and GDP-Man whereas the next seven sugars are derived from the lipid intermediates, Dol-P-Man and Dol-P-Glc (Katz, et al., 1977; Hanover and Lennarz, 1980). These reactions are all catalysed by membrane-bound glycosyl transferases on the cytoplasmic and lumenal surfaces of the ER.

(ii) Transfer of the lipid-linked oligosaccharide

The oligosaccharide is then transferred in the lumen of the ER, from this lipid carrier to specific asparagine residues that are in the consensus sequence, Asn-X-Ser / Thr (Pless and Lennarz, 1977). However, this consensus sequence alone is not sufficient for glycosylation to occur. In many cases an Asn does exist in the consensus sequence but is not glycosylated, indicating the involvement of other factors in glycosylation.

Several studies have shown that the glucose residues on the lipid-linked oligosaccharide facilitate the *in vitro* transfer of the oligosaccharide to protein (Spiro et al., 1979; Murphy

and Spiro, 1981; Sharma et al., 1981). However, the presence of glucose residues is not an absolute requirement for transfer, which vary with the cell type.

(iii) Processing of the oligosaccharide

Once the oligosaccharide is transferred to the protein, the oligosaccharide chain begins to undergo a series of processing reactions. The reactions involved in modification of the oligosaccharide are outlined in Fig. 1.8. The first reactions occur in the ER where two membrane-bound α-glucosidases remove all three of the glucose residues from the oligosaccharide chains of the protein (glucosidase I removes the outer glucose and glucosidase II can remove the next two glucoses). These reactions result in glycoproteins in the ER with high-mannose oligosaccharides (Chen and Lennarz, 1978; Grinna and Robbins, 1979).

Oligosaccharide processing optionally continues in the compartments of the Golgi, with a variety of exoglycosidase and glycosyltransferase reactions leading to the generation of "complex type "oligosaccharide structures. Once the protein is transferred to the Golgi apparatus, mannosidase I removes one to four of the α1,2-linked mannoses from $Man_9GlcNAc_2$-protein. The product of mannosidase I is $Man_5GlcNAc_2$-protein (Tulsiani and Touster, 1988; Tabas and Kornfeld, 1979). This oligosaccharide is the acceptor for a GlcNAc that is donated from UDP-GlcNAc. This reaction is catalysed by the enzyme called N-acetylglucosaminyl-transferase (GnT) I which is probably located in the medial Golgi apparatus of all eukaryotic cells.

Once this GlcNAc has been added, this oligosaccharide becomes a substrate for another medial Golgi enzyme, mannosidase II, that can remove the α1,3- and α1,6-linked mannoses to give GlcNAc-$Man_3GlcNAc_2$-protein.

The product of α-mannose II action, $GlcNAcMan_3GlcNAc_2$-Asn, serves as the substrate for GnT II catalyzed addition of an GlcNAc residue in β1,2 linkage to the terminal mannose1,6 residue. This tranferase cannot act on $Man_3GlcNAc$ or $Man_5GlcNAc$. Its

product, $GlcNAc_2$-$Man_3GlcNAc_2$-Asn, is the precursor to complex oligosaccharides having two, three, or four outer branches.

This oligosaccharide can then be elongated by the addition of various sugars, such as GlcNAc, galactose, sialic acid and fucose, to give a great variety of complex structures (Beyer et al., 1981). Most of the glycosyl transferases that catalyze these reactions reside in the trans-Golgi apparatus.

If GnT III acts on the product to attach a "bisecting" GlcNAc residue to the β mannose then the oligosaccharide cannot be further branched. In the absence of prior action by GnT III on $GlcNAc_2Man_3GlcNAc_2$-Asn, other GnT can act to add GlcNAc in β1,4 linkage to the α 1,3 mannose (transferase IV) or in β1,6 linkage to the α1,6 mannose (transferase V) to produce structures with three or four outer branches.

(b) O-linked glycosylation

The initial step in O-glycosylation by mammalian cells is the covalent attachment of GalNAc to ser or thr via a α 1 linkage in ER. This reaction is catalyzed by the enzyme UDP-GalNAc-polypeptide N-acetylgalactosaminyltransferase. Fig. 1.9 shows a common mammalian O-linked glycosylation pathway. This pathway is evident in the O-linked oligosaccharides of IL-2, EPO, IgA, G-CSF and plasminogen (Goochee et al., 1991).

No O-glycosylation sequence on polypeptides has been identified analogous to the Asn-X-Ser/Thr template required for N-glycosylation. No clear rules have yet emerged as to how the cell selects specific Ser and Thr residues for O-linked glycosylation (Lehle and Tanner 1995). In further contrast to N-glycosylation, O-glycosylation does not require dolichol-linked intermediates since UDP-GalNAc and UDP-Gal serve as the substrates for the first and all subsequent steps in O-linked processing (Carraway and Hull, 1989).

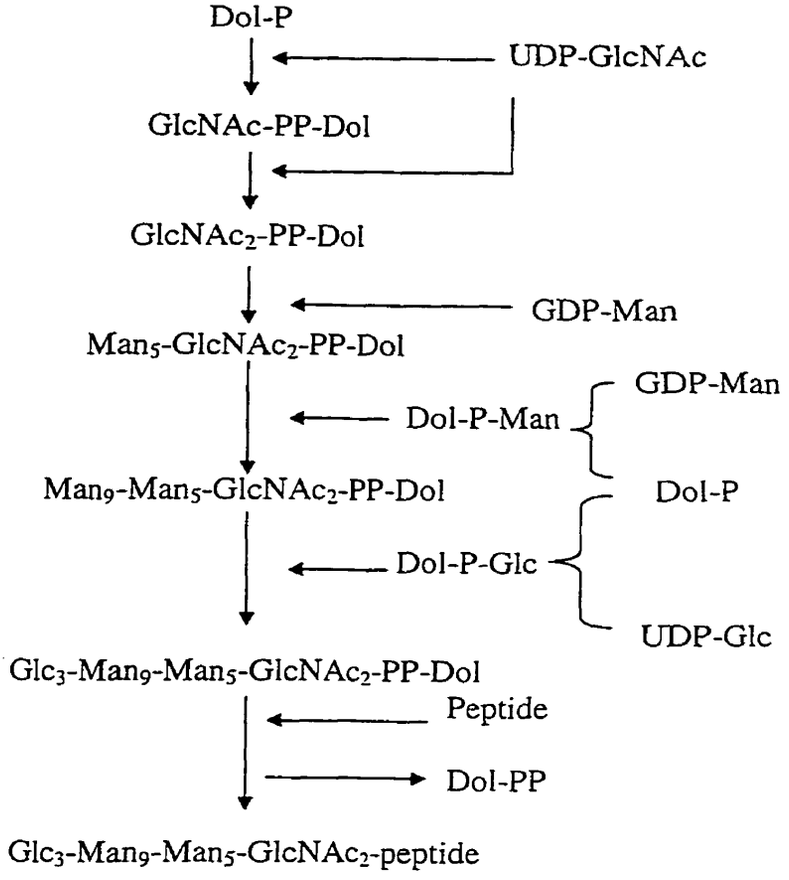


Fig. 1.7 Pathway of lipid-linked oligosaccharide synthesis in the rough endoplasmic reticulum (Snider, 1984).

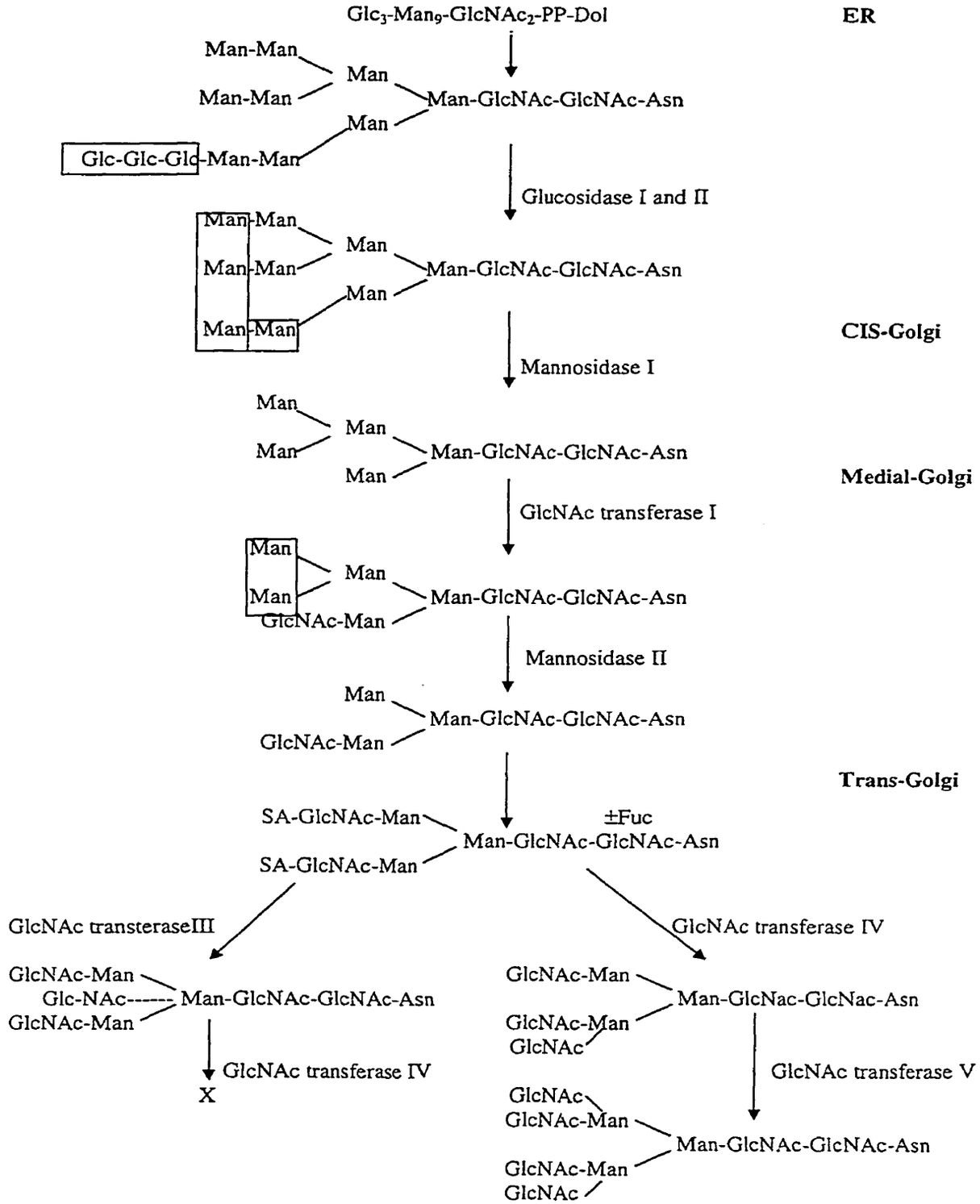


Fig. 1.8 The processing pathway for modification of the N-linked oligosaccharides. This pathway involves the removal of a number of sugars by ER and Golgi-bound glycosidases and the addition of various sugars by Golgi glycosyltransferases.

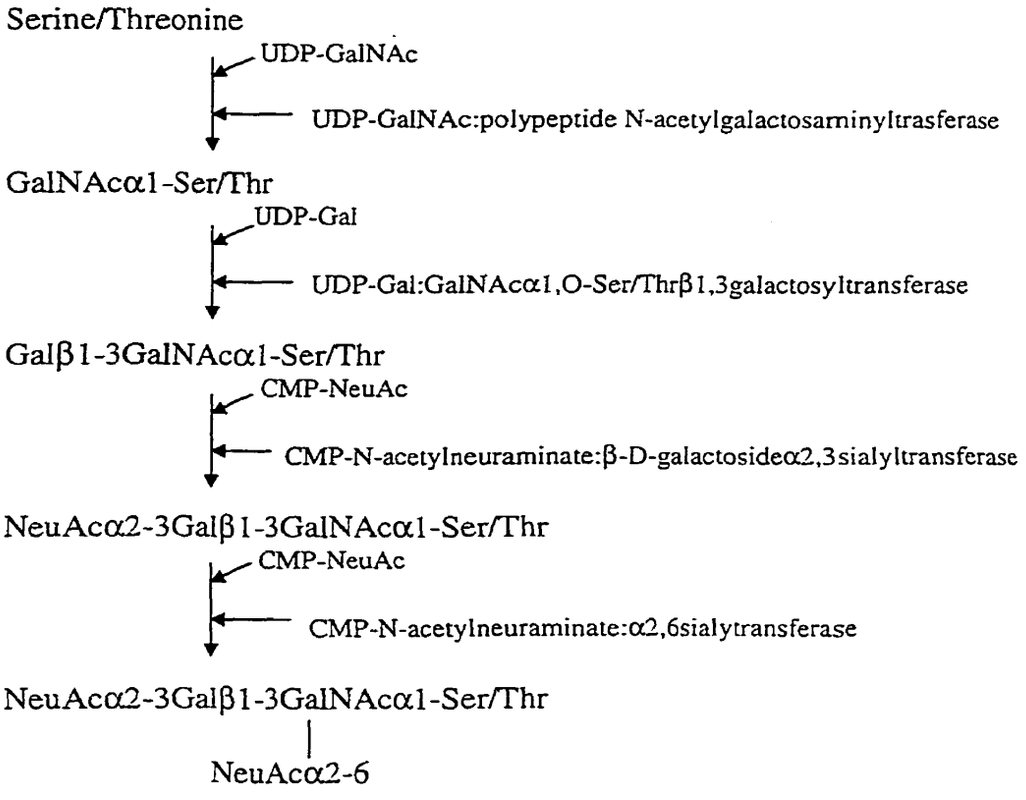


Fig. 1.9 A common mammalian O-linked glycosylation pathway (Goochee et al., 1991).

1.6.4 Variability of glycosylation

A characteristic of glycosylation is the variability of the structure because of the lack of template in the biosynthesis. Characteristics of polypeptide glycosylation include the following:

(a) The same glycoprotein from different cells may contain different oligosaccharide structure;

(b) Glycoproteins containing identical protein structures have different oligosaccharide structures, termed "glycoforms";

(c) An individual polypeptide usually carries several different glycosylation sites (N- and O-linked glycosylation);

(d) Many oligosaccharide structures are found at the same glycosylation site, termed "site microheterogeneity";

(e) The pattern of oligosaccharide heterogeneity at a single glycosylation site under constant physiological conditions is reproducible and not random.

The variability in oligosaccharide structure at glycosylation sites appears very common. This can lead to the production of a non-glycosylated protein. This is clearly evident in experiments involving tunicamycin, which blocks synthesis of the oligosaccharide precursor. The resulting protein is free of carbohydrates (Elbein, 1987).

Glycosylation site heterogeneity may take several forms. Variability in the initial attachment of N-linked or O-linked oligosaccharides termed variable site occupancy leads to glycoforms with different numbers of oligosaccharide structures. For example, cultured mammalian cells secrete two types of tPA, processing either two or three N-linked oligosaccharides due to variable site occupancy at Asn glycosylation sites (Pohl et al., 1984).

Additionally, once initiated at a particular site, glycosylation typically leads to a variable outcome. For example, for secreted mammalian glycoproteins, the N-linked glycosylation pathway does not always proceed to completion leading to the production of a mixture of different structures at a given glycosylation site.

Another source of microheterogeneity results from competing glycosyltranferases in the Golgi (Schachter, 1986). For example, consider the competition of GnT III and GnT IV for an oligosacchride substrate. If GnT III acts first, the resulting oligosaccharide structure is no longer an acceptable substrate for GnT IV, and the oligosaccharide is committed to a processing pathway leading to a bisected, bi-antennary complex-type structure. However, if GnT IV acts before GnT III, the resulting structure is committed to a processing pathway potentially leading to a tri-antennary complex-type structure. Variability in oligosaccharide branching may also occur in the absence of glycosyltranferase competition, presumably due to incomplete processing of the oligosaccharide substrate by particular GlcNAc transferases (Goochee, 1992).

The other type of microheterogeneity is due to variability in terminal processing reactions. The variabilities in sialylation, fucosylation and lactosamine repeats are observed for human IFN-β1, tPA and EPO from CHO cells (Spellman et al., 1989; Takeuchi, 1988).

Site microheterogeneity is also observed for O-linked oligosaccharides. For example, the mono- and di-sialylated versions of this same structure are found at the single O-glycosylation site in IL-2 produced by human lymphocytes and CHO, BHK and Ltk cells (Conradt et al., 1989).

1.7 Factors influence protein glycosylation

Since oligosaccharide chains play an important role, it is important to investigate and understand the factors that influence glycosylation. There are several potential factors that determine the structure of sugar chains on glycoproteins: peptide structures of glycoproteins, glycosylation systems of host cells, and culture environments.

1.7.1 Peptide structure

The protein exerts considerable influence upon its own oligosaccharide processing. Sheares and Robbins, (1986) analyzed the sugar chains of ovalbumin produced in mammalian cells, and suggested that the polypeptide chain was responsible for both the sugar chain structure and the glycosylation site of ovalbumin. It was observed that protein folding could effect protein glycosylation. Once the protein has folded, glycosylation sites may not be accessible to glycosyltransferase (Pless and Lennarz, 1977).

An examination of protein sequences has revealed that only about one third of potential Asn-X-Ser/Thr sites in proteins are actually glycosylated (Kronquist and Lennarz, 1978). It appears that, to be glycosylated, the Asn must be in an exposed region, such as a β-turn of the protein (Marshall, 1972). The presence of the consensus sequence Asn-X-Ser/Thr does not, by itself, initiate N-glycosylation. Thr at position 3 leads to an increased chance of glycosylation compared to Ser at this position, and a proline residue within or near this sequence reduces the likelihood of glycosylation (Gavel and Von Heijne, 1990).

The interesting finding is that the tetra-antennary structures of rHuEPO secreted by different mammalian cell types are well conserved (Takuechi et al., 1988; Sasaki et al., 1987; Goto et al., 1988; Nimtz et al., 1993; Ohashi et al., 1989). This suggests that the EPO polypeptide chain can act to promote the processing of oligosaccharides to tetra-antennary structures, regardless of the cell type. This fact suggests that a certain region of HuEPO polypeptide may give a signal for building a tetra-antennary sugar chain structure in host cells. It is likely that the polypeptide moiety of a glycoprotein determines the branching part. The core part is common to all of the Asn-linked sugar chains.

1.7.2 Host cell type

The cell-type plays a role in oligosaccharide processing. Significant differences are observed between the N- and O-linked oligosaccharide structures from yeast, plant, insect, and mammalian cells (Kaushal et al., 1988; Kukuruzinska, et al., 1987; Kuroda et

al., 1990; Jenkins et al., 1996). Among mammalian cells, oligosaccharide processing is species dependent and cell-type dependent within a given species, even between different cells in one tissue type and among individuals of the same species. The influence of cell type on glycosylation appears to be related primarily to the presence, concentration, kinetic characteristics, and compartmentalization of the individual glycosyltransferases and glycosidases (Pauson and Colley, 1989). Host cells may determine the structure of the terminal part of oligosaccharides.

Hela cells are a representative human cell line which originated from a cervical carcinoma. At first, it was widely believed that a human cell line should be superior to murine cell lines as a host for human EPO production because of species-specific sugar chain formation. However, there are no human-specific sugar chain structures in uHuEPO.

Since the kidney is the major organ that produces HuEPO (Jacobson et al., 1957), BHK cells, which originated from baby hamster kidney cells, were expected to be a suitable host for HuEPO production. The specific activity, carbohydrate composition, and oligosaccharide structure of rHuEPO produced in BHK cells is, in fact, comparable to that of uHuEPO (Goto et al., 1988; Tsuda et al., 1988).

Among mammalian cells, CHO cells have been used extensively for the production of therapeutic proteins. CHO cell lines have three advantages: (i) high productivity, (ii) ability to synthesize oligosaccharide chain structures resembling those of the natural product and (iii) CHO cells which adapt to growth in suspension culture avoid the complexities involved with microcarrier technology. For these reasons, many recombinant proteins are expressed in CHO cells. Extensive characterization of the carbohydrate structure and composition has been performed on recombinant glycoproteins. The oligosaccharide structural components of recombinant glycoproteins produced in CHO cells, including EPO, tPA, IFN-γ and IL-2, are remarkably similar to those isolated from humans or produced in human diploid cells. In contrast, other host cell lines have not been studied in sufficient depth to completely define their

glycosylation capabilities (Jenkins and Curling, 1994). The focus on CHO cells for recombinant gene expression has led to advances in production technology and the use of these cells to produce human pharmaceuticals on a commercial scale (Table 1.4).

However, CHO cells do possess deficiencies that may limit their applicability in specific cases, such as limited capability for γ-carboxylation and inability for oligosaccharide sulfation (Goochee et al., 1991). In addition, recombinant CHO cells generate N-linked oligosaccharides containing only the NeuAcα2,3Gal linkage, but not NeuAcα2,6Gal linkage suggesting that only one of the two sialytransferases is active in each cell type (Takeuchi et al., 1988).

Table 1.4 Recombinant proteins produced by CHO cells

Recombinant protein	Reference
Granulocyte colony-stimulating factor (G-CSF)	Andersen et. al., 1985
Mouse placental lactogen I (mPL-I)	Borys et al., 1993
Human follicle stimulating hormone (huFSH)	Chotigeat et al., 1994
Human interleukin-2 (huIL-2)	Conradt et al., 1989
Tissue-type plasminogen activator (tPA)	Hansen and Emborg, 1994
Human erythropoietin (huEPO)	Lin et al., 1985
Human interleukin-6 (huIL-6)	Orita et al., 1994
Human interferon-γ (huIFN-γ)	Scahill et al., 1983
Human tissue kallikrein (huTK)	Watson et al., 1994
Human antithrombin III (huAT III)	Yamauchi et al., 1992

1.7.3 Culture environment

Glycosylation is an enzymatic process and is governed by (a) cell energy state; (b) the availability of lipid-linked oligosaccharide donor and (c) the level of oligosaccharyltransferase activity. For these reasons, glycosylation can vary with culture conditions, even in one given polypeptide as well as cell type. The synthesis of correctly glycosylated proteins needs to be validated in a culture process. Many studies analyzing glycoproteins demonstrated that culture environments could affect oligosaccharides in recombinant glycoproteins (Goochee, 1991; Gawlitzek et al., 1995; Jenkins, 1995). Thus, reproducible glycosylation requires rigorous control of culture conditions.

(a) Glucose

Several studies have shown that glucose limitation results in incomplete protein glycosylation. The low glucose concentration affects the degree of glycosylation of monoclonal antibodies produced by human hybridomas in batch culture (Tachibana et al., 1994). Moreover, CHO, NIH-3T3, vesicular stomatitis virus-infected BHK cells and mouse myeloma cells showed an altered lipid-linked oligosaccharide profiles in glucose starved cultures (Gershem and Robbins, 1981; Stark and Heath, 1979; Turco, 1980).

Glucose starvation leads to two distinct abnormalities in the synthesis of glycoproteins: (1) in some cases, abnormally small dolichyl precursor oligosaccharides are added to asparaginyl glycosylaton sites in the initial glycosylation step. (2) In other cases, the absence of oligosaccharide moieties at asparaginyl sites, which are normally glycosylated was observed (Stark and Heath, 1979; Davidson and Hunt, 1985; Elbein, 1987). These effects observed under glucose starvation are probably related to both energy depletion of the cell and the shortage of glucose-derived oligosaccharide precursors.

(b) Lipids

Lipids such as dolichol act as key carriers in the glycosylation process, and lipid supplements alone or in combination with lipoprotein carriers can improve the N-glycosylation site occupancy of IFN-γ (Castro et al., 1995; Jenkins et al., 1994). The lipid composition of the medium was found to significantly influence the extent of IFN-γ glycosylation. The lipoprotein supplement 'ExCyte' was shown to minimize the glycosylation deterioration in batch culture, and partially substituting the BSA content of the medium with a fatty-acid-free preparation had a similar effect. The latter result indicates that oxidized lipids carried in Cohn fraction V-derived BSA may damage the glycosylation process (Jenkins et al., 1994).

(c) Ammonia

Various effects of ammonia on monoclonal antibody and recombinant protein glycosylation have been reported. Addition of 10 mM NH_4Cl to cultured plasma cells results in the secretion of IgM deficient in terminal sialylation (Thorens and Vassalli, 1986). Increased concentration of ammonia in the culture medium resulted in reduced O-linked sialylation in G-CSF produced by CHO cells (Andersen and Goochee, 1994). Borys et al., (1994) indicated that ammonia has the potential for affecting the entire glycosylation process of recombinant mPL-I by CHO cells in a pH dependent manner. Grammatikos et al., (1998) and Gawlitzek et al., (1998) reported that ammonia induces N-glycan complexity and decreases the sialylation of recombinant protein by BHK cells. Ammonia inhibited neural cell adhesion molecule (NCAM) polysialylation in CHO as well as small cell lung cancer (SCLC) cells (Zanghi et al., 1998a) and sialylation of immunoadhesin tumor necrosis factor-IgG (TNFR-IgG) produced by CHO cells (Gawlitzek et al., 2000).

(d) Dissolved oxygen

The effect of dissolved oxygen on the glycosylation of some recombinant proteins has been reported previously. Chotigeat et al., (1994) reported that there was a shift in the recombinant human follicle stimulating hormone isoforms to the lower pI fractions corresponding to increased sialic acid content as the dissolved oxygen increased from 10 to 90%. By contrast, there was little change in the N-glycosylation of tPA from CHO cells in perfusion cultures under different dissolved oxygen levels (Lin et al., 1993). Another study found that anoxic conditions in perfusion cultures for short time periods did not alter the N-glycosylation of an IL-2 variant from BHK cells (Gawlitzed et al., 1995). A definite shift towards decreased galactosylation of a monoclonal antibody was observed as dissolved oxygen concentration was reduced in a continuous culture (Kunkel et al.,1998).

(e) Hormones

Glycoproteins are the substrates for oligosaccharyltransferase and for the variety of exoglycosidase and glycosyltransferase enzymes in the ER and Golgi. The enzyme activities are regulated by a variety of mechanisms. For example, stimulation of thyroid cells by thyroglobulin leads to increase per cell activity of several glycosyltransferases, including oligosaccharyltransferase. Durham et al., (1983) observed an increased activity of specific galactyl, fucosyl, and sialyltransferases and decreased activity of a fucosyltransferase in mouse myeloid cells treated with retinoic acid. Regulation of IgE binding factor oligosaccharide structure has also been observed to be dependent upon external protein factors (Ishizaka, 1988).

(f) Degradation by glycosidase

Mammalian cells possess endogenous glycosidases raising the possibility for glycosidase release upon cell lysis (Conzelmann and Sandhoff, 1987) or secretion from live cells during cell culture (Hasilik and Neufeld, 1980). Fucosidase, β-galactosidase, and β-

hexosaminidase activities can accumulate in the extracellular medium of CHO cells after overnight incubation, demonstrating that glycosidases are synthesized by CHO cells and that they may be released under cell culture conditions (Hall et al., 1986).

Changes in the glycan structure have been analyzed during the course of many culture systems. The presence of extracellular sialidase has been reported to be responsible for the loss of terminal sialic acid during the course of anti-thrombin III (Munzert et al., 1996) and DNase (Ferrari et al., 1998) in batch culture of CHO cells. A progressive loss of sialic acid caused by an increased level of sialidase in the culture fluid has been observed (Gramer et al., 1995). The sialidase has been purified from CHO culture supernatant and can degrade glycans from proteins such as recombinant gp120 at neutral pH (Gramer and Goochee, 1993).

Sialidase, β-galactosidase, β-hexosaminidase, and fucosidase can be detected at low levels in supernatants from mouse 293, NS0, and hybridoma cells, and the sialidase activity is much lower than that found in CHO cells (Gramer and Goochee, 1994).

1.8 Ammonia in mammalian cell cultures

The accumulation of ammonium ions during *in vitro* cultivation of mammalian cells is one of the main causes of uncontrolled growth inhibition and glycosylation heterogeneity. Ammonia can be rapidly removed by the kidney *in vivo*. However, no such mechanism exists *in vitro*.

1.8.1 Ammonia production

In vitro cultured mammalian cells obtain ammonia from two major sources. In both cases glutamine is involved. Glutamine, an essential amino acid is the nitrogen source as well as the main energy source of mammalian cells.

The main source of ammonia in mammalian cell cultures is from glutamine metabolism. Before glutamine enters the citric acid cycle, the ammonia groups are released via

glutaminolysis due to glutaminase and glutamate dehydrogenase (Fig. 1.10). Thus, one mole of glutamine yields up to 2 moles of ammonia. However the ratio is normally lower because of the competing amido- and aminotransferase reaction (Mckeehan, 1982; McQueen and Bailey, 1990a). In all cases ammonia is secreted into the medium and may act as a growth inhibitor.

Secondly, ammonia is generated by the temperature-dependent degradation of glutamine to a five-member ring structure, pyrrolidone carboxylic acid, and the formation of NH_3 (Tritsch and Moore, 1962). The glutamine degradation rate increases with the increased temperature. The half-life of glutamine at 37 °C is 6.5 days (Tritsch and Moore, 1962). In addition, pH and the chemical composition of the medium may have a considerable influence on the reaction kinetics (Seaver et al., 1984; Ozturk et al., 1990).

Due to the high pKa of ammonium (pKa is 9.3 at 37 °C), NH_4^+ is the predominant form under physiological conditions, less than 1% is in the form of NH_3. Unionized ammonia is the toxic species, rather than the ammonium ion. Doyle and Butler, (1991) observed that the toxicity of ammonium chloride added to the culture medium is enhanced at higher pH values which favors the presence of unionized ammonia.

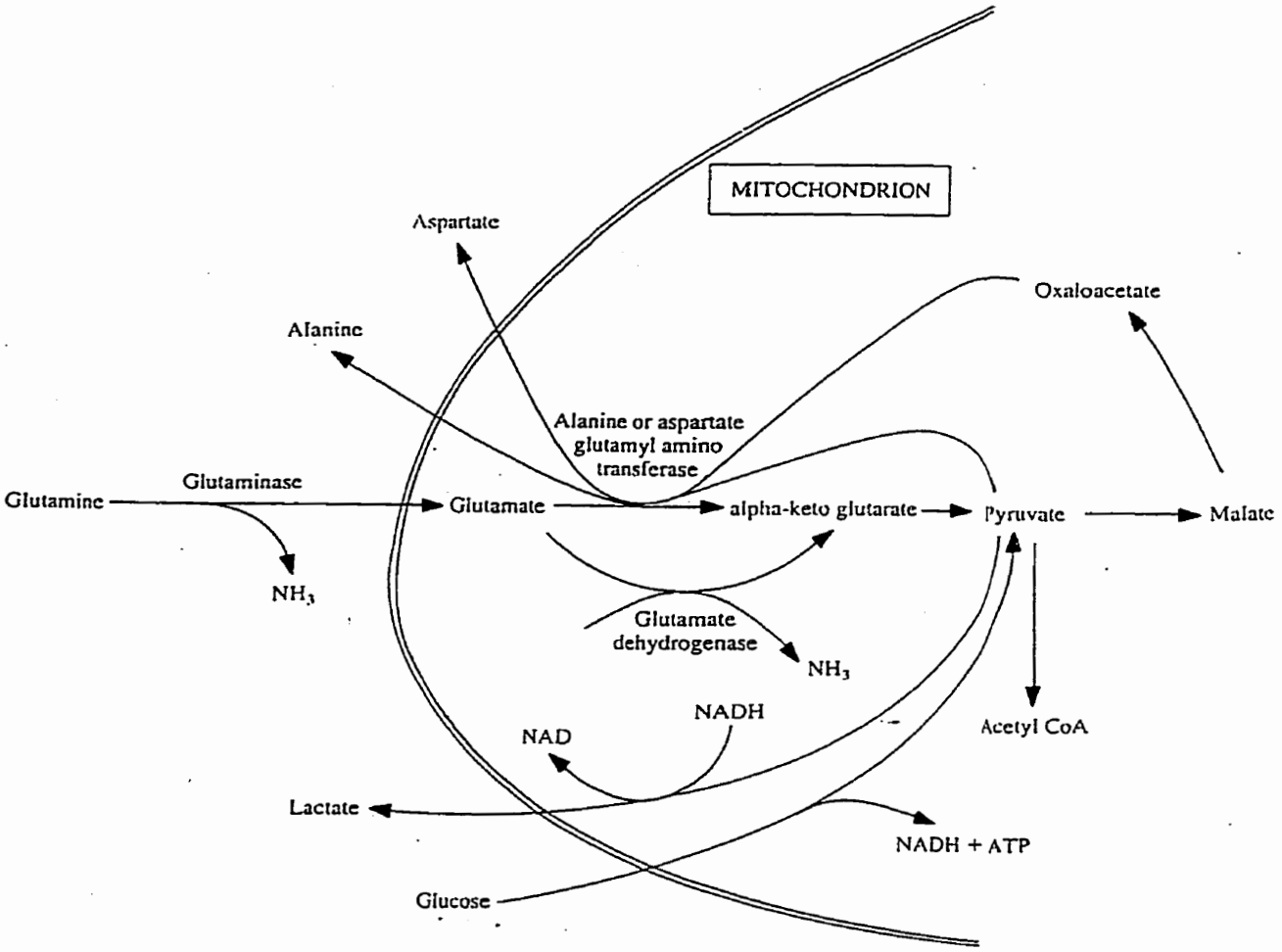


Fig. 1.10 Ammonium production pathway from glutamine by cell metabolism (Adapted from Cartwright, 1994b).

1.8.2 Mechanisms of ammonia effects on mammalian cell culture and protein glycosylation

(a) Increase of intracellular pH

Ammonia and ammonium enter into cells through two different systems. (1) Ammonia, the neutral form, diffuses through the cell membrane (Knerpper et al., 1989). (2) The charged ammonium ion is carried into the cell by surface transporter proteins like the Na^+/K^+-ATPase and the $Na^+K^+2Cl^-$ -cotransporter (Martinelle and Häggström, 1993). Ammonium transport across cellular membranes is extremely slow. Once inside the membrane, the ammonia ionizes by capturing a proton resulting in a local pH rise. The combination of passively transported NH_3 and actively transported NH_4^+ generates intracellular and extracellular pH changes (Martinelle and Häggström, 1993).

The physiological consequences of adding ammonia extracellularly to the medium are very different to those resulting from ammonia produced intracellularly. The metabolism of glutamine and glutamate via glutaminase and glutamate dehydrogenase in the mitochondria results in the production of ammonium. In this case, the NH_4^+ rapidly equilibrates with NH_3 and H^+ according to the mitochondrial pH. The NH_3 subsequently diffuses across the mitochondrial membranes into the cytoplasm and then into other organelles or the extracellular environment. Once outside of the cell, NH_3 equilibrates with NH_4^+ which can be transported back into the cytoplasm by the Na^+K^+-ATPase pump. Thus, H^+ accumulates in the mitochondria because NH_3 diffuses out leaving H^+ in the matrix. The cytoplasmic proton concentration also increases because of the outward flux of NH_3 and inward transport of NH_4^+. While both the mitochondrial matrix and the cytoplasm are acidified, other organelles such as lysosomes, Golgi compartment and ER suffer elevated pH as NH_3 diffuses into them.

Alternatively, ammonium originating from an extracellular source such as the decomposition of glutamine or the direct addition of ammonium salts to culture medium is predicted to increase the mitochondrial pH. The cytoplasmic pH is also decreased in

this case as the NH_4^+ is transported into the cell. However, NH_4^+ rapidly equilibrates with NH_3 which subsequently diffuses into other organelles including the mitochondria (Schneider et al., 1996).

In general, glycosylation enzymes have pH optima that range between neutral and acidic. Inside the cell ammonium tends to accumulate in pH-sensitive, acidic intracellular compartments to concentrations in excess of their extracellular concentration. As a result, the pH of these compartments is raised, resulting in inhibition of pH-sensitive enzyme activity and disruption of receptor-ligand interactions. Andersen and Goochee (1995) suggested than an ammonium-induced increase in the trans-Golgi pH is responsible for decreased sialylation of G-CSF O-glycans. Barasch et al., (1991) reported that defective acidification of intracellular organelles (e.g. trans-Golgi) in cells from cystic fibrosis patients resulted in reduced sialylation of glycoproteins and lipids. Recently, Gawlitzek et al., (2000) found that the ammonium effect on TNFR-IgG oligosaccharide structures could be mimicked by chloroquine, another weak base. pH titration of endogeneous CHO α2,3-sialyltransferase and β-1,4GnT revealed a sharp optimum at pH 6.5, the reported trans-Golgi pH. Thus, at pH 7.0 to 7.2, a likely trans-Golgi pH range in the presence of 10 to 15 mM ammonium, activities for both enzymes are reduced to 50-60%. Reduced glycotransferase activities due to the alkalinization of the compartment by ammonium seems to alter the oligosaccharide synthesis.

(b) Waste of metabolic energy

The energy metabolism of the mitochondria, lysosomal digestion of macromolecules and glycosylation of proteins, may all be affected by accumulation of ammonium and ammonia. It was found that energy deprivation of thyroid slices resulted in depletion of $Glc_3Man_9GlcNAc_2$-P-P-Dol, an accumulation of the $Man_9GlcNAc_2$ lipid-linked species and a concomitant decrease in protein glycosylation (Spiro et al., 1958).

The Na^+/K^+-ATPase system requires ATP hydrolysis to provide energy to pump K^+ ions into the cell and to maintain the K^+-rich intracellular environment. The increase in

maintenance energy required to pump excess NH_4^+ may be a major factor in reducing cell viability and product generation capacity. Animal cells typically expend over half of their total energy consumption on maintenance energy. The Na^+/K^+-ATPase pump is particularly costly in energy and has been estimated to account for the consumption of over 50% of the total energy production of rabbit renal cells (Harris et al., 1981).

In addition, it has also been suggested that ammonia may be involved in the so-called "futile metabolic cycles" in which ammonium ions are cyclically added to and released from glutamate with a net hydrolysis of ATP, again exerting toxicity by depleting the high-energy phosphate pool (Fig. 1.11) (Tagler et al., 1975). Clearly, significant waste of metabolic energy could seriously reduce the capacity of the cells to proliferate or to produce protein products.

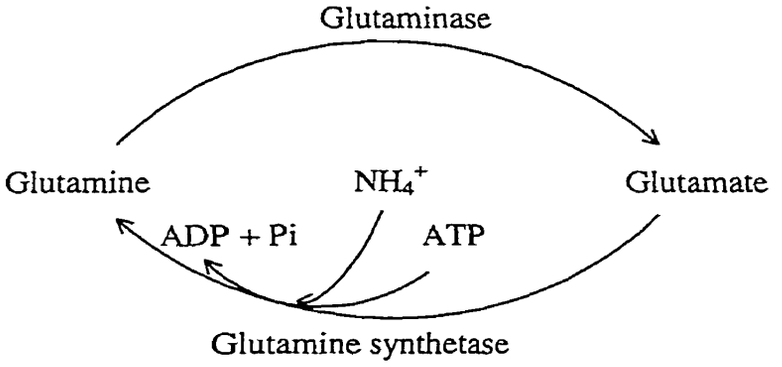


Fig. 1.11 Diagram of a futile cycle.

(c) Imbalance of intracellular nucleotide sugar pools

A new comprehensive hypothesis for the growth inhibition and glycosylation effects of ammonium ions on mammalian cells is proposed to be the imbalance of intracellular nucleotide sugar pools.

The nucleotide sugars represent the activated forms of the corresponding sugars. Nucleotide sugar pools are present as two major structural isomers within the cell, namely UDP-N-acetylglucosamine (UDP-GlcNAc) and UDP-N-acetylgalactosamine (UDP-GalNAc), collectively abbreviated to UDP-GNAc. The two forms of UDP-GNAc can be interconverted by an isomerase enzyme. Synthesis starts with the amination of fructose-6-phosphate (Frc-6-P), from the glycolytic pathway, with glutamine or ammonium to form glycosamine-6-phosphate catalyzed by glutamine-fructose-6-phosphate transaminase or glucosamine-6-phosphate deaminase, respectively, in the cytoplasm of the cells. Glucose is needed for the Frc-6-P supply. UDP-GlcNAc is formed by acetylation of glucosamine-6-phosphate followed by activation with UTP (Fig. 1.12).

Ammonium, a direct precursor of the UDP-GlcNAc pool, is incorporated into frc-6-P to synthesize glycosamine-6-phosphate. Ryll et al., (1994) demonstrated a rapid dose-dependent increase in the intracellular UDP-GNAc levels in four different cell lines (BHK, CHO, Ltk$^-$ 929, and hybridoma) in response to exogeneously applied ammonium chloride. Gawlitzedk et al., (1999) and Valley et al., (1999) demonstrated the incorporation of ammonium into intracellular UDP-activated N-acetylhexosamines and into carbohydrate structures in the recombinant glycoproteins.

The animal cell intracellular sugar-nucleotide pool has been correlated to several cellular events. Increase in UDP-N-acetylhexosamine levels were concomitant with the inability of human colon cancer cells to differentiate (Wice et al., 1985), growth inhibition (Ryll et al., 1994) and cell ultrastructure modifications such as ER distentions (Morin et al., 1983).

In addition, the nucleotide sugars are substrates in the synthesis of N- and O-linked oligosccharides on secreted or membrane bound glycoproteins. Furthermore, UDP-GlcNAc is also a substrate for intracellular O-GlcNAcylation (Haltiwanger et al., 1992). Pels-Rijcken et al., (1995) suggested that elevated UDP-GNAc impaired the transport of CMP-NeuAc into the trans-Golgi compartment, resulting in reduced sialylation of glycoprotein. Increased branching of IL-2 N-glycosylation variants produced in BHK-21 cells was found to correlate with increased levels of UDP-GNAc (Gawlitzek et al., 1998; Grammatikos et al., 1998).

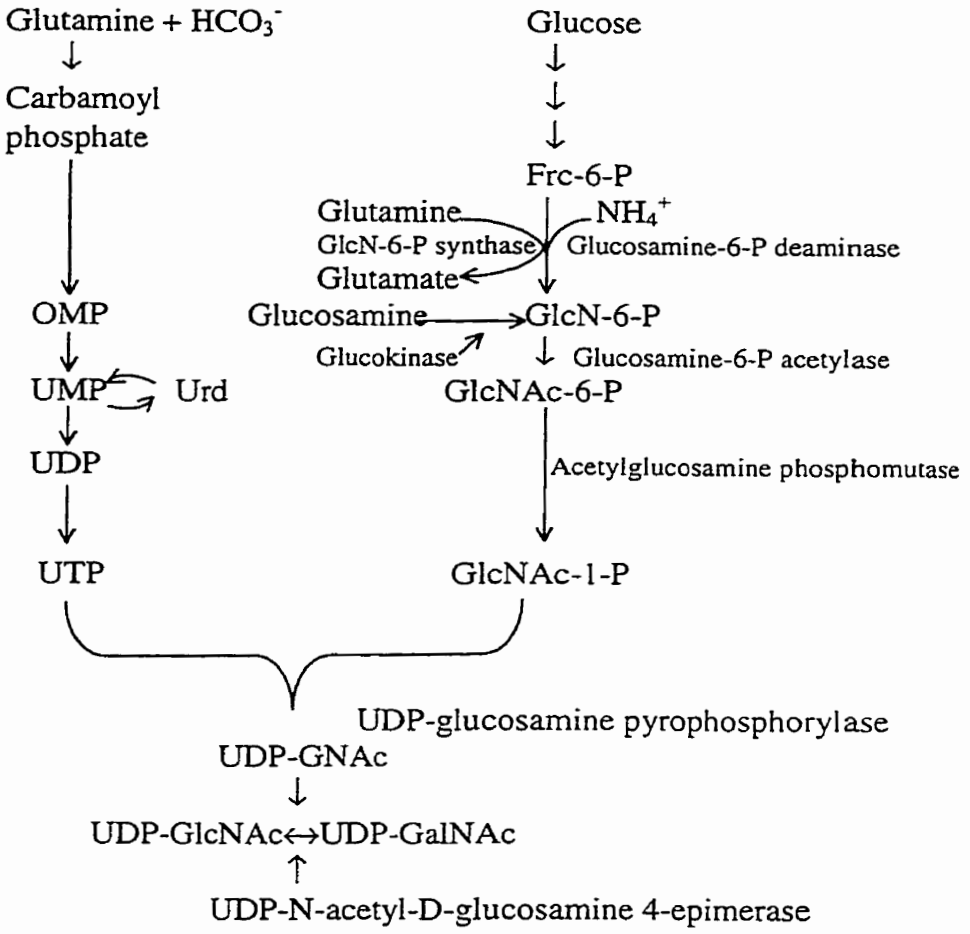


Fig. 1.12 Pathway of UDP-GNAc formation (Adapted from Ryll and Wagner, 1992; Zanghi et al., 1998b).

1.9 Objectives of the present study

Ammonia is an accumulated waste product and a potent inhibitor in mammalian cell cultures. The effects of ammonia on some cell lines such as hybridomas are well characterized in terms of cell growth and protein production (McQueen and Bailey, 1990a). However, less information is available on the influence of ammonia on CHO cell growth and productivity. Since these are the most frequently used mammalian host cells for biomedical products, it is important to elucidate ammonia effects on CHO cell culture and protein production.

Previous studies have shown different effects of ammonia on monoclonal antibody and recombinant protein glycosylation. Ammonia inhibited the sialylation of IgM, G-CSF and TNFR-IgG (Thorens and Vassalli, 1986; Andersen and Goochee, 1995; Gawlitzek et al., 2000), affected the entire glycosylation process of recombinant mPL-I (Borys et al., 1994) and increased the oligosaccharide structure complexity of mutant IL-2 (Gawlitzek et al., 1998; Grammatikos et al., 1998).

The mechanism by which ammonia interferes with glycosylation of recombinant proteins is not clear. Gawlitzek et al. (1998) and Grammatikos et al. (1998) suggested that an increase of the intracellular UDP-GNAc pool caused by elevated concentrations of NH_4^+ or glucosamine in the culture medium contributed to the formation of more complex oligosaccharide structures. Another report indicated that the high UDP-GNAc level can impair the transport of CMP-NeuAc, resulting in decreased sialylation (Pel Rijken et al., 1995). It is possible that the intracellular UDP-sugar pools might mediate the glycosylation changes induced by ammonia. An understanding of the mechanisms by which glycosylation can be controlled is important for the development of bioprocesses leading to consistent recombinant glycoprotein production.

The cloned CHO-81 cell line transfected with the HuEPO gene was used for this study. The recombinant EPO was used as a model glycoprotein. Ammonia was chosen as the culture parameter to investigate. The objectives of the project are summarized below:

(a) Determine the effects of NH_3/NH_4^+ on cell yield and cell metabolism.

(b) Investigate the effects of ammonia on EPO production.

(c) Explore the effects of ammonia on EPO glycosylation.

(d) Examine the heterogeneity of EPO during batch culture.

(e) Determine whether the intracellular sugar pool has significant effect on EPO glycosylation.

The observations can be used to monitor the variability of EPO glycosylation during production. These observations may also be applicable to other glycoproteins to produce less variation in protein glycoforms and obtain consistent glycosylation.

CHAPTER 2

Materials and methods

2.1 Chemicals

All chemicals and reagents were obtained from Sigma Chemical Company unless indicated. All additions to the culture medium were cell culture grade or of the highest purity available. All aqueous solutions were prepared with double distilled water obtained after reverse osmosis followed by Milli-Q (Millipore) filtration. Cell culture procedures were carried out in a laminar flow cabinet (Nuaire Class II, Nuaire).

2.2 Cell culture

2.2.1 Cell line

A cloned stable transfectant (EPO-81) which expresses human EPO was provided by Cangene Corp. for this work. This was derived from a CHO-K1 cell line co-transfected with a plasmid containing the gene for huEPO and scaffold attachment region (SAR) elements. The function of the SAR elements is to increase the expression level of the EPO gene. This cell line has been selected in preference to CHO cell line transfected with the dhfr gene amplification system because the specific production rate of the SAR cell line is higher and more stable.

2.2.2 Culture medium

A serum-free medium designated CHO-SFM2.1 was developed by incremental improvements at the University of Manitoba. The formulation of the medium is proprietary.

2.2.3 Cultures

The transfected CHO cells were maintained in 75 cm^2 T-flasks in humidified incubators at 37°C and 10% carbon dioxide. Routine sub-culturing of stock cells was carried out between 72-96 hours. Cells were detached from the growth surface by trypsin and viable cell concentrations were determined. 1 X 10^5 cells / ml were inoculated into fresh medium.

2.2.4 Trypsinization of CHO cells

Trypsin:	0.5% in D.D.W	
Soybean trypsin inhibitor:	0.9 mg/ml in PBS	
PBS:	Sodium phosphate monobasic	1.93 g
	Sodium phosphate dibasic	9.66 g
	NaCl	8.5 g

Added DDW to 1 liter and filtered through a 0.2 μm filter to sterilize.

PBS / EDTA: added EDTA 0.3722 g to 1 liter PBS

CHO cells grown in T-flasks were detached by trypsin. For 75 cm^2 T-flask, the medium containing the cells was removed from the flask and 3 ml PBS/EDTA was added. The cell suspension was centrifuged at 150 rpm for 5 min. The supernatant was removed and the cells were resuspended in 3 ml PBS/EDTA. The cell suspension was transferred back to the flask. 0.3 ml 0.5% trypsin was added and incubated for 1-3 min. After incubation, 0.3 ml 0.9 mg/ml trypsin inhibitor was added followed by 3 ml PBS. Cell clumps are dispersed by pipetting. The single cell suspension was centrifuged. The cell pellet was resuspended in the fresh medium.

2.2.5 Viable cell determination

Samples of cell suspension were diluted 1:1 with 0.2% trypan blue in PBS / 0.05% NaN_3. Both grids of a Neubauer haemocytometer slide were loaded with the cell suspension and

the number of cells present in four large squares of each grid were counted under microscopy. Cell concentration was calculated from 8 squares and multiplied by 2 X 10^4 (cells/ml). Cells which did not take up the dye were determined as live cells, those that appeared blue were counted as non-viable cells.

The viability of a culture was determined by:

$$\frac{\text{Viable cell number}}{\text{Total cell number}} \text{ X100\%}$$

2.3 Analysis of media components

2.3.1 Glucose

Glucose was determined by an assay based on a kit from Sigma (Glucose Trinder 315-100). The enzymatic reaction involved:

Reaction 1:

$$\text{Glucose} + H_2O + O_2 \xrightarrow{\text{Glucose Oxidase}} \text{Gluconic Acid} + H_2O_2$$

Reaction 2:

$$H_2O_2 + \text{4-Aminoantipyrine} + \text{p-Hydroxybenzene Sulfonate} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine Dye} + H_2O$$

Quinoneimine Dye has an absorbance maximum at 505 nm. The intensity of the color produced is directly proportional to the glucose concentration in the sample.

Procedure

Glucose standard was prepared from 0.02 to 5 mM in 2 fold dilutions. 30 µl of standard or culture supernatant samples (1:5 and 1:10 diluted in water) was added to a 96-well plate. 180 µl Glucose Trinder Solution was added to each well and the plate was

incubated at room temperature for 18 minutes. The absorbance was determined with the THERMOmax plate reader (Molecular Devices) at 490 nm.

2.3.2 Lactate

Lactate dehydrogenase was used to determine lactate concentrations. The reaction catalyzed by the enzyme is as follows:

$$\text{L-lactate} + NAD^+ \xrightarrow{\text{LDH}} \text{pyruvate} + NADH + H^+$$

The production of NADH measured spectrophotometrically at 340 nm is proportional to the initial lactate concentration present in the sample (Gutmann and Walhefeld, 1974).

(a) Reagents and solutions

1. Glycine/Hydrazine buffer: 0.83 M glycine and 5.1% hydrazine hydrate (v/v) / 0.05% NaN_3. The solution was stored at 4 °C.
2. NAD solution: 17.2 mM NAD stored at -20 °C. Aliquots of the solution were thawed as needed.
3. LDH solution: Concentrated L-lactate dehydrogenase was diluted to 119 units /ml in Glycine/Hydrazine buffer before use.
4. Lactate standard: 2 mM lactate (sodium salt) was stored at -20 °C.

(b) Procedure

Lactate standard (0.062 - 2 mM) was prepared in 2 fold dilutions. Culture supernatant samples were diluted 1:80 and 1:160 in water, within the concentration range of the standards. 40 µl of standards or samples were added into a 96-well plate, followed by 40 µl NAD solution, 130 µl Glycine/Hydrazine buffer and 40 µl of LDH solution. The plate was incubated at 37°C for 30 minutes. The absorbance of each well was read (at 340 nm) with the THERMOmax plate reader.

2.3.3 Glutamine

The enzymatic method for glutamine determination was performed according to the procedure described by Lund (1985).

$$\text{L-glutamine} + H_2O \xrightarrow[H^+]{\text{glutaminase}} \text{L-glutamate} + NH_4^+$$

$$\text{L-glutamate} + NAD^+ + H_2O \underset{\text{ADP}}{\overset{\text{GDH}}{\rightleftharpoons}} \alpha\text{-ketoglutarate} + NH_4^+ + NADH$$

The glutamine present in the sample is proportional to the production of NADH and the increase in absorbance at 340 nm. The glutaminase reaction has a pH optimum of 5.0 and is carried out under acidic conditions. The equilibrium of the glutamate dehydrogenase reaction favors the production of glutamate and NAD^+. However, the forward reaction may be forced in a basic buffer with a high NAD concentration and a trapping agent for α-ketoglutarate. ADP is included to activate the enzyme.

(a) Reagents

1. Acetate buffer: 0.5 M acetate buffer was prepared by mixing 0.5 M sodium acetate and 0.5M glacial acetic acid in appropriate proportions to obtain pH 5. The solution was stored at 4 °C.
2. Glutaminase solution: Lyophilized glutaminase (Sigma, G 5382) was di ssolved in 10 fold diluted Acetate buffer to 10 U/ml. Aliquots were stored at -20 °C.
3. Hydoxylamine solution: 20 mM hydroxylamine, stored at 4°C.
4. Tris/Hydrzine buffer: 0.15 M Trizma base, 3 mM EDTA and 7.5% hydrazine hydrate (v/v) was prepared and the pH adjusted to 9.0. The solution was stable at 4 °C for one week.
5. NAD solution: 30 mM NAD, stored at –20 °C.

6. ADP solution: 100 mM ADP (sodium salt) prepared in D.D.W and neutralized with 2 M NaOH. Aliquots were stored at –20 °C.

7. GDH solution: 2500 U/ml glutamate dehydrogenase (Sigma, G2626) was stored at 4°C.

Reagent A: 2160 µl Acetate buffer
1080 µl Hydroxylamine solution
1155 µl D.D.W. mixed and warmed at 37 °C.
108 µl Glutaminase solution was added before use.

Reagent B: 2160 µl Acetate buffer
1080 µl Hydroxylamine solution
1265 µl D.D.W. mixed and warmed at 37 °C.

Reagent C: 9.6 ml Tris/Hydrazine buffer
3.1 ml D.D.W. mixed and warmed at 37 °C.
1440 µl NAD, 144 µl ADP and 90 µl GDH were added just before use.

(b) Procedure

Glutamine standard with a concentration up to 0.5 mM was prepared in two-fold dilution. Samples were diluted 1:4 and 1:8 with water to fall within the concentration range of the standards for part A and 1:1 for part B. 70 µl standards and samples were added into the plate. 50 µl reagent A (hydroxylamine + Glutaminase in 0.5 acetate buffer) or reagent B (without glutaminase) was added to the wells for part A or part B of the plate respectively. The plate was incubated at 37 °C for 75 minutes. 120 µl reagent C was added and the plate was incubated at 37 °C for 30 minutes. The absorbance of each well was read at 340 nm with the THERMOmax plate reader.

Glutamine concentration = (glutamine + glutamate) (part A) – glutamate (part B)

2.3.4 Ammonia

The ammonium ion concentration was measured by an Orion 9512 ammonia probe connected to a Fisher Scientific pH meter 25. The ammonium concentration includes ammonium (NH_4^+) and ammonia (NH_3). The electrode is comprised of sensing and reference elements immersed in a filling solution separated from the sample by a hydrophobic membrane. A sample was made basic by addition of 10 N NaOH, converting ammonium ion to ammonia gas. The ammonia from the sample diffuses through the membrane and into the filling solution until the partial pressure on either side of the membrane is equal. The partial pressure of ammonia is proportional to its concentration in the alkaline sample. The ammonia reacts with the water in the filling solution as shown below:

$$NH_3 + H_2O \rightleftharpoons NH_4^+ + OH^-$$

The reaction results in a change in potential between the sensing and reference elements that is related to the ammonia concentration in the sample.

Procedure

Standards from 10^{-3} M to 10^{-5} M were prepared by 10 fold serial dilutions. Samples were diluted 1:5 in water for a total volume of 1ml. 10 µl of 10N NaOH was added to 1ml of standards or samples just before each measurement. The electrode was immersed in the stirred sample and the millivolt was read after the meter stabilized. Between each measurement, the electrode was washed extensively with water.. The sample concentration was determined from a calibration curve of the electrode potentials for a series of standard ammonium solutions.

2.4 EPO sample preparation for analysis

2.4.1 De-salting and changing buffer

Column PD-10 (bed vol. 9.1 ml, 1.5x5 cm; Pharmacia Biotech) containing Sephadex G-25 M was used for rapid desalting and buffer exchange. Culture supernatant samples were desalted by Column PD-10 before 2-dimensional electrophoresis analysis and deglycosylation.

Procedure

The gel bed was equilibrated with 25 ml of water or the required buffer. 2.5 ml sample was loaded onto each column. The sample was eluted with 3.5 ml water or the required buffer. The column was washed with 25 ml PBS / 0.05% NaN_3 and stored at 4 °C.

2.4.2 Sample concentration

Ultrafree-4 Centrifugal Filter Unit (M.W. cut off 10 kDa) (Millipore) was used to concentrate samples. Briefly, the sample was poured into the Ultrafree-4 filter unit and the cap was closed tightly. The Ultrafree-4 filter unit was placed into a centrifuge tube and centrifuged at 7,500 g. After centrifugation, the device was removed and the sample was recovered from the inside of the device bottom. The unit was rinsed with water and stored in PBS / 0.05% NaN_3 at 4 °C.

2.4.3 Enzymatic release of oligosaccharides

(a) Reagents

10% SDS

2-mercaptoethanol (2-ME)

12.5% NP-40

50 mM phosphate buffer pH 7.2: 50 mM Na_2HPO_4

50 mM NaH_2PO_4

The pH of NaH_2PO_4 was adjusted with Na_2HPO_4

Recombinant peptide-N-glycosidase F (PNGase F), 1 unit/μl, (Boehringer Mannheim)

Clostridium perfringens neuraminidase (α2-3,6,8, linked N-acetylneuraminic acids) (sialidase), 2 mU/μl, (Sigma)

Diplococcus pneumoniae O-glycan-peptide hydrolase (O-glycosidase), 1mU/μl (Boehringer Mannheim)

(b) Procedure

Samples (50 μl) of desalted and concentrated culture supernatants (10X) were denatured by boiling for 3 min with 1 μl SDS (10%) and 1 μl 2-ME. After denaturation, 5 μl NP-40 (12.5%) and 40 μl 50 mM phosphate buffer (pH 7.2) were added to each sample. Enzymes for individual digests were added as follows: 2 μl PNGase F for removing N-linked glycans; 2 μl sialidase for removing sialic acids; 2 μl of PNGase F, 1 μl O-glycosidase plus 2 μl sialidase to remove all oligosaccharides from EPO. The mixtures containing enzymes were incubated at 37 °C for 16 hours. After incubation, samples were ready for Western blot and 2-dimentional (2-D) electrophoresis analysis.

2.5 EPO analysis

2.5.1 EPO concentration determination by ELISA

(a) Reagents

Microtiter plates (Nalgene Nunc International)

Polyclonal anti-human EPO (Sigma)

Recombinant human EPO 1 U/μl (R&D Systems Inc.)

Monoclonal mouse anti-EPO antibody (purified from the culture supernatant of a murine hybridoma (5F12 AD3), 0.3 mg/ml)

Alkaline phosphatase (AP) conjugated anti-mouse IgG (adsorbed with rat serum protein) (Sigma)

SIGMA FAST p-Nitrophenyl Phosphate (pNPP) Substrate Tablet Set (N-1891)

Coating buffer: 0.1M sodium bicarbonate buffer (pH 8.3)

Blocking buffer: 3% BSA / PBS

Diluting buffer: 1% BSA / PBS / 0.05% Tween $_{20}$

Washing buffer PBS / 0.05% Tween $_{20}$

(b) Procedure

Microtiter plates (96 well) were coated with 100 μl polyclonal anti-human EPO (4 μg / ml) in coating buffer and incubated overnight at 4 °C. The plates were blocked with 200 μl blocking buffer for 2 hours at room temperature. 100 μl of two fold diluted EPO standard or culture supernatant samples were added to the wells and incubated for 4 hours at room temperature. The bound EPO was incubated with 100 μl monoclonal mouse anti-EPO antibody (1 μg / ml) at 4 °C overnight. 100 μl AP-conjugated anti-mouse IgG (1:15,000 diluted in diluting buffer) was added to each well and incubated for 3 hours at room temperature. For detection of the antigen-antibody reaction, SIGMA FAST pNPP Substrate Tablet was added as the substrate, and incubated overnight at 4 °C. The optical absorbance at 405 nm was measured by the THERMOmax plate reader. Each incubation step was followed by washing three times with washing buffer.

2.5.2 SDS-polyacrylamide gel electrophoresis (PAGE)

(a) Reagent and buffer

1. Acrylamide/bis (30% T, 2.67% C):

Acrylamine	29.2 g
N'N'-bis-methylene-acrylamide	0.8 g

Made to 100 ml with DDW and stored at 4 °C in the dark.

2. Separating gel buffer: 1.5 M Tris-HCl, pH 8.8

Tris base	18.5 g
D.D.W.	80 ml

Adjusted pH to 8.8 with HCl and made to 100 ml with water (kept at 4 °C)

3. Stacking gel buffer: 0.5 M Tris-HCl, pH 6.8

Tris base	6 g
D.D.W.	60 ml

Adjusted pH to 6.8 with HCl and made to 100 ml (kept at 4 °C)

4. 10% SDS: dissolved 10 g SDS in DDW and brought the volume to 100 ml.

5. Sample buffer:

D.D.W.	4 ml
0.5 M Tris-HCl, pH 6.8	1 ml
Glycerol	0.8 ml
10% SDS	1.6 ml
2-ME	0.4 ml
0.05% bromophenol blue	0.2 ml

6. 5 X electrode buffer (pH 8.3):

Tris base	15 g
Glycine	72 g
SDS	5 g

Made to 1 liter with DDW and stored at 4 °C.

7. Separating and stacking gel preparation

	14% separating gel	3% stacking gel
D.D.W.	2.65 ml	6.1 ml
Separating gel buffer	2.5 ml	
Stacking gel buffer		2.5 ml
10% SDS	100 µl	100 µl
Acrylamide/Bis (30%)	4.7 ml	1.3 ml
10% ammonium persulfate	50 µl (fresh)	50 µl
TEMED	5 µl	10 µl

(b) Procedure

Electrophoresis was performed in a discontinuous polyacrylamide gel under reducing conditions according to the method of Laemmli (1970). Gels were run using the Mini-Protein II apparatus (Bio-Rad). Samples containing EPO were mixed 1:1 with sample buffer and boiled for 5 minutes. EPO was analyzed by 14% SDS-PAGE separating gel and 3% stacking gel. Electrical current was supplied by a power supply (Model 1000/500, Bio-Rad).

2.5.3 Protein stain, molecular weight determination and quantification

Silver stain: SDS-polyacrylamide gel was stained with the Silver Stain Kit (Sigma) according to the manufacture's instruction.

Coomassie blue stain: The gel was stained for 1 hour with Coomassie blue staining solution (0.1% Coomassie blue R-250, 40% methanol, 10% acetic acid) and destained with the destaining solution (40% methanol and 10% acedic acid).

The pre-stained protein molecular weight standard (GIBCO) was used to determine the molecular weight of protein bands. Quantitative analysis of protein bands was based on

the number of pixels in the bands subtracted by background values using Gel Doc Analytic Software (Bio-Rad)).

2.5.4 Western blot analysis

(a) Reagent and buffer

Transfering buffer:	25 mM Tris	3.03 g
	192 mM glycine	14.4 g
	20% methanol	200 ml

Made the volume to 1 liter with D.D.W.

Diluting buffer: 1% BSA / PBS / 0.05% $Tween_{20}$.

Blocking buffer: 3% BSA / PBS

Monoclonal mouse anti-EPO antibody

Alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma)

SIGMA FAST 5-Bromo-4-Chloro-3-Indolyl Phosphate/Nitro Blue Tetrazolium (BCIP/NBT) alkaline phosphatese substrate Tablet (Sigma)

Washing buffer: PBS / 0.05% $Tween_{20}$

Non-glycosylated EPO sample produced from *Streptomyces* was kindly provided by Dr. Stewart (Binnie et al, 1997).

(b) Procedure

Proteins separated by SDS-PAGE were electrophoretically transferred onto nitrocellulose (NC) membrane. The membrane was blocked by blocking buffer for 2 hours and incubated with purified monoclonal anti-human EPO (3 μg/ml) antibody diluted in a diluting buffer at room temperature for at least 3 hours. Antibody binding was detected by incubation with 1 : 30,000 diluted alkaline phosphatase-conjugated goat anti-mouse IgG for 2 hours. A tablet of SIGMA FAST BCIP / NBT was dissolved in 10 ml water and

used as the substrate. The enzyme-substrate developing reaction was stopped by PBS / EDTA. The membranes were washed 4 times for 5-10 minutes with washing buffer between each step.

2.5.5 Two-dimensional electrophoresis

The 2-D electrophoresis technique provides exceptionally high resolution of the protein components in a complex sample. It is capable of resolving large amount of individual protein species. The first dimension is isoelectric focusing (IEF) during which proteins are separated according to their isoelectric points. The separation is in a non-sieving polyacrylamide gel in the presence of carrier ampholytes, which establishes a pH gradient increasing from anode to the cathode. During electrophoresis the protein reaches a position in the pH gradient where its net charge is zero (defined as isoelectric point (pI)). At this point, migration will cease, and a concentration equilibrium of the focused protein band is established. The first dimension is performed using tube gels (Mini-PROTEIN II 2-D cell, Tube cell, and Tube Module) (Bio-Rad). The second dimension is SDS-PAGE in which proteins are separated by molecular size.

(1) Reagents and solutions

First dimension acrylamide stock solution:

30% Acrylamide	28.38 g
5.4% Bis	1.62 g

Made up to 100 ml with DDW and stored at 4 °C in the dark.

10% Triton X-100 solution: Diluted 10 g Triton X-100 detergent to 100 ml with DDW.

First dimension sample buffer:

9.5 M urea	5.7 g
2% Triton X-100	2 ml 10% stock
5% 2-ME	0.5 ml

2% ampholyte	0.5 ml (preblended pH 4-6)

Made up to 10 ml with DDW and stored at –70 °C.

First dimension overlay buffer:

9 M urea	5.41 g
1% ampholyte	0.25 ml (preblended)
Bromophenol blue	0.5 ml of a 0.05% solution

Diluted to 10 ml with DDW and stored at –70 °C.

SDS sample equilibration buffer:

62.5 mM Tris / HCl	12.5 ml 0.5 M Tris / HCl, pH 6.8
2.3% SDS	23 ml 10% SDS
5% 2-ME	5 ml
10% glycerol	8 ml
Bromophenol blue	2.5 ml 0.05% stock solution
DDW	49 ml

Upper chamber buffer (cathode electrode solution): 100 mM NaOH

Lower chamber buffer (anode electrode solution): 10 mM H_3PO_4

(2) Procedure

1. One end of the casting tube was sealed with a piece of parafilm and filled it with capillary gel tubes.
2. First dimension tube gel solution was prepared as below:

9.2 M urea	5.5 g
4% acrylamide	1.33 ml 30% acrylamide / 5.4% bis
20% Triton X-100	2 ml 10% Triton X-100
2% ampholyte	0.5 ml (preblended pH 4-6)
0.01% ammonium persulfate	10 µl 10% ammonium persulfate (fresh)
0.1% TEMED	10 µl TEMED

3. The first dimension gel solution was pulled into casting tube. The mixed solution was drawn into each gel tube and allowed to polymerize overnight at room temperature.
4. After polymerization, the tubes were connected to a Mini-protein II 2-D Cell.
5. Desalted and concentrated samples were prepared by adding an equal volume of sample buffer. The mixture was incubated at room temperature for 10 minutes. Following centrifugation, samples (40-60 µl) were loaded into the sample reservoir connected to the capillary tube gel.
6. The sample was overlaid with 30 µl overlay buffer.
7. The cathode reservoir was filled with the cathode electrode solution (100 mM NaOH) and the anode reservoir was filled with the anode electrode solution (10 mM H_3PO_4).
8. Electrofocusing was performed at 750 V for 3.5 hours at room temperature.
9. After the first dimension, the tube gel was removed by the tube gel ejector and equilibrated in SDS sample equilibration buffer for 10 minutes or immediately frozen at -70°C for later analysis.
10. The tube gel was then placed on top of the slab gel for electrophoresis in the second dimension.
11. SDS-PAGE and Western blot detection were performed as described 2.5.2 and 2.5.4.
12. The pI was calculated according to the 2-dimensional electrophoresis protein maker (Sigma).

2.6 EPO purification

2.6.1 Monoclonal anti-human EPO antibody

There were two hybridoma cell lines, 5F12 AD3 (mouse / mouse hybridoma) and BF-11 (rat / mouse hybridoma) which produce anti-human EPO antibody were available from the American Tissue-type Culture Collection (ATCC). Both these hybridomas were grown in a serum free medium. The culture supernatants containing Mabs were purified by a Protein G Sepharose 4 fast flow column (Pharmacia). Since BF-11 had very low productivity and poor binding to our EPO, we chose 5F12 AD3 using in EPO ELISA and purification.

2.6.2 Affinity chromatography preparation

(a) Reagents and solutions

Affi-prep 10 (Bio Rad): N-hydroxysuccinimide ester activated ligand

10 mM sodium acetate (pH 4.5): Sodium Acetate 13.6 mg
Added DDW to 100 ml.

0.2M $NaHCO_3$ (pH8.0) / 0.3 M NaCl: $NaHCO_3$ 33.6 g
NaCl 35.1 g
Brought the volume to 2 liter.

0.1M Ethanolamine HCl (pH8.0): Ethanolamine HCl 0.61 ml
Adjusted pH to 8.0 and made to a volume of 100 ml.

0.5M NaCl: NaCl 2.9 g
Added DDW to 100 ml

PBS (pH7.2)

(b) Procedure

10 ml gel slurry was transferred to a plastic tube and sedimented by a brief centrifugation. The gel was washed with 30-50 volume of cold 10 mM sodium acetate pH 4.5 and mixed with an equal volume of cold purified monoclonal anti-EPO antibody (2mg/ml) dialyzed against 0.2 M $NaHCO_3$ (pH8.0) / 0.3 M NaCl. The mixture was rotated end over end at 4°C for 5 hours. The remaining active esters was blocked by mixing with an equal volume of 0.1 M ethanolamine HCl (pH8.0) and rotated end over end at room temperature for 10 min. The gel was washed with 2-4 volumes of 0.5 M NaCl to remove uncoupled ligand. The coupled support was transferred to a column and rinsed with PBS until the OD of the effluent is stable.

2.6.3 EPO purification by immuno-affinity column

(a) Reagent and buffer

1st washing buffer:	PBS	
2nd washing buffer:	10 mM sodium phosphate monobasic	1.38 g
	0.5 M NaCl	29 g
	Adjusted pH to 7.4 and brought the volume to 1 liter.	

Elution buffer:	3M potassium thiocyanate (KSCN)	1.2 g
	3 mM EDTA	0.5 g
	20 mM Tris	146 g
	Adjusted pH to 7.0 ± 0.2 and brought the volume to 500 ml.	

(b) Procedure

The immunoadsorbant column containing monoclonal anti-human EPO antibody fixed on Affi-Prep 10 was equilibrated with PBS. 1 liter culture supernatant was filtered through 0.22 μm filter and loaded onto the column. After loading, the column was extensively washed with 50 bed volumes 1st washing buffer and 8 bed volumes 2nd washing buffer. The bound EPO was eluted with 2 bed volumes of elution buffer. The EPO fraction (second bed volumes of the eluant) was collected and kept at –20 °C. Following elution, the affinity column was rinsed with PBS / 0.05% NaN_3 and stored at 4 °C. All of the purification procedures were carried out at 4 °C.

2.7 Oligosaccharide analysis

2.7.1 FACE analysis

Fluorophore-assisted carbohydrate electrophoresis (FACE) technology was first described by Jackson, in 1990. It combines the high resolution and simplicity of polyacrylamide gel

electrophoresis with the sensitivity and visibility of fluorescence. Carbohydrates with a reducing terminus react with a fluorophore, [flourophore 8-aminonaphthalen-1, 3, 6-trisulfonic acid (ANTS)]. ANTS has a primary amino group and three sulfonic acid groups (Fig. 2.1). ANTS labeling confers fluorescence and adds three negative charges that enable the migrate in an electric field. Separation of ANTS-labeled oligosaccharides is based both on charge and size. The derivatives are separated by PAGE and followed by detection of the fluorescence bands under UV illumination. ANTS has an excitation maximum at 365 nm and an emission peak at 515 nm. All of the reagents, polyacrylamide gel and electrophoresis buffer were supplied by N-linked Oligosaccharide Profiling Kit (Bio-Rad). The procedure was performed according to Bio Rad's instruction.

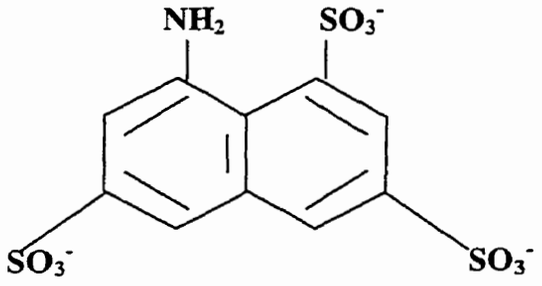


Fig. 2.1 Chemical structure of ANTS

2.7.1a N-linked oligosaccharide profile analysis

1. An equal volume of 2X releasing buffer was added to 45 µl of purified glycoprotein (50 –200 µg) sample.
2. 1.0 µl of 5% SDS and 1.5 µl of 1:10 diluted 2-ME were added to the sample. The sample was heated for 5 minutes at 95 °C.

3. 4 µl of 10% NP-40 and 2 µl of PNGase F were added to the denatured sample. The sample was mixed by gently tapping the side of the tube and centrifuged for 5 seconds.
4. Samples were incubated at 37° C for 16 hours.
5. After incubation, 3 volumes of cold 100% ethanol were added to each tube. The tubes were placed on ice for 10 minutes and centrifuged for 5 minutes to pellet proteins.
6. The supernatant was transferred to a clean tube and dried using a Speed-Vac.
7. 5 µl of labeling reagent ANTS was added to each sample and incubated at 37° C for 16 hours.
8. After incubation, the sample was dried until the sample reaches a viscous-gel stage.
9. The labeled sample was dissolved in 10 µl water and 2 µl of the sample mixed with equal amount of sample buffer was loaded onto polyacrylamide gel.
10. Fluorescently-labeled oligosaccharides were electrophoretically separated in an N-linked oligosaccharide gel using the Mini-Protein II electrophoresis cell.
11. Polyacrylamide gels were subjected to an electric field at 15 mA/gel at 4°C.
12. Images were acquired with the Glyco Doc imager and analyzed by Glyco Doc analytical Software (Bio-Rad).
13. Glycan standards, asialo-agalacto-biantennary with core fucose; asialo biantennary with core fucose; asialo triantennary and asialo tetraantennary were obtained from Oxford Glyco-Systems and used to identify unknown oligosaccharide bands.

2.7.1b N-glycan sequence determination

The N-linked Oligosaccharide Sequencing Kit (Bio Rad) is based on recombinant glycosidases with precisely defined monosaccharide and linkage specificities. Sequence determination of the oligosaccharides was accomplished by a sequential exoglycosidase digestions. Exoglycosidase digestion results in a stepwise release of the monosaccharides from the intact structure eventually to the tri-saccharide core structure. The products of each digestion were separated by PAGE. The values of glucose units serve to correlate mobility shifts measured in the sequencing gel with the number of monosaccharides

released from a glycan structure. Hence, the oligosaccharide sequence can be deduced from a knowledge of the general structural features of N-linked oligosaccharides.

Procedure

1. The N-linked oligosaccharides were released using PNGase F and labeled with the ANTS fluorophore.
2. After labeling, the sample was dried and dissolved in 10 μl water.
3. The sequencing experiment involved setting up four separate enzyme digests. Each of tubes received 2 μl of ANTS labeled glycans, reaction buffer and one or more of the exoglycosidases supplied in the kit.
4. The samples are incubated in 37°C for 16 hours. Extra reaction buffer (2 μl) and 1 μl of enzymes (Table 2.1, 2-4) were added and incubated for 2-4 hours in order to obtain complete digestion.
5. The sequence was determined by band shifts and related to a standard glucose ladder.

Table 2.1. Exoglycosidases used in N-linked glycan sequence determination.

Enzymes used in sequence analysis	Enzyme specificity
1. Neuraminidase	(α2-3,6,8) linked N-acetylneuraminic acids
2. β-galactosidase	(β1-4) linked galactose
3. β-N-Acetylhexosaminidase	(β1-2,3,4,6) linked N-acetylglucosamine
4. α-Mannosidase	(α1-2,3,6) linked mannose

2.7.2 HPLC analysis

The HPLC system used mainly includes a high pressure dual-pump and a supply of mobile phase, an injector unit for introducing samples into the column, a column packed with a high efficiency stationary phase, and an online detector. HPLC is used for a very wide variety of separations. In addition to different columns with different separation

mechanisms the composition of the mobile phase offers a further spectrum of possibilities for controlling separations. The use of HPLC has many advantages over the classical technique of gas chromatography, since the liquid mobile phase allows the separation and recovery of substances which are not volatilized. In addition, liquid chromatography is to be preferred for molecules which have high polarity and high molecular weight. Such features are characteristic of most biological macromolecules, e.g. peptides, proteins and glycans which have caused many difficulties in other chromatographic studies.

2.7.2.1 Oligosaccharides preparation

(a) Enzymatic release

The elution buffer containing purified EPO was either dialyzed against or changed with PBS (0.1 X) / 0.01% Tween $_{20}$. Then the sample was concentrated to 50-100 µl by a Ultrafree-4 Centrifugal Filter Unit. The procedure for the enzymatic release of oligosaccharides from purified EPO is described in 2.4.3.

(b) Ethanol precipitation

After deglycosylation, the oligosaccharides were separated from protein by ethanol precipitation. 3 volumes of cold 100% ethanol were added to each deglycosylated sample. Samples were placed on ice for 10 minutes and centrifuged for 5 minutes to pellet the proteins. The supernatant was transfered to a tube and dried by a Speed-Vac.

(c) Carbohydrate clean up

GlycoClean S Cartridge (Oxford Glyco-Systems) contains a hydrophilic glycan adsorption disc that binds glycans. GlycoClean S Cartridge can be used for the purification of glycans after procedures including derivatization with 2-AB and enzyme digestion. Glycans bind to the disc in a non-selective manner while hydrophobic peptides,

detergents, and other contaminants were removed with aqueous acetonitrile washes. The bound glycans were eluted using water.

Procedure

The ethanol precipitated and dried sample was dissolved in 10 µl water. The sample was applied to the cartridge and incubated for 15 minutes at room temperature. Non-glycan material was eluted using 96% acetonitrile (5 X 1ml). Glycans were eluted using water (3 X 0.5ml). The cleaned oligosaccharides were dried and ready for derivatization.

2.7.2.2 Sialylated oligosaccharide analysis

(a) Fluorescent labeling with 2-Aminobenzamide (2-AB)

The procedure of 2-AB labeling was based on Bigger et al., (1995).

(1) 350 µl of DMSO and 150 µl of glacial acetic acid were added into a 1.5 ml microtube.
(2) 200 µl of this mixture was added to a tube containing 10 mg 2-AB (0.35M) and mixed by vortex until dissolved.
(3) 100 µl of the above mixture was added to a tube containing 6.2 mg of sodium cyanoborohydride (1M $NaBH_3CN$) and mixed until dissolved.
(4) 5 µl of labeling reagent was added to the dried glycan sample, mixed thoroughly, and incubated at 65°C for 2 hours.
(5) After labeling with 2-AB, labeled glycans were recovered by GlycoClean S Cartridge with the procedure as described in 2.7.2.1 (c).

(b) Glycan analysis by anion exchange column

Anion-exchange HPLC was carried out using a GlycoSep C column (4.6X100mm; Bed volume: 1.7mm; Oxford Glycosystems Ltd.). Neutral oligosaccharides eluted in the void

volume. Negative charged glycans were separated into mono-, di-, tri-, or tetra-sialylated peaks by a linear gradient. Buffers and gradient conditions used were as follows:

(1) Buffer

Buffer A: 20% acetonitrile : 80% water

Buffer B: 20% acetonitrile : 80% 250 mM ammonium acetate pH 4.5

(2) Gradient

(a)

Time (min)	%A	%B	Flow rate (ml/min.)
0	100	0	0.4
2	100	0	0.4
30	0	100	0.4
35	100	0	0.4
45	100	0	0.4

(b)

Time (min)	%A	%B	Flow rate (ml/min.)
0	100	0	0.4
2	100	0	0.4
20	60	40	0.4
36	0	100	0.4
41	100	0	0.4
51	100	0	0.4

(3) Procedure

Sample and standard preparation: the dried 2-AB labeled glycans from 1liter culture supernatant or 3-5 μg of each glycan standard were redissolved in 120 μl water and centrifuged for 5 minutes. Oligosaccharide standards used in HPLC analysis were 3'

sialylactose (Sigma); di-sialylated-galactosylated-biantennary and tri-sialylated galactosylated triantennary (Glyko). A typical oligosaccharide standard chromatogram is shown in Fig. 2.2.

A Glyco Sep-C column was purged with both buffer A and buffer B in HPLC system. The autoinjector was purged with 20% ethanol. 100 µl of sample was injected by the autoinjector. Oligosaccharide elution was monitored by a Pharmacia LKB fluorescent detector. The excitation filter was 330 nm and the emission filter was a cut off filter, 408 nm.

To store the column, buffer A was replaced with D.D.W. The system was flushed at 0.4ml/min for 20 min with water. Buffer B was then replaced with 25% acetonitrile and run for 10 min. The column was removed and stored at room temperature.

2.7.2.3 Asialo-glycan analysis

An amide adsorption normal phase column (4.6 X 250mm; Phenomenex) was used for separation of neutral oligosaccharides. Retention of asialo-glycans on this column was based on the hydrophilicity of the molecule, which is related to molecular size. Generally, small glycans are eluted early followed by larger structures by normal phase column.

The baseline from anion exchange chromatography was high and truncated the base of the peaks. As our fluorescent detector aged, the sensitivity of the detection was significantly reduced and could not set the proper baseline. Although the peak areas and heights were proportionally less, the relative peak areas and proportions as analyzed could still provide useful informations. Since the peak could not be integrated by computer software following normal phase chromatography separation, 4-aminobenzonitrile (4-AB) was used as the labeling reagent for oligosaccharide (Schwaiger et al., 1994). The advantages of using 4-AB as oligosaccharide derivatizer are that they can it be detected by UV detector plus the clean up procedure could be eliminated.

(a) Oligosaccharide derivatization by 4-AB

1. The reagent solution was prepared freshly prior to derivatization.

6% 4-AB	120 mg
5% Acetic acid	100 µl
50% Methanol	1 ml
DDW	900 µl

2. The labeling reagent was formed by dissolving 10 mg of $NaBH_3CN$ in 1 ml of the above reagent solution.

3. 5 µl of the labeling reagent was added to each dried glycan sample.

4. The sample was incubated at 65°C for 2 hours and dried by Speed Vac.

(b) Oligosaccharide analysis by normal phase column

(1) Buffer

Buffer A: 250 mM ammonium formate pH 4.4

Buffer B: 80% acetonitrile: 20% buffer A

(2) Gradient

Time (min.)	% of Buffer B	Flow rate (ml/min.)
0	100	0.4
0.1	100	0.4
2	100	0.4
184	59	0.4
187	0	1.0
189	0	1.0
194	0	1.0
199	100	1.0
204	100	1.0

(3) Procedure

The standard and glycan sample were dissolved into 25 µl DDW and diluted with 95 µl of buffer B. Samples were centrifuged for 5 min at 13,000 rpm. Glucose ladder and asialo-oligosaccharide standards (asialo-agalacto-biantennary with core fucose; asialo biantennary with core fucose; asialo triantennary and asialo tetraantennary) were used to calibrate the normal phase column (Fig. 2.3 and 2.4). Oligosaccharide peaks were identified by comparing retention times with a standard mixture under the same conditions. The autoinjector was purged with 20% ethanol. 100 µl of sample was injected by the autoinjector. Oligosaccharide elution was monitored with an UV detector (Linear Instruments Model 2000 Detector) at 285 nm.

To store the column, the buffer A was replaced with water and the system was flushed at 1ml/min for 20 min with water. The buffer B was then replaced with 100% isopropanol and run for 10 min. The column was removed and stored at room temperature.

2.8 Intracellular nucleotide sugar pool analysis

2.8.1 Nucleotide extraction

The CHO–81 cells cultured under different conditions were collected on day 4. The cell pellet (1.5-2 X 10^6) was resuspended in 100 µl cold 6% trichloroacetic acid and sonicated for 15 seconds (Micon Ultrasonic cell Disruptor, Mandel). The suspension was kept on ice for 15 min. The cell/trichloroacetic acid was transferred to a centrifuge tube and centrifuged for 10 min. at 13,000 rpm. Supernatants (100 µl) were collected and neutralized with 27 µl 0.5 M Tris (pH 9.0) and 2.3 µl 10 N NaOH. Neutralized extracts were kept at –20°C.

2.8.2 Intracellular nucleotides analysis by HPLC

The analysis of nucleotides was based on Ryll and Wagner, (1991); Barnabé and Butler, (1994). The procedure involves separation of intracellular nucleotides using a reversed phase chromatography (Adsorbosphere C-18 column, 200mm X 4.6 mm, 5 μm particle size, Alltech) with a guard column containing the same adsorbent (Waters). The spectral detector was set to read absorbance at 254 nm. Nucleotide peaks were identified by comparing retention times with standard mixtures. Figure 2.5 shows the typical nucleotide chromatograms of the nucleotide standard mixture (Fig. 2.5a) and a CHO cell extract (Fig. 2.5b). UDP-GalNAc and UDP-GlcNAc are represented by a single but well resolved peak at a retention time of 11 min.

(1) HPLC buffers

Buffer A:	KH_2PO_4	46.8 g
	K_2HPO_4	9.6 g
	Tetrabutyl ammonium hydrogen sulfate	10.88 g

Adjusted pH with 85% phosphoric acid to 5.3, brought the volume to 4 liter, and filtered through a 0.2 μm filter.

Buffer B: 70 % buffer A / 30% methanol
The pH was adjusted to 5.9 with 2.5 M KOH.

(2) Nucleotide standard and sample preparation

The nucleotide standards include: NAD, UDP-Glc, UDP-GalNAc, UDP-GlcNAc, AMP, GDP, CTP, UTP, ADP, GTP and ATP. The standard mixture solution was prepared by adding 40 μl of 1 X 10^{-3} M stock of each nucleotide to 560 μl water and 1 ml buffer A for a total volume of 2 ml. The concentration of each standard was 2 X 10^{-5} M. Nucleotide extract samples were diluted 1 : 1 with Buffer A and centrifuged at 13,000 rpm for 10 min.

(3) Gradient for nucleotide analysis

% Buffer B	Time (min.)	Flow rate (ml/min.)
0	0	1
20	5	1
24	10	1
60	15	1
100	23.5	1
100	35.5	1
0	39.5	1
0	41.5	1

(4) Procedure

The 20% ethanol in the purging system of the autoinjector (Shimadzu SIL-9A) was replaced with DDW. Autoinjector lines were purged with DDW for 5 times. To avoid precipitation water was flushed though the HPLC system before the addition of buffer A and buffer B. The methanol content was reduced in the C-18 column from 70% to 0% by replacement with DDW. All lines were purge with water for 5-10 min and followed by buffer A and buffer B. 100 µl of standard solution or sample was injected by the auto-injector. The peaks were monitored and detected by an UV detector at 254 nm.

To store the column, buffer A and buffer B in the solvent conditioner were replaced with D.D.W. The system was flushed at 1 ml/min for 20 min with water. Buffer B was then replaced with 100% methanol and a gradient was run from 0-100% buffer B (a step increase of 20% buffer B each 10 min was used). 100% buffer B was then run for 10 min and decreased to 70% buffer B for another 10 min. The column was removed and stored at room temperature.

2.8.3 Determination of nucleotide concentrations

Response factors (RF) were calculated for each nucleotide in the standard run.

RF = Cstd / Astd

Cstd: concentration of nucleotide in the standard solution (2×10^{-5} M).

Astd: area of nucleotide standard.

RF values are shown in Table 2.2.

The concentration of nucleotides in the sample was calculated based on the peak area of each nucleotide and the response factor.

C = RF x A x df

C: concentration of the nucleotide of interest;

RF: response factor determined from the standard run;

A: area of nucleotide of interest;

df: dilution factor of sample with buffer A (=2).

2.9 Determination of specific growth rate, specific consumption and production rates

2.9.1 Specific growth rate

The specific growth rate (μ) is a growth parameter which is measure of the rate increase of cell number at a certain cell concentration.

$$\mu\ (h^{-1}) = \frac{In\ N - In\ No}{t}$$

where No = initial cell concentration, N = cell concentration at time t, and t = elapsed time from the start of cell growth.

2.9.2 Specific consumption and production rates

Calculation #1:

Specific rates of consumption or production were calculated by a plot of the concentrations of EPO, glucose, ammonia, lactate or glutamine against the integral values of the growth curve (Renard et al., 1988). The slope of the line was equivalent to the specific consumption or production rate during this period of growth. Data were obtained from the supernatant collected every day during exponential growth (day 0 to day 4). Each time point is based on data from two independent cultures.

The integral of the cell number (viability index) was determined by the following equation:

Viability index ($X10^6$ cell-day/ml) $= \int_0^t X_v dt$

Where X_v is the viable cell concentration at time point t. The slope was calculated using SigmaPlot software.

Calculation #2

Specific rates of nutrient consumption or waste production (q) were calculated using the equation:

$$q = [\Delta C / T] / [(N-No) / lnN-lnNo)]$$

Where ΔC is the change in concentration over the time period, T.

Where No and N are the initial and final cell concentrations, respectively.

This method was used during the exponential growth of cells.

2.10 Statistical analysis

Analysis of data was performed using Sigma Plot software. Student's *t* test was used for group comparisons. Although the error determination from two replicates for each group in this thesis, however, all the experiments were repeated at least twice and the results were consistent.

Table 2.2 Response factors calculation for nucleotide concentration determination. RFs were calculated according to the peak area of the standards by computer integration and used to determine the concentration of unknown nucleotide concentrations.

Nucleotide	Concentration ($X 10^{-5}$ M)	Area	RF ($X 10^{-10}$)
NAD	2	440097	0.45
UDP-Glc	2	220825	0.91
UDP-GalNAc+ UDP-GlcNAc	4	489570	0.82
AMP+GDP	4	732371	0.55
CTP	2	86092	2.3
UTP	2	169716	1.18
ADP+GTP	4	622558	0.64
ATP	2	318219	0.63

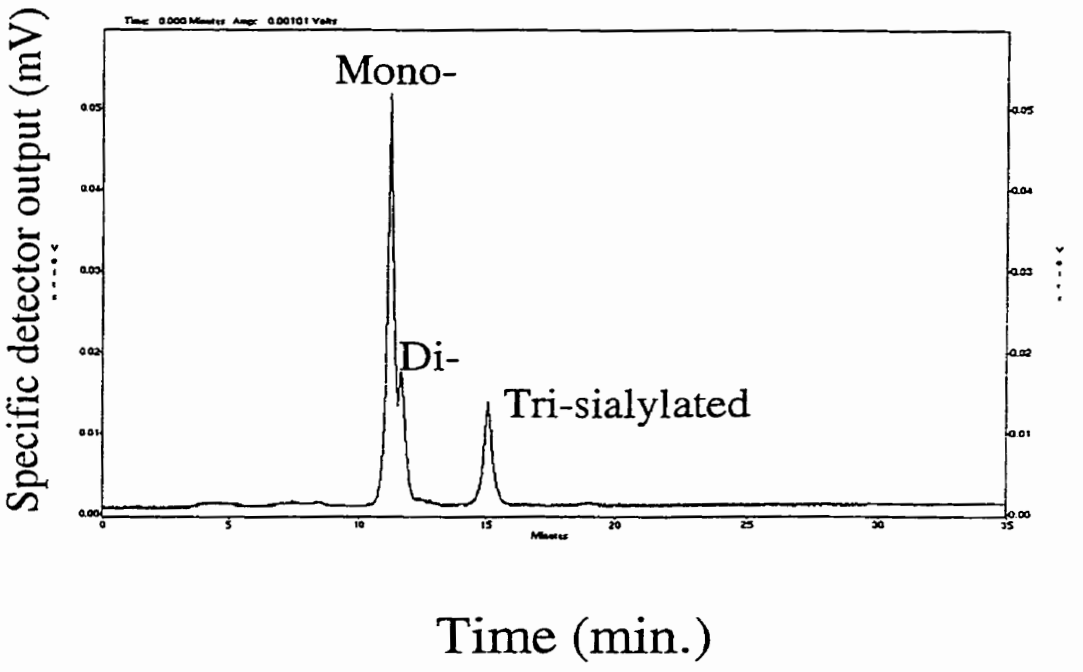


Fig. 2.2 Anion exchange profile of 2-AB labeled sialylated glycan standards. 3' sialylactose; di-sialylated-galactosylated-biantennary and tri-sialylated galactosylated triantennary were labeled with 2-AB followed by GlycoClean S cartridge to remove free dye and analyzed by GlycoSep C column using gradient (a).

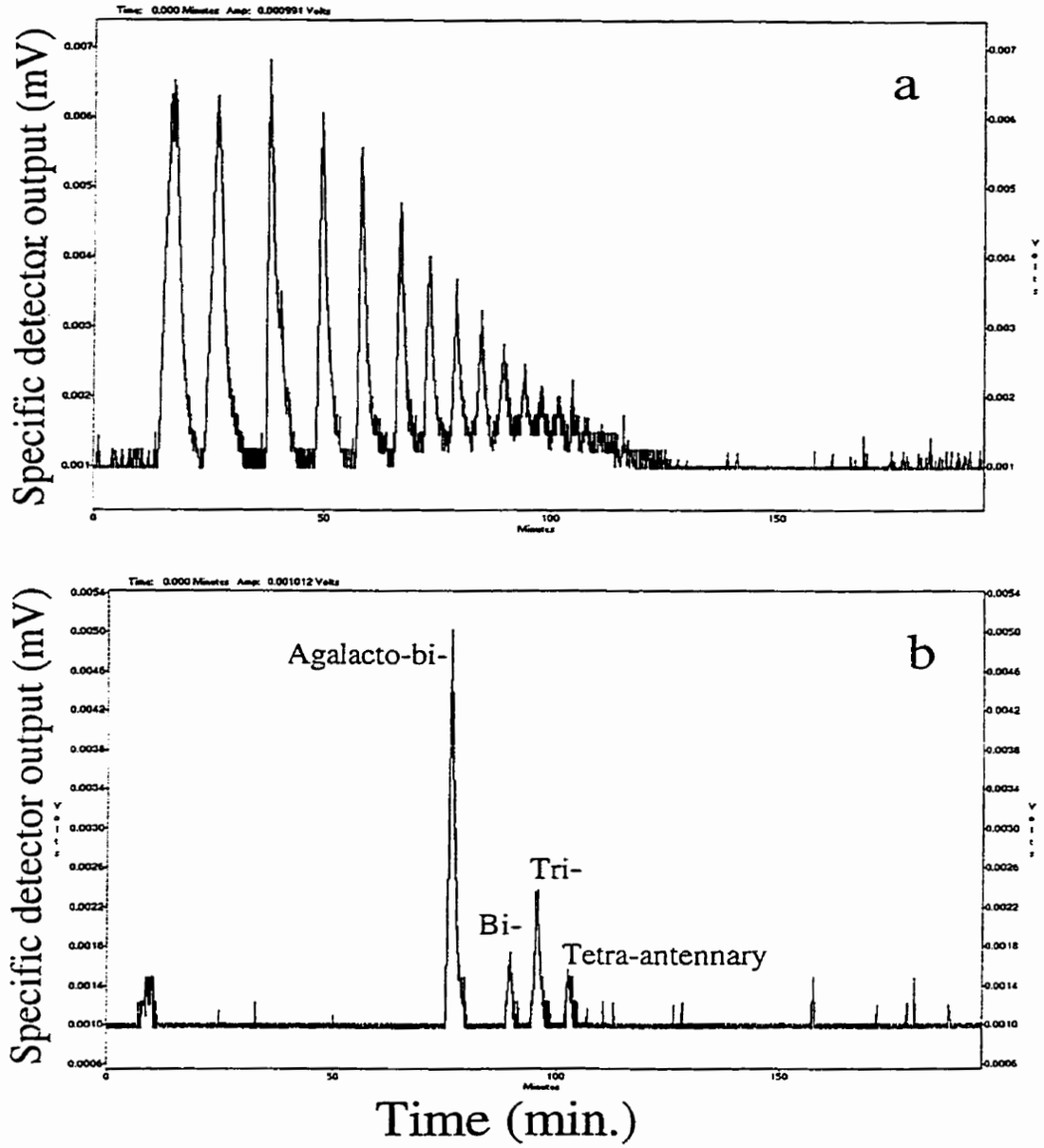


Fig. 2.3 Analysis of 2-AB derivatized standards by normal phase column using HPLC. Glucose ladder (a) and asialo-oligosaccharide standards (b) were labeled with 2-AB and analysed using normal phase column.

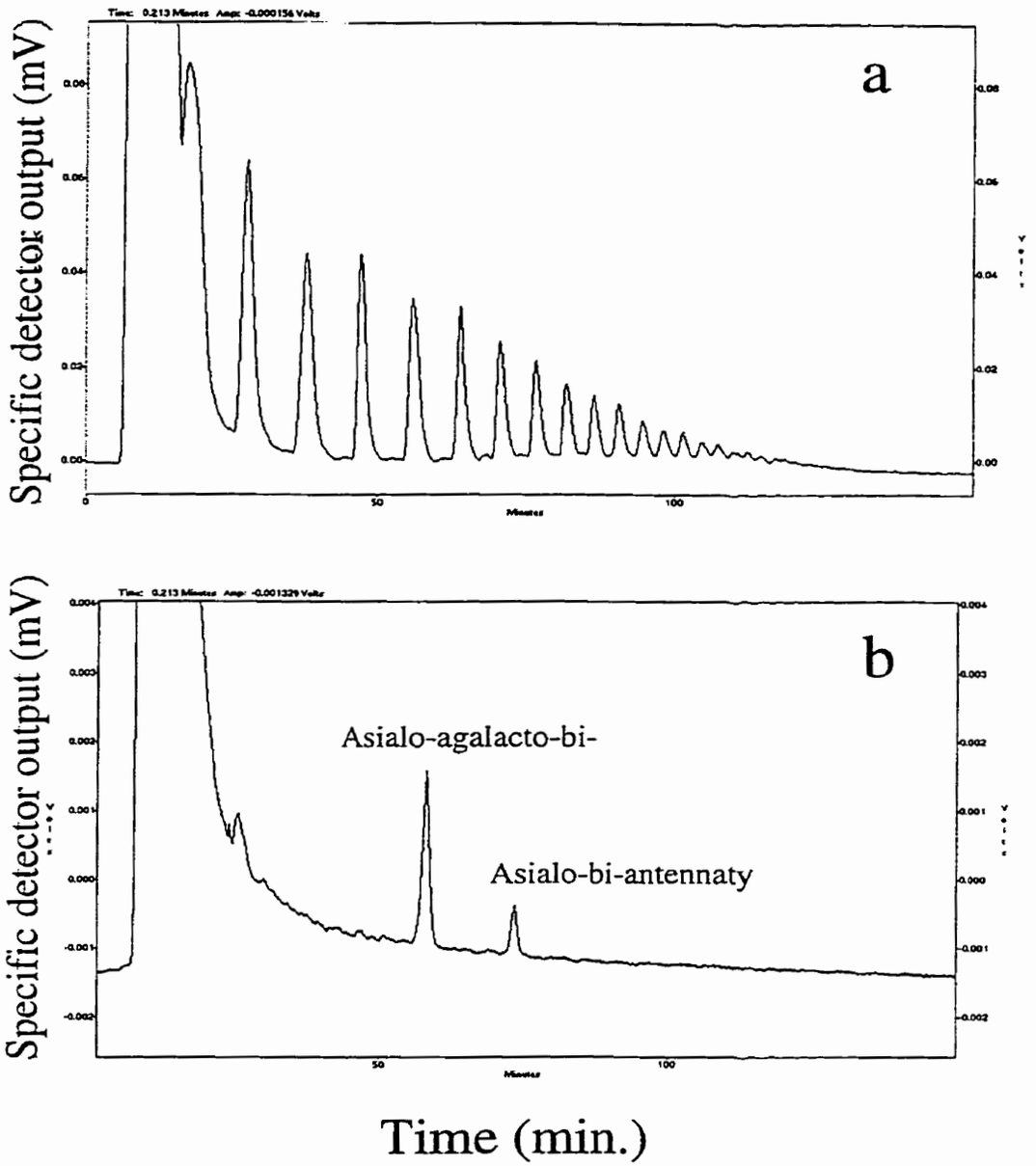


Fig. 2.4 Analysis of 4-AB derivatized standards by normal phase column using HPLC. Glucose ladder (a) and asialo-oligosaccharide standards (b) were labeled with 4-AB and analysed using normal phase column.

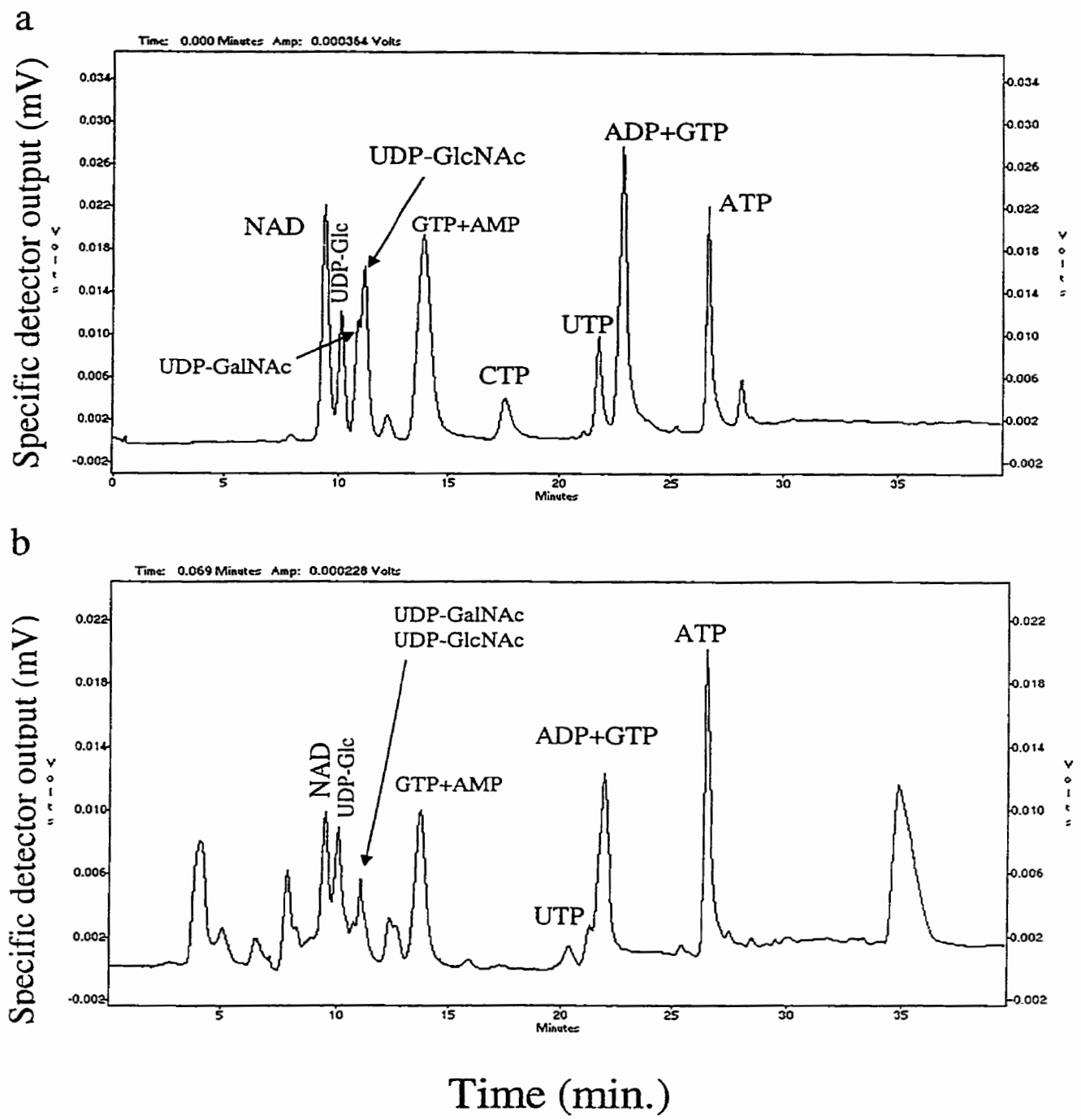


Fig. 2.5 Nucleotide analysis by a reverse phase column using HPLC. (a) Nucleotide standards; (b) Nucleotide extract from CHO cells. CHO-81 cells cultured in CHO-SFM2.1 were collected and the cell yields were determined on 4 day. The nucleotide was extracted and analyzed using reversed phase HPLC. The peaks were identified by comparing retention times with standard mixtures and by spiking samples with standards.

CHAPTER 3

EPO immuno-assay development and standardization

3.1 Introduction

It was a high priority to develop a routine fast detection assay suitable for multiple cell culture samples containing EPO. Two distinct bioassay methods, *in vivo* and *in vitro* systems, are available for EPO. The *in vivo* activity of EPO is measured by the incorporation of ^{59}Fe into erythroblasts of polycythaemic mice (Cotes and Bangham, 1961) or rats (Goldwasser and Gros, 1975). The *in vitro* activity of EPO is measured by the incorporation of ^{59}Fe into culture bone marrow cells or by the growth of EPO dependent cell lines (Goldwasser and Gros, 1975; Kitamura et al., 1989). However, bioassays are difficult, expensive, and require either animal or continuous culture of EPO responsive cells for optimization of their growth prior to assay. The significant disadvantage of *in vitro* bioassay is that it detected more activity for not fully glycosylated EPO. It has been reported that asialo-EPO (Goldwasser et. al.,. 1974), a less sialylated EPO (Goto et al., 1988) and a glycosidase-treated EPO (Dordal et al., 1985) showed similar or even higher activity by *in vitro* assay in spite of a lower activity by *in vivo* assay. The most important activity of EPO as a medicine is the *in vivo* activity. RIA is sensitive, precise, specific and reproducible. However, radioimmunoassay (RIA) for EPO is time-consuming, variable and associated with potential radiation hazard.

ELISA was first introduced by Engvall and Perlmann in 1971. This procedure has become an important method for quantifying antigen or antibody because of its high sensitivity. Therefore, we developed a specific, sensitive, and reproducible sandwich ELISA for detection of EPO production from culture supernatants. In this ELISA, EPO is captured by immobilized polyclonal anti-EPO and identified with mouse monoclonal anti-EPO and AP-conjugated anti-mouse IgG antibodies. The assay can be performed in a

single microtitre plate. The sole limitation of this assay, in common with all ELISAs, is that it does not directly assess biological activity.

In order to demonstrate that the CHO-81 EPO has a similar molecular weight and antibody binding capacity to a commercial EPO, the two EPO samples were compared by ELISA and Western blot analysis.

3.2 Results and discussion

3.2.1 Development of ELISA

A sandwich ELISA protocol was employed for detection of EPO in culture supernatant. When a rat monoclonal anti-huEPO antibody (ATCC BF-11) was coated onto microtiter plate, the sensitivity of detection was very low. In order to increase the sensitivity, a polyclonal rabbit anti-huEPO was tested because of the potential to bind more EPO through different binding sites. The concentration of coating antibody was titrated. As shown in Fig. 3.1, as the coating antibody concentration increased, the sensitivity of the ELISA increased. At 4 μg / ml polyclonal anti-EPO antibody gave a sensitive response without significantly increasing the non-specific binding. Optimal concentrations for monoclonal anti-EPO (1 μg / ml) and conjugated anti-mouse IgG antibodies were chosen by titration in a multi-well plate. The AP-conjugated rabbit anti-mouse IgG absorbed with rat serum showed higher binding compared to AP-conjugated goat anti-mouse IgG antibody.

To determine the reproducibility of the ELISA, an intraassay study in which twenty samples in triplicates (intra-well) and three different dilutions (intra-dilution) were tested. The results obtained from the calculation of the coefficients of variation (CV) between wells and dilutions are shown in Table 3.1. The interassay reproducibility was also determined. Four samples containing EPO at different concentrations were assayed on five different occasions (Table 3.2). The calculated CV were 8.1 –20.8 % for the five assays. This indicated that the EPO ELISA was reproducible between assays.

Most importantly, this assay is highly specific for EPO. The assay was tested for specificity by OD measurement from a series of samples. A commercial sample of recombinant human EPO, CHO-81 cell culture supernatant containing EPO, culture medium only (control) and CHO parent cell culture supernatant (control) were tested by ELISA. The OD values of medium and CHO parent cell culture supernatant were 0.12 and 0.17. Whereas, rHuEPO and culture supernatant containing EPO showed more than five times higher OD values than controls (Fig. 3.2).

3.2.2 Standardization of EPO

ELISA results between assays were standardized by a reference culture supernatant. This supernatant was standardized by a recombinant human EPO (R&D) to a defined value of 825 U/ml.

A standard commercial sample of EPO was also obtained from Amgen: 4,000 U/ml with a specific activity of 124.6 U / μg. The units are expressed as WHO units. However these units are different from those of R & D System. The R & D Systems unit = 1.2 WHO units as stated in the literature.

Samples of Amgen rHuEPO and a CHO-81 cell culture supernatant were serially diluted (X 2) and detected by ELISA. As seen in Fig. 3.3, CHO-81 EPO produced a response curve that was not significantly different to that obtained with Amgen EPO. The Amgen EPO concentration determined using the CHO-81 supernatant standard was 3,800 WHO U/ ml (=3,200 R&D U / ml) compared to the Amgen stated value of 4,000 WHO U / ml. The dose agreement of values validated the ELISA for routine detection of EPO.

The EPO concentration from CHO-81 culture supernatant was also determined using Amgen EPO as a standard. The calculated EPO concentration in the supernatant was 1,200 WHO U / ml and equates to 9.76 μg / ml (Amgen Std. 125 U = 1 μg). This compares with a value of 990 WHO U / ml for the EPO as standardized by the R &D

Systems EPO (= 825 R&D U / ml). This includes a percentage discrepancy of 9.6 % between standards and using this ELISA.

3.2.3 Variable response of ELISA in the determination of glycosylated and deglycosylated EPO

The ELISA developed for EPO was used to measure EPO concentration in culture supernatant. Moreover, the binding capacity of the monoclonal anti-EPO antibody to glycosylated and non-glycosylated EPO was determined.

A desalted culture supernatant was divided into three aliquots and treated separately. An untreated aliquot was compared with denatured and deglycosylated aliquots. The samples (100 µl) were denatured by boiling for 3 min. in the presence of 1 µl 10% SDS and 1 µl 2-ME. Deglycosylation was accomplished by incubation at 37 °C for 16 hours with PNGase F, sialidase and O-glycosidase. Each aliquot was tested by the ELISA. For each aliquot, a measured OD and the corresponding EPO concentration based on our previously standard curve are shown in Fig. 3.4. The observed OD value and apparent EPO concentration increased significantly after denaturation and deglycosylation. The result corresponded to the observations by Goto et al., (1989). They compared the antibody binding curves with glycosylated and deglycosylated EPO. The result showed that deglycosylated EPO had an increased binding affinity to two of the four monoclonal anti-EPO antibodies. They suggested that carbohydrates were near the peptide sequences recognized by these antibodies. Our results indicated that denaturation and deglycosylation could enhance the exposure of the antibody binding site (epitope) of the EPO molecule. This is an important observation because it means that denatured and / or non-glycosylated EPO has a greater response in this ELISA.

3.2.4 EPO detection by Western blot

In order develop a rapid method to detect EPO heterogeneity in the culture supernatant, the murine anti-EPO monoclonal antibody was used in Western blot analysis of

electrophoretically separated samples. Culture supernatant samples were concentrated (X 10). The resulting samples were subjected to SDS-PAGE separation. Fig. 3.5a (lane 1). illustrates an electrophoretogram of concentrated supernatant from a 4 day CHO-81 cell culture supernatant. Several protein bands in the culture supernatant are shown. However, it is difficult to determine EPO band. The purified non-glycosylated EPO produced from *Streptomyces* could be distinguished by Coomassie Brilliant blue. The molecular weight of the non-glycosylated EPO was 18 kDa as determined by the molecular weight maker. By Western blot analysis using monoclonal anti-EPO antibody, a single protein band corresponding to EPO with a determined molecular weight of 33-39 kDa was shown from the culture supernatant (Fig. 3.5b). The result indicated that the Western blot analysis is sensitive and specific.

EPO produced by CHO-81 cell was compared with commercial EPO from Amgen by SDS-PAGE and immunoblotting (Fig. 3.6a). EPO from both sources were concentrated 10 times by centrifugal filter units (Millipore). Since the sample from Amgen contains a high concentration of sugar, the buffer was changed to PBS. The Western blot analysis showed that, Amgen EPO has a molecular weight of 35-39 kDa, while the molecular weight of EPO produced by CHO-81 is 33-39 kDa (Table 3.3). The broader band of the CHO-81 EPO indicated greater heterogeneity. It is possible that the narrower range of molecular weight found in the Amgen sample is due to selective elution from an ion exchange column. This procedure can be performed in the commercial purification to reduce the proportion of EPO with a low degree of sialylation.

The N-linked glycans of the EPO samples were selectively removed by incubation with PNGase F and the resulting compounds were analyzed by electrophoresis (Fig. 3.6b). SDS-PAGE resulted in a protein band with molecular weight of about 19 kDa for the CHO-81 EPO whereas the Amgen EPO showed a predominant band with a higher molecular weight at 20 kDa. The deglycosylation step was specific for N-glycans. Therefore the differences between the two deglycosylated samples could be due either to a difference in the size of the O-linked glycan or to a difference in the size of the peptide.

3.3 Conclusion

A specific, sensitive, and reproducible assay was developed for the detection of EPO in culture supernatants. Deglycosylated and denatured EPO had a higher binding affinity to the antibody in this ELISA system. This suggested that the mAb binding epitope was either near the carbohydrates or partially buried in the interior of the native EPO molecule. Denaturation and deglycosylation could enhance the exposure of the antibody binding site (epitope) of the EPO molecule. The Western blot analysis using monoclonal anti-EPO antibody is very specific and sensitive. EPO produced by CHO-81 cells has a similar molecular weight and antibody binding property compared with commercial Amgen EPO.

Table 3.1 Coefficients of variation (%) in ELISA for the measurement of EPO

Sample numbers (N)	Intra-wells Mean ± SEM	Intra-dilutions Mean ± SEM
20	6.3 ± 1.6	21.4 ± 2.3

Twenty samples containing different concentrations of EPO were assayed with ELISA. The mean ± SEM is the mean of the twenty CV from triplicate wells and dilutions of each sample.

Table 3.2 Interassay variance of EPO ELISA.

Samples	No of runs	Mean EPO (U/ml)	± SEM	CV (%)
1	5	280.54	21.0	16.7
2	5	155.74	14.5	20.8
3	5	527.8	19.2	8.1
4	5	438.8	19.0	8.8

Four samples containing EPO at different concentrations were assayed on five different experiments with ELISA. The mean is the mean of EPO concentrations determined on five different occasions. Triplicate dilutions of each sample were done on each assay.

Table 3.3 Comparison of Amgen EPO and CHO-81 EPO by ELISA and Western blot.

	Concentration (U/ml)	ELISA determined concentration (U/ml)	Molecular weight ($X10^3$ kDa) Glycosylated	N-linked de-glycosylated
Amgen EPO	4000	3800 (using a culture supernatant std.)	35-39	20
EPO (CHO-81)	990	1200 (using Amgen EPO as a std.)	33-39	19

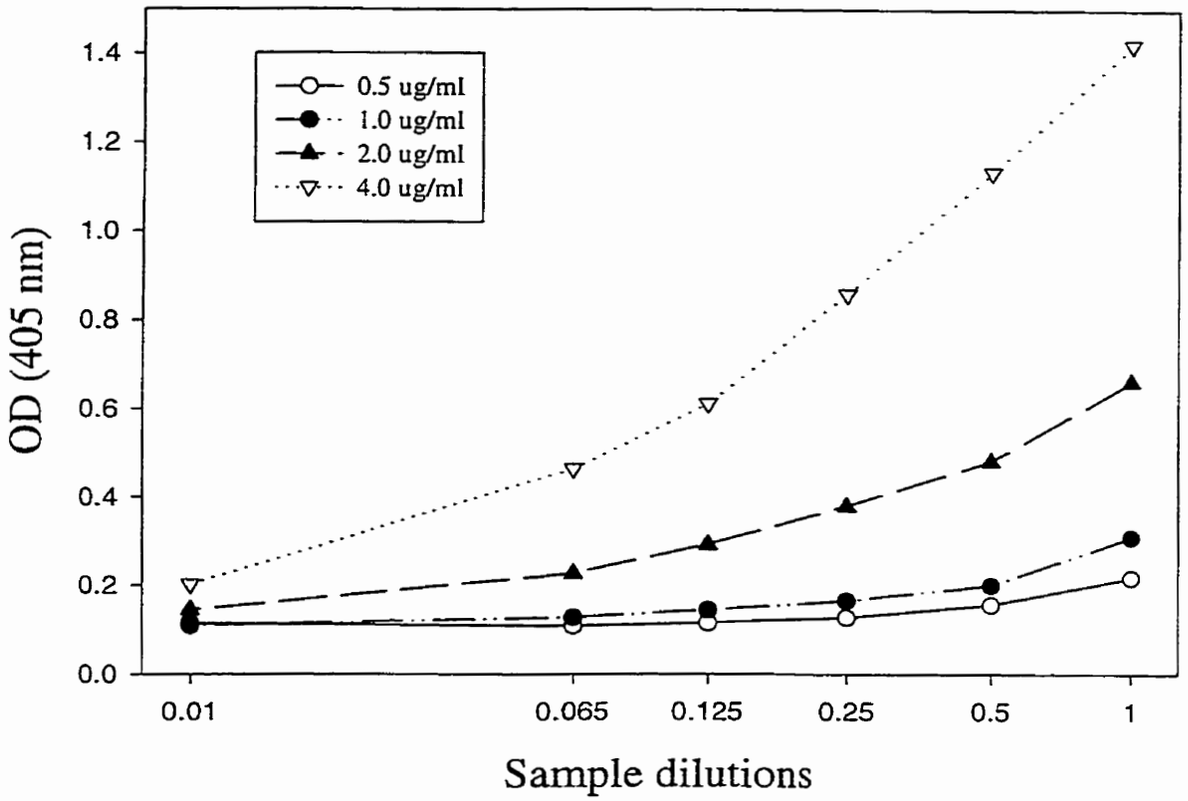


Fig. 3.1 Titration of coating antibody concentration. Polyclonal anti-EPO antibody was coated onto microtiter plate at different concentrations. A culture supernatant from CHO-81 cells was tested by the ELISA procedure.

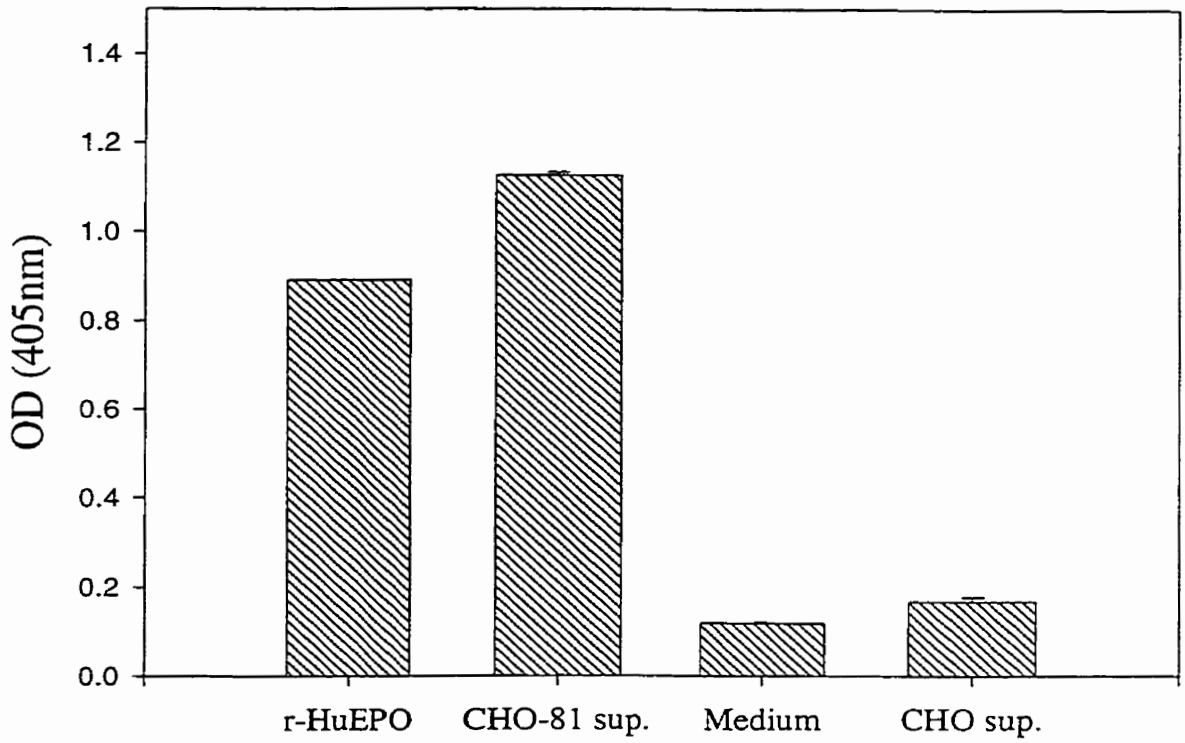


Fig. 3.2 Specificity of EPO ELISA. A recombinant human EPO (200 U/ml, R & D), culture supernatant from CHO-81 cells, CHO-SFM2.1 (control) and CHO culture supernatant (control) were tested by EPO ELISA. rHuEPO and culture supernatant containing EPO showed more than five times higher OD values than controls

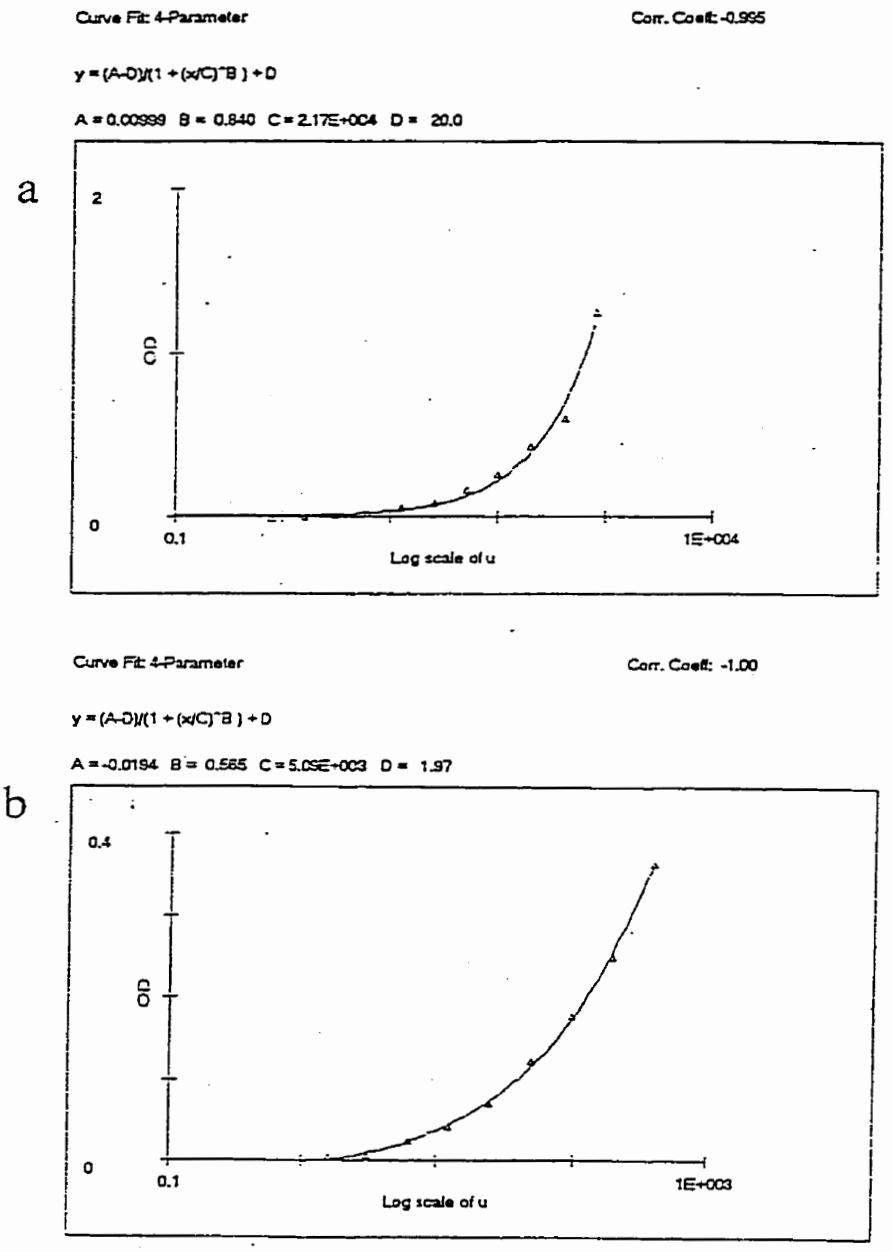


Fig. 3.3 ELISA standard curves of EPO produced by CHO-81 cells and Amgen EPO. A standardized culture supernatant (825 U/ml) (a) and EPO from Amgen (400 U/ml) (10 times dilution of the original) (b) were analyzed by ELISA.

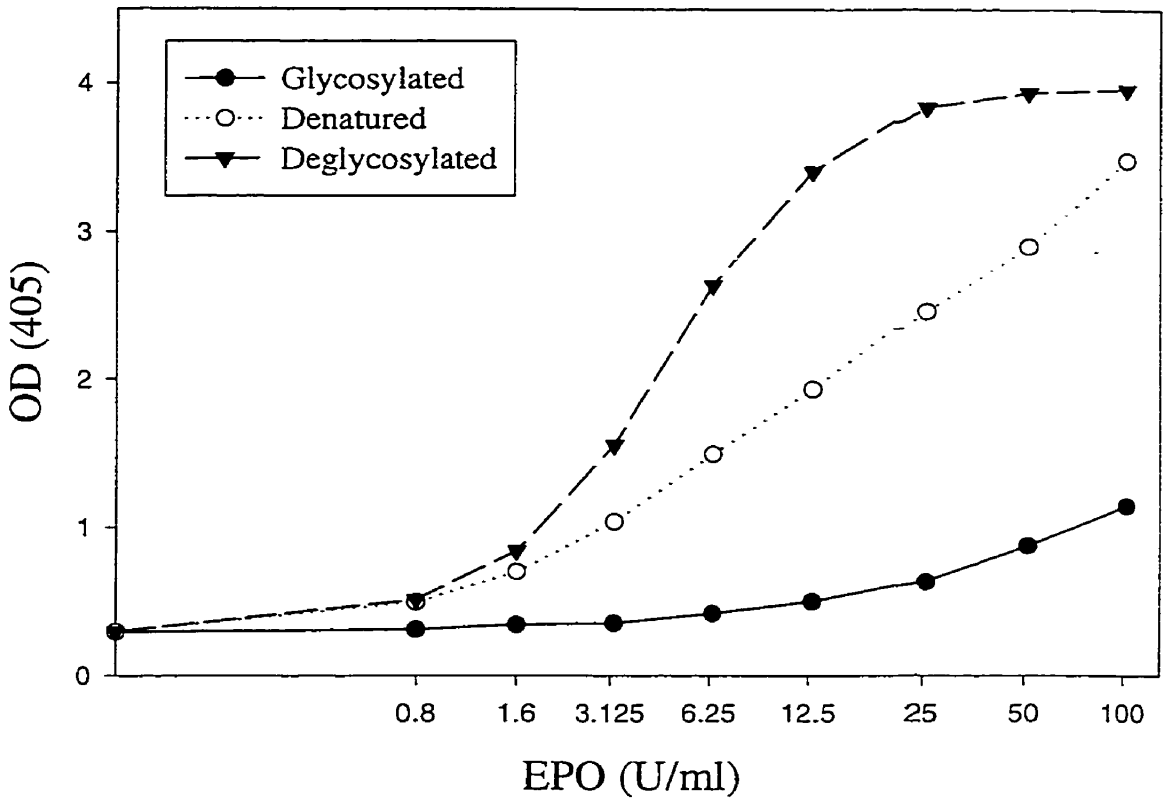


Fig. 3.4 Analysis denatured, deglycosylated and glycosylated EPO by ELISA. A culture supernatant was divided into three aliquots and treated separately. An untreated (glycosylated), denatured and deglycosylated aliquots were tested by the ELISA. The observed OD value and apparent EPO concentration increased significantly after denaturation and deglycosylation.

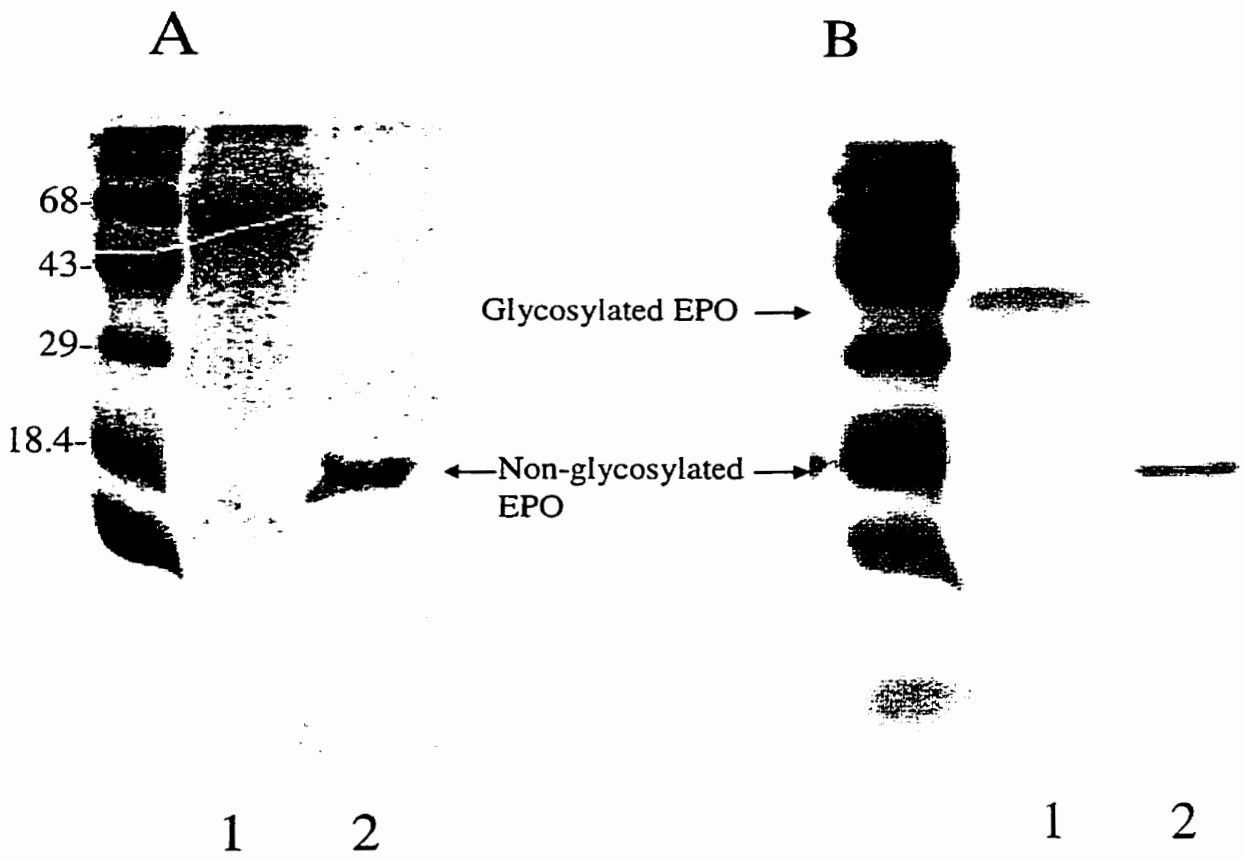


Fig. 3.5 SDS-PAGE and Western biot analysis of glycosylated and non-glycosylated EPO. EPO produced by CHO cells and Streptomyces cells were separated by 14 % SDS-PAGE and stained with Coomassie Brilliant blue (a). The separated protein was transferred onto a NC membrane which was detected by mouse monoclonal anti-EPO antibody (b). Lane 1 is glycosylated EPO in culture supernatant produced by CHO cells. Lane 2 is purified non-glycosylated EPO sample produced by *Streptomyces* cells. Protein molecular weight maker as indicated ($X10^3$ kDa).

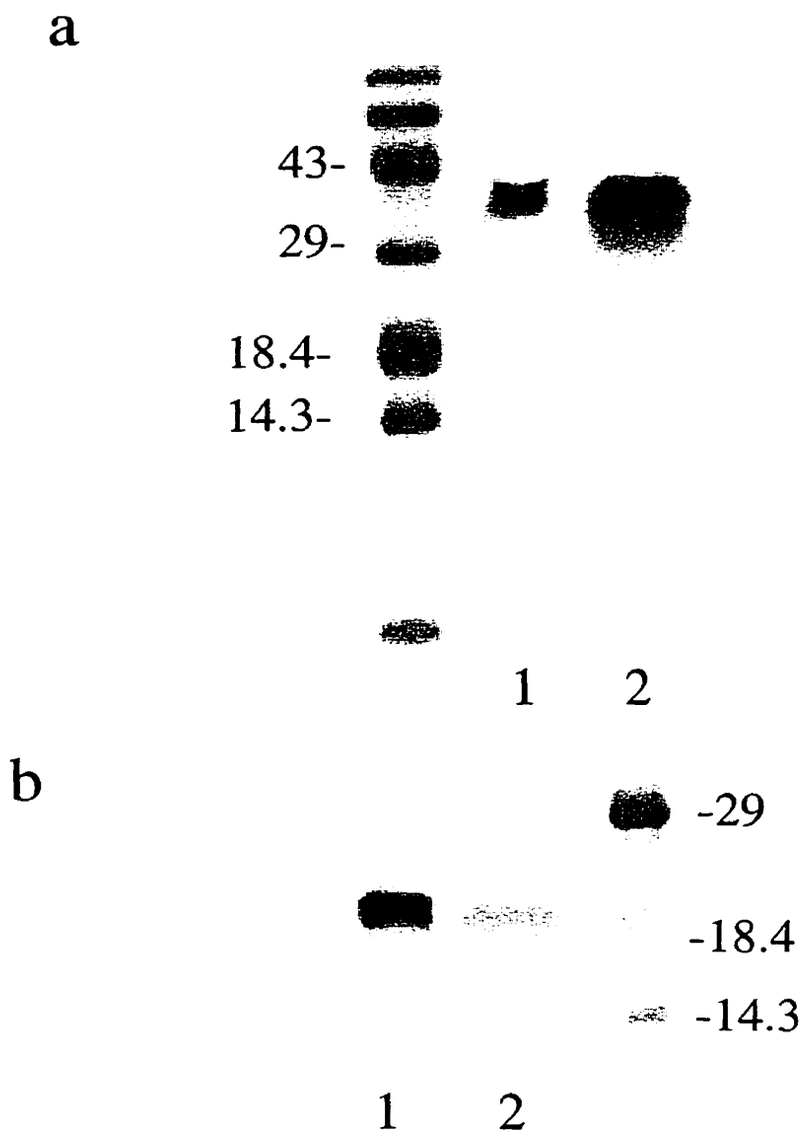


Fig. 3.6 Western blot analysis of EPO. EPO from Amgen and CHO-81 culture supernatant were separated by 14% SDS-PAGE. The protein was transferred to NC membrane and detected by mouse monoclonal anti-EPO antibody. (a) Glycosylated EPO. (b) EPO without N-linked oligosaccharides. Lane 1, Amgen EPO. Lane 2, CHO-81 culture supernatant. Protein molecular weight maker as indicated ($X10^3$ kDa).

CHAPTER 4

EPO Purification from CHO cell culture supernatant

4.1 Introduction

A rapid and efficient preparative separation procedure suitable for recovery of EPO from culture supernatant was required. It was essential to develop a procedure to avoid conditions that modified protein physicochemical properties. It has been reported that exposure to alkali condition can cause cleavage of O-glycosidic bonds between protein and carbohydrate in glycoproteins. Acidic conditions can lead to hydrolysis of susceptible glycosidic linkages between sugars, especially those involving sialic acid (Beeley, 1985). EPO has been purified from urine of aplastic anemic patients by affinity chromatography using a monoclonal antibody (Miyake et. al., 1977; Yanagawa et. al., 1984).

This chapter describes the development of a simple purification procedure with a relative high yield and purity of EPO using a monoclonal anti-human EPO antibody. Adequate amounts of pure EPO were obtained by the immunoaffinity chromatography. These were used for further studies on EPO carbohydrate analysis.

4.2 Results and discussion

4.2.1 Selection of elution buffer for affinity chromatography

The starting material for purification of EPO was CHO-81 cell culture supernatant from batch culture of CHO cells grown in CHO-SFM2.1. For the first step in purification, cell culture supernatant was filtered and loaded onto an immunoaffinity column consisting of purified monoclonal anti-EPO antibody bound on to the Affi-prep 10 matrix. To eliminate non-specific adsorption caused by weak hydrophobic or ionic interactions, the column was washed extensively with PBS followed by the second washing buffer which contained high concentration of salt prior to elution of the retained EPO.

The criterion for the most suitable elution buffer is one that dissociates antigenic EPO from the column but which minimizes bleeding of the bound antibody. Of the various elution buffers tested were those at low pH, high pH, protein denaturants and chaotropic agent are shown in Table. 4.1.

Initially, a generally used elution buffer, 0.2 M acetate buffer pH 2.5 was tested for dissociation the bound EPO on the column. Analysis of the eluted sample by SDS-PAGE indicated a single protein band with an estimated molecular weight of 33-39 kDa as determined from standards. However, Western blot analysis using monoclonal anti-EPO antibody failed to reveal any bands on the NC membrane. It is possible that low pH elution buffer caused damage of the EPO antigenic determinants recognized by the antibody.

The protein denaturant, guanidine hydrochloride (GuHCl) was tested to elute EPO from the affinity column. EPO eluted with 6 M GuHCl from the immunadsorbant column was recognized as a main band corresponding to EPO molecule weight on SDS-PAGE and Western blot (Fig. 4.1, lane 1). However, some contaminant protein bands were present and the determined molecular weights were 52 kDa and 26 kDa which correspond to antibody heavy and light chains. To confirm this, the same sample was separated by SDS-PAGE and transferred to NC membrane. The membrane was directly incubated with the second antibody, goat anti-mouse IgG-AP conjugated, but omitted the first incubation step with anti-EPO antibody. The result showed identical high and low molecule weight bands from both membranes which indicated that the presence of the heavy and light chains of the mouse anti-EPO antibody (Fig. 4.1). This suggested that the bound anti-EPO antibody in the column was co-eluted with EPO.

The relatively effective elution protocol involved the use of 0.1 M acetate buffer pH 4.0 containing 0.15 M NaCl. The result demonstrated that the physicochemical and chemical properties of EPO eluted with this buffer were not altered. EPO purified by the immunoadsorbent column was recognized as a major band with M.W. 33-39 kDa on SDS-PAGE. The EPO was identified by anti-EPO antibody using Western blot. A very

small amount of the antibody was co-eluted with EPO compared with the EPO eluted by protein denaturants.

The elution buffer, 3 M KSCN / 20 mM Tris / 3 mM EDTA, pH 7.0 resulted in significantly higher quantities of EPO (X 1.5) (Fig. 4.2). A comparison of various dissociating agents showed that this elution buffer was the most effective desorbent for EPO bound to sepharose based immunosorbers. It has been reported that the immunosorbers treated with 3M sodium thiocyanate appear to retain their binding capacity to a great degree with repeated usage than immunosorbers desorbed with other buffers (Beeley, 1985). The dissociating capacity of 3 M KSCN was greater than other elution buffers. This method was adopted for subsequent of elution EPO from the immunoaffinity column.

4.2.2 Gel filtration chromatography

Analysis of the concentrated and purified EPO eluted by 3 M KSCN / 20 mM Tris / 3 mM EDTA, using SDS-PAGE and Western blot showed some high molecular weight protein band (80-90 kDa) (Fig. 4.2). A further purification step with a Sephadex G-100 column was used in order to ensure separation from any contaminated protein which co-eluted with EPO from the immunoaffinity column. Analysis of the 40 (0.5 ml) fractions eluted with the gel filtration column indicated one major protein peak. It was found that EPO presented in this peak as measured by ELISA. Comparison with the column calibration showed that the EPO peak corresponded to molecular weight of 33-39 kDa (Fig. 4.3). Two peak fractions #20 and #21 were analyzed by SDS-PAGE and detected by silver stain. The result showed a protein band corresponding to EPO at 33-39 kDa but a high molecular weight band was also observed at around 80-90. The gel filtration step should be sufficient to remove any protein contaminant at this molecular weight. It is possible that the higher molecular weight band is a dimer of EPO arising from the purification procedure. Sasaki et. al., (1987) observed the similar result during EPO purification. It was reported that EPO dimers or trimers occurred using heterobifunctional crosslinking reagents exhibited a markedly enhanced plasma survival *in vivo* (Sytkowski

et al., 1998). It was concluded that the gel filtration step is not necessary for EPO purification.

4.2.3 EPO recovery

A sandwich ELISA was used to determine EPO concentrations in the samples before, after loading and eluant from the immuno-affinity column. As shown in Table 4.2, 70% of EPO were eluted from the column. 30% of EPO did not bind to the column and remained in the supernatant. The EPO binding capacity was not increased by repeated loading to the column. It is possible that the remaining EPO is because of either over loading or epitope blockage by attached oligosaccharides.

In order to increase column binding capacity, SDS-treated EPO was applied to an immunoaffinity column (Yanagawa et al, 1984). In this case, the antibody can bind directly any epitope that may be buried in the interior of the native EPO molecule. A concentrated culture supernatant (10 X) was boiled for 3 min after the addition of solid SDS (final concentration 2 %) and applied onto a 1ml column in which monoclonal anti-EPO was fixed on Affi-Prep 10. After extensively washing, the bound EPO was eluted with 6M GuHCl. Although the EPO binding capacity increased 5 % compared with untreated EPO, this method is undesirable because of the need to denature the EPO by SDS prior to purification and require protein denaturants to dissociate the bound EPO.

4.2.4 Selection of a solution to dissolve the purified EPO

EPO is a hydrophobic molecule and has low solubility. The purified EPO in the absence of other protein tends to form aggregates. Some glycoforms of EPO may be lost from these aggregates and change their composition. Hence a protocol is needed to dissolve the purified EPO.

EPO was dialyzed against several different solutions and the effects on EPO solubility are shown in Table 4.3. In 10 mM ammonium acetate pH 4, EPO appeared insoluble even

in the diluted form. This pH was close to the isoelectric point of EPO and increased the possibility of protein aggregation. In 10 mM ammonium acetate pH 7.2, EPO was maintained in solution at low concentration but precipitated when it was concentrated. The best result was obtained with PBS (0.1 X normal concentration) containing 0.1% Tween 20 with the maintenance of EPO solubility even after concentrating. This suggests that because of the high polarity of the EPO molecule, a hydrophobic environment at neutral pH is necessary to maintain the solubility of the molecule.

4.2.5 Effect of concentration

After the dissociation from immunoaffinity column, the EPO was concentrated in order to characterize its properties and purity. Unfortunately the methods widely used for protein concentration led to the formation of EPO molecular aggregates with a low solubility. Re-constitution of dried EPO by either lyophilization or speed vacuum resulted in precipitates in the samples and this became unacceptable for further analysis. The purified EPO concentrated using a Centrifugal Filter Device gave the best outcome. This avoids the complete drying of the sample which tends to produce EPO aggregates and is difficult to re-dissolve.

4.3 Conclusion

Human EPO was isolated from culture supernatant in a relatively high yield with a simple purification procedure using an immunoaffinity column adsorbent with monoclonal anti-EPO antibody. One of the advantages of the immunoaffinity purification is that the sample does not need pre-treatment, e.g. concentration or changing buffer. Among various dissociating agents tested, 3 M KSCN / 20 mM Tris / 3 mM EDTA, pH 7.0 was found to be the most effective for eluting EPO from an immunoaffinity column. About 70 % of EPO were recovered as determined by a sandwich ELISA. Analysis of the eluant from the column showed a major protein band which corresponded to EPO. A high molecule weight band which was identified as an EPO dimer. The SDS-treated EPO in culture supernatant increased 5 % binding capacity. However a strong elution buffer, like GuHCl was needed to dissociate the tightly bound EPO from the column which increased

the possibility of dissociation of antibody coupled to the matrix. Neutral pH buffer containing detergent was required to dissolve the purified EPO.

The disadvantage of using an immunoaffinity column for purifying EPO is that some of the highly glycosylated EPO may not bind to the column, whereas low or non-glycosylated EPO may have high binding capacity to the antibody which have lower or non-activity *in vivo*.

Table 4.1. The effect of different buffers for elution of EPO from an immunoaffinity column.

Method	Elution buffer	Contents of elution buffer	Results
1	Low pH	0.2 M Acetate/0.15 M NaCl pH 2.5	EPO eluted from the column could not be detected by Western blot. Antibody might not recognize a degraded EPO.
2	High pH	0.1 M Triethylamine pH 11.5	EPO and a lot of non-specific proteins presented on SDS-PAGE.
3	Protein denaturants	3 M and 6 M GuHCl	EPO and coupled anti-EPO antibody were co-eluted from the column.
4	Relatively low pH + high salt	0.1 M Acetate / 1 M KCl pH 4.0	EPO could not be detected by SDS-PAGE. It is possible that EPO was washed out during the washing with 1 M KCl.
5	Relatively low pH	0.1 M Acetate/ 0.15 M NaCl pH 4.0	EPO was recognized as a major band on SDS-PAGE. A limited amount of antibody co-eluted from the column.
6.	Chaotropic agent	3 M KSCN/ 20 mM Tris/ 3 mM EDTA, pH 7.0	More EPO was eluted from the column compared with other elution buffers. There was no detectable antibody co-eluted from the column.

Concentrated culture supernatant (X10) was loaded onto an immunoadsorbant column (1 ml bed volume) containing anti-human EPO. After extensive washing, the bound EPO was dissociated from the column by different elution buffers. The washing buffers were (a) phosphate buffered saline (50 bed volume) and (b) 10 mM sodium phosphate + 0.5 M NaCl pH 7.4 (8 bed Volume) except in method 4 where the washing buffer was 0.1 M phosphate buffered saline + 1 M KCl pH 7.5. The eluted EPO was analyzed by SDS-PAGE and detected by silver stain and Western blot.

Table 4.2 EPO recovery from immunoaffinity column

Samples	Total EPO (units)	Percentage of total EPO (%)
Before loading (culture supernatant)	196000	100
After loading (flow through)	60000	30
Eluant	141306	70

1 liter supernatant was loaded onto a 10 ml Affi-prep column containing monoclonal anti-EPO antibody. EPO was eluted with 3 M KSCN / 20 mM Tris / 3 mM EDTA, pH 7.0 buffer. EPO concentrations in the samples before, after loading and eluted from the column were determined by ELISA.

Table 4.3 The effect of different buffers for dissolving EPO

Solution	Before concentration	After concentration
Distilled water	Dissolved	Aggregated
10 mM Ammonium acetate pH 4	Aggregated	
10 mM Ammonium acetate pH 7.2	Dissolved	Aggregated
PBS (0.1X)	Dissolved	Aggregated
PBS (0.1 X) 0.1% tween 20	Dissolved	Dissolved

EPO was purified by immunoaffinity column. The eluant was dialyzed against different solutions for 24 hours. The dialyzed samples were concentrated by Centrifugal Filter Device (Millipore).

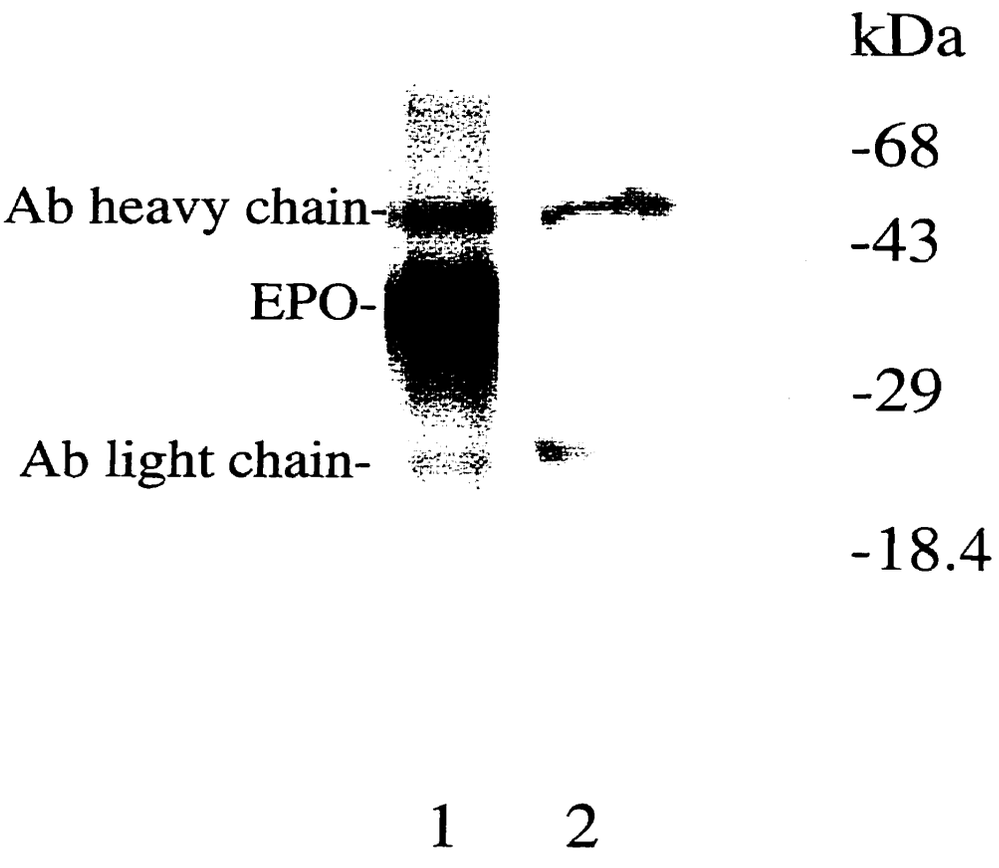


Fig. 4.1 Analysis of purified EPO by SDS-PAGE and Western blot. EPO eluted with 6M GuHCl was concentrated and analyzed by 14% SDS-PAGE. The protein was transferred to NC membrane. Lane 1. Protein was detected with anti-EPO antibody followed by a goat anti-mouse IgG-AP. Lane 2. Protein was detected by a goat anti-mouse IgG-AP. A pre-stained protein molecular weight marker was used and the molecule weights as indicated (X 10^3 kDa).

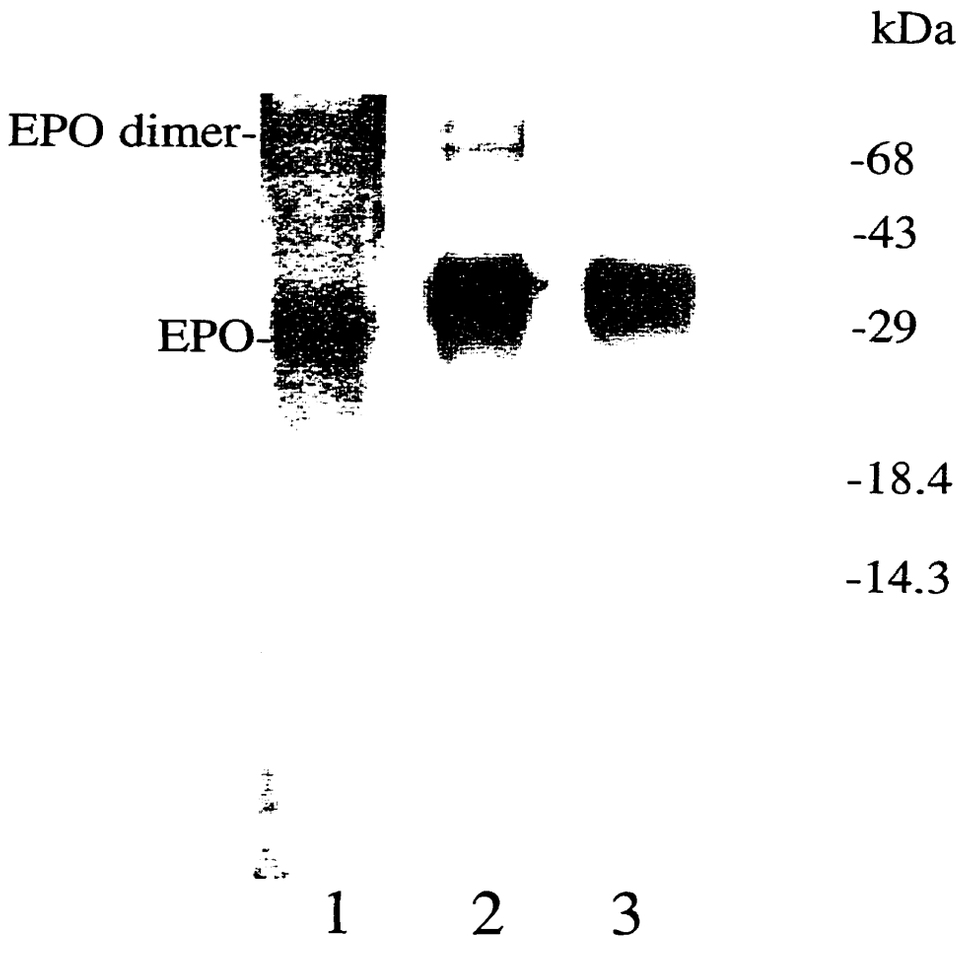


Fig. 4.2 EPO analysis by SDS-PAGE and Western blot. The bound EPO in the immunoaffinity column was eluted with 3 M KSCN / 20 mM Tris / 3 mM EDTA, pH 7.0, concentrated and analyzed by 14% SDS-PAGE. Lane 1. Purified EPO detected by silver stain. Purified EPO (lane 2) and 10 X concentrated culture supernatant containing EPO (lane 3) were analyzed by Western blot. A pre-stained protein molecular weight marker was used and the molecular weights as indicated (X 10^3 kDa).

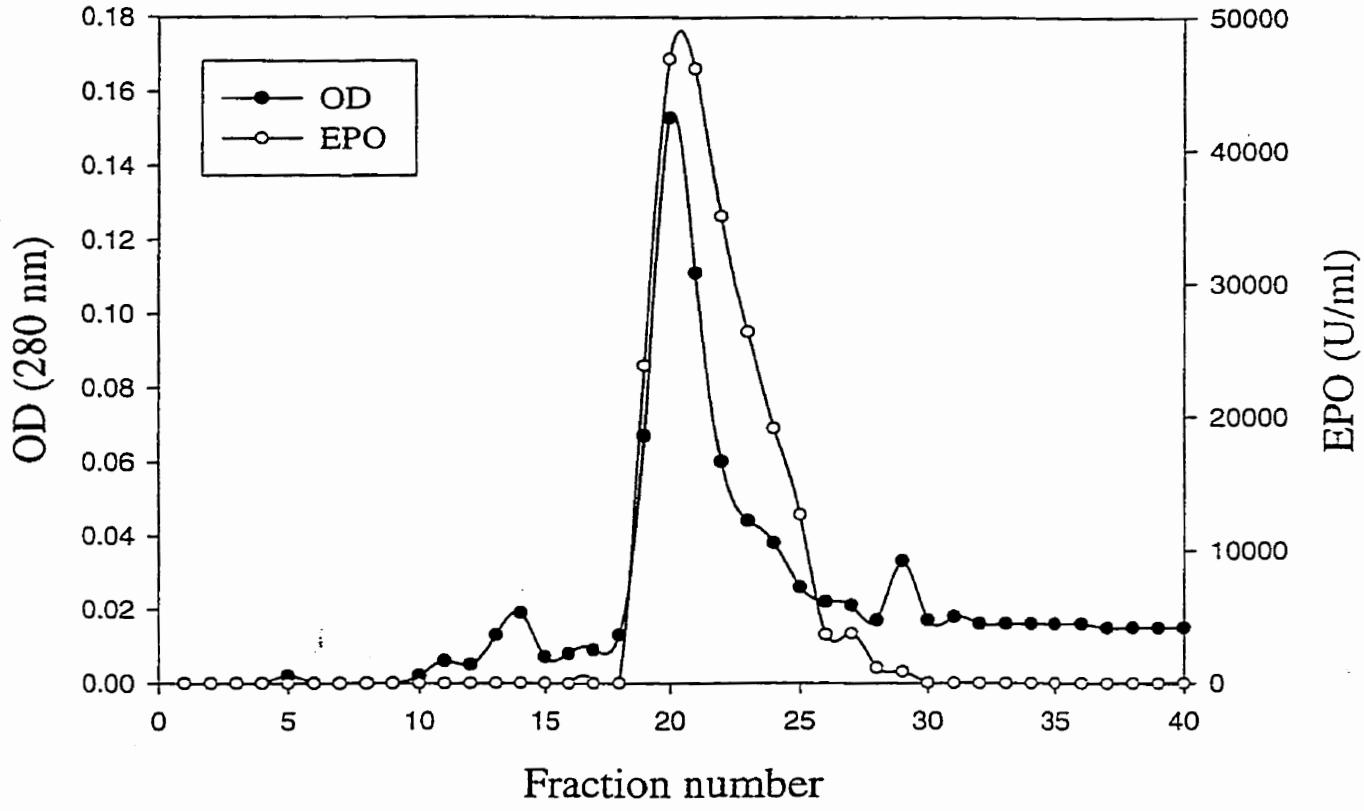


Fig. 4.3 Sephadex G-100 chromatography of EPO fraction purified with the immunoaffinity column. The EPO fraction eluted from the immunoaffinity column was dialyzed against PBS and dried. The dried material was dissolved in 250 µl PBS and centrifuge. The clear solution was loaded on a Sephadex G-100 column. The volume of each fraction was 0.5 ml. The absorbance at 280 nm and EPO concentration were determined by a spectrophotometer and ELISA.

CHAPTER 5

Effects of ammonia on CHO cell growth, metabolism, EPO production and heterogeneity*

5.1 Introduction

Recombinant protein production processes depend on high cell yield, consistent productivity and product quality. However, it is clear that the culture parameters that influence these processes are not well understood, particularly with respect to protein glycosylation (Gawlitzek et al., 1995; Goochee et al., 1991).

It is widely recognized that one of the most important inhibitory substances accumulating in cell cultures is ammonia (NH_3 or NH_4^+). Many different effects of elevated concentrations of ammonia on mammalian cell cultures have been reported. These include the cessation of cell growth (Butler et al., 1983; Butler and Spier, 1984; McQueen and Bailey, 1990a; 1990b; 1991; Kurano et al., 1990; Ryll et al., 1994), a decline in productivity (Hansen and Emborg, 1994), the inhibition of virus proliferation in cells (Farias et al., 1988; Koyama and Uchida, 1989) and specific alterations of protein glycosylation (Andersen and Goochee, 1995; Borys et al., 1994; Thorens and Vassalli 1986).

Studies of glycoprotein hormones have demonstrated widely varying functions for the carbohydrate chains. The carbohydrate chains of EPO may affect its conformation leading to changes in biosynthesis, secretion (Dubé et al., 1988), solubility, susceptibility to protease and other denaturing conditions (Goldwasser et al., 1974; Narhi et al., 1991; Uchida et al., 1997). Asialo-EPO has limited therapeutic value because it is rapidly accumulated in the liver as a result of specific binding to a lectin (Fukuda et al., 1989; Spivak and Hogans, 1989).

* The contents of this chapter were included in a paper: M. Yang and M.Butler. 2000. Effects of ammonia on CHO cell growth, erythropoietin production and glycosylation. Biotechnol. Bioeng. 68: 370-380.

The degree of sialylation of the oligosaccharides from 4 batches of rHuEPO from transfected CHO cells was determined by Rice et al., (1992) to be 80-88%. However, they did not report on the characteristics of the cell culture from which the EPO was purified nor did they explore the culture parameters that could give rise to variability in the degree of sialylation. A small amount of asialo-glycoprotein was reported by Gu et al., (1997) under normal culture conditions of CHO cells and was attributed to incomplete intracellular sialylation. However, the parameters that cause low or incomplete sialylation are not well understood.

An understanding of the culture conditions that can affect cell growth and protein glycosylation is important for the development of an effective production process for a therapeutic glycoprotein. Since EPO contains both N- and O-linked glycans, it is an ideal glycoprotein for studying the control of glycosylation. In this chapter, the effect of the accumulation of ammonia on the cell growth, metabolism, secretion and pattern of protein glycosylation was studied in cultures of a CHO cell line transfected to secrete a recombinant human EPO.

5.2 Results

5.2.1 Effect of ammonia on CHO cell growth and EPO production

The effects of added ammonium chloride (NH_4Cl) on CHO cell growth and EPO production were examined (Figs. 5.1-3). CHO cells were inoculated into 7 ml medium with the addition of NH_4Cl, NaCl or chloroquine as indicated. A small culture volume (7 ml) was chosen for experiments, since it was found that the experimental results were more consistent and reproducible with 25 cm^2 T-flask than the large T-flask. Viable cell yields were determined after 4 days growth by trypan blue exclusion. EPO in the culture supernatants were deglycosylated by PNGase F and the concentrations were determined by ELISA. All cultures were initiated at an inoculum of $2x10^5$ cells/ml and a culture pH of 7.25 which decreased gradually over 4 days of culture. There was no significant observable difference in the pH

of the cultures treated with NH_4Cl.

As shown in Fig. 5.1a, cell yields decreased at higher NH_4Cl concentrations. The cell yield was reduced by 25 % at 20 mM NH_4Cl and 56% at 40 mM NH_4Cl. The inhibitory concentration for a 50% decrease in growth (IC-50) was estimated in this system at 33 mM NH_4Cl. In order to determine the effect of increased osmolality, control cultures were established in media containing added NaCl up to 40 mM (Fig. 5.1b). Although there was apparently no significant effect of the addition of 20 mM NaCl, the cell yield was significantly reduced by 30% in the presence of an added 40 mM NaCl. However, this decrease was considerably less than that observed in the equivalent concentration of NH_4Cl, which indicated that the effect of NH_4Cl on cell growth was not due to osmolality alone. The specific production of EPO in the presence of added NaCl did not change significantly (data not shown).

For time course experiments, a series of parallel flasks were set up for control cultures and cultures containing 10 mM NH_4Cl. Every 24 hours cell numbers were determined following trypsinization. After counting, cells were discarded and supernatants were stored until analysis. The time course of cell growth and EPO production is shown in Fig. 5.2 for a control culture and one containing 10 mM NH_4Cl. There was a gradual increase in viable cell concentration up to a maximum at day 4 in both the control and ammonia-supplemented cultures, after which time the cell concentration decreased. The total and viable cell yields were significantly lower (19 %) in the culture containing 10 mM NH_4Cl compared to the control ($p = 0.036$ and 0.035 for total and viable cells respectively). Measurement of EPO in the culture supernatant shows that the extracellular concentration increases gradually up to day 5 of culture. The yield of EPO was significantly higher in the control culture than in the ammonium-supplemented culture ($p = 0.045$) (Fig. 5.2b).

The final yield of EPO was compared between cultures containing different concentrations of added NH_4Cl (Fig. 5.3). The EPO concentration was higher in the control culture compared to the ammonium-supplemented cultures. However, the specific EPO productions

calculated in over 10 mM ammonium containing cultures were higher compared to control culture. The EPO specific productivity was slightly lower when the ammonium concentrations were 2.5–5 mM.

5.2.2 Cell metabolism

Table 5.1 shows that the specific rates of glucose and glutamine utilization as well as the specific rate of lactate production increased progressively as the added NH_4Cl concentration increased up to 40 mM. The fourth column of data in Table 5.1 shows that there was no equivalent increase for the addition of 10 mM NaCl to the cultures. Although the specific lactate production increased in the presence of ammonia, the measured lactate concentration generated in each culture was not significantly different. The ammonium concentrations were examined in the culture supernatants from day 1 to day 5. The ammonium level increased to about 4 mM in the control culture on day 5. The concentration of ammonium in the culture with added NH_4Cl (30 mM) was consistently high and reached a level at day 3 of 37 mM (Fig. 5.4).

The amino acid content of the medium was analyzed by HPLC during the growth phase (day 0-4) of control and 10 mM NH_4Cl-supplemented cultures (Table 5.1). The effect of the added NH_4Cl was to alter significantly the pattern of utilization and production of the amino acids analyzed. There was an increased rate of production of 3 amino acids (glu, ala and gly). A summation of the amino acid data showed that for the 18 amino acids analyzed there was a net consumption of 3.82 $\mu mol/10^6$ cell-day in the control culture compared with a net production rate of 1.46 $\mu mol/10^6$ cell-day in the NH_4Cl-supplemented culture. A major contribution to this net change was the two fold increase in the production rate of alanine and glutamate.

5.2.3 The effect of ammonia on EPO heterogeneity

The effect of NH_4Cl on the heterogeneity of EPO produced by CHO cells was examined by

SDS-PAGE and immunoblotting of samples from the culture supernatant (Fig. 5.5). The data showed that, as the NH_4Cl concentration increased from 5mM to 40mM, there was an increase in the width of the EPO band which suggested a greater heterogeneity of glycoforms at higher levels of NH_4Cl. The molecular weight of EPO from the control culture was 33-39 kDa (lane 1). The sample analyzed from the culture containing 40 mM NH_4Cl had a much broader band which appeared to extend from 27 kDa to 37 kDa (lane 6).

EPO samples were enzymatically deglycosylated by PNGase F and O-glycosidase to remove both N-linked and O-linked carbohydrates. The immunoblot analysis of all the enzyme-treated samples showed a single protein band with molecular weight of 18 kDa (Fig. 5.6). The position of this band corresponded to a standard non-glycosylated EPO produced by *Streptomyces* cells. This indicated that the effect of NH_4Cl was on the heterogeneity of the oligosaccharide side chains and not the EPO polypeptide.

5.2.4 Two dimensional electrophoresis of EPO samples

The heterogeneity of the glycosylated EPO samples was analyzed further with protein separation by two-dimensional electrophoresis and band detection by Western blotting (Fig. 5.7). EPO samples were analyzed from CHO cultures containing 0, 20 or 40 mM NH_4Cl. The result showed a significant increase in the pI of EPO isoforms with an increase in the ammonia concentration of the culture. The EPO analyzed from the control culture showed seven protein spots distributed over a range of pI values of 4.06 to 4.67 (Table 5.2). With the addition of NH_4Cl to the cultures there was a distinct shift of the pI range to higher values. At 20 mM NH_4Cl nine bands were detected. The band at the lowest pI (4.06) in the control sample disappeared and the upper range of pI was extended to 5.59. At 40 mM NH_4Cl the number of observed bands increased to ten with the appearance of an additional band at a pI value of 6.05. EPO from the 40 mM NaCl supplemented culture showed the same pattern as that of the control culture (Fig. 5.8a). This showed that the altered osmolality of the culture medium did not have effect on protein glycosylation.

In order to determine if the pI change of the isoforms of EPO protein was caused by variable sialylation, the sialic acids were removed by incubation of the samples with neuraminidase. After this treatment, EPO samples from both control and 20 mM NH_4Cl-containing cultures were analyzed by the same 2-dimensional electrophoresis technique (Fig. 5.7b). The results showed identical profiles for the two samples with two bands at pI values between 5.59 and 6.05. These bands were within the pI range of the extra bands which appeared in samples from analysis of the untreated samples from NH_4Cl-containing cultures (Fig. 5.7a). This indicates that the altered heterogeneity of EPO isoforms induced by NH_4Cl is caused by variable sialylation.

5.2.5 Effect of ammonia on O-linked glycosylation

The N-linked glycans of EPO were selectively removed by incubation with PNGase-F and the EPO containing only O-linked oligosaccharides was analyzed by electrophoresis. SDS-PAGE resulted in two protein bands with molecular weights of 18 kDa and 19 kDa which represented non-glycosylated EPO and EPO with an O-linked oligosaccharide (Fig. 5.9). The lower molecular weight band corresponded to the position of the non-glycosylated EPO band resulting from treatment with both PNGase-F and O-glycosidase (Fig. 5.6). As the NH_4Cl concentration of the culture was increased so did the proportion of the lower protein band. Analysis of these bands by a densitometer indicated that the ratio of the non-glycosylated to the O-linked glycosylated EPO bands increased proportionally to the concentration of NH_4Cl from 2.5 to 40 mM (Table 5.3). This analysis showed that ammonia might prevent O-linked oligosaccharides attachment to the protein.

The PNGase-F treated samples of EPO were also analyzed by 2D-electrophoresis in order to determine any change in the pI distribution of the O-linked glycosylation EPO isoforms. The results showed that the pI of non-glycosylated EPO did not change for either the control or ammonia containing cultures. However, the 19 kDa molecular weight band from EPO extracted from cultures with 40 mM NH_4Cl was shown to have a higher pI range than the equivalent sample from the control culture. This indicated that ammonia might inhibit

sialylation of the O-linked glycans as well as the N-linked glycans of EPO.

5.2.6 The effect of a sialidase inhibitor

The variable sialylation caused by ammonia could be a result of an altered intracellular enzymic activity or an increased sialidase activity in the culture supernatant. Gramer et al., (1995) reported that the percentage of asialo-gp120 in a cell culture decreased from 14% to <0.1% after the addition of the sialidase inhibitor at the start of the culture period. Moreover, Gu et al., (1997) found that the introduction of this sialidase inhibitor to a culture prevented loss of sialic acid from IFN-γ. In an attempt to maintain the sialic acid on EPO this sialidase inhibitor was added to our CHO cell cultures. The sialidase inhibitor (2,3-dehydro-2-deoxy-N-acetylneuraminic acid) (Sigma) was added at 1 mM or 2 mM on day 0 or day 3 of cultures growing in the presence of 20 mM or 40 mM NH_4Cl. In each culture, samples were taken at day 4 and analyzed for EPO heterogeneity by 2-dimensional electrophoresis. However, the results showed that the sialidase inhibitor had no effect on the EPO banding patterns which were identical in all cases.

5.2.7 Effect of chloroquine on cell growth and EPO glycosylation

Chloroquine has been shown to increase intracellular pH and affect glycosylation of recombinant proteins (Thorens and Vassalli, 1986; Andersen and Goochee, 1995). This effect has been compared previously to the effect of ammonia. Chloroquine was added to the CHO cultures at a concentration of 0.32 to 40 μM . Over this concentration range the chloroquine reduced the cell yield after 4 days in a dose dependent manner (Fig. 5.10). The cells were unable to grow at concentrations of chloroquine higher than 50 μM. The glycosylation pattern of EPO extracted from the chloroquine-treated cultures was analyzed by 2-D electrophoresis. However, the banding pattern of EPO did not change from that of the control cultures (Fig.5.8b). This indicated that under these conditions chloroquine had no apparent effect on the glycosylation of EPO and did not mimic the previously found effects of NH_4Cl.

Table 5.1 Specific production (-) or consumption (+) by cells in control and NH_4Cl-treated cultures ($\mu mol/10^6$ cell-day). Each value is a mean based on two independent cultures analyzed over the growth period (0-4 days). nd = not determined.

	NH_4Cl concentration			
	0 mM	10 mM	40 mM	10 mM NaCl
Glucose	7.64	8.30	9.30	7.51
Lactate	-15.5	-16.8	-25.0	-13.3
Glutamine	2.00	2.13	2.87	1.81
Glutamate	-0.20	-0.55	nd	nd
Glycine	-0.58	-0.98	nd	nd
Alanine	-2.34	-4.57	nd	nd

Table 5.2 The effect of NH_4Cl on the heterogeneity of EPO isoforms. Data were obtained from Fig. 5.7a. The pI and molecular weight were calculated based on 2D-electrophoresis markers and pre-stained M.W. standards.

NH_4Cl (mM) in culture	pI range	Number of detectable bands	M.W. range (kDa)
0	4.06-4.67	7	33-39
20	4.18-5.59	9	30-38
40	4.18-6.05	10	27-37

Table 5.3 Effect of ammonia on O-linked glycosylation. Data were obtained from Fig. 5.8. The relative percentage of each band was determined by Gel Doc analytical Software (Bio-Rad).

	NH_4Cl concentration (mM)						
	0	2.5	5	10	20	40	+10 mM NaCl
% of EPO with an O-linked glycan	77.1	76.3	74.5	66.7	55.6	53.1	77
% of non-glycosylated	22.9	23.7	25.5	33.3	43.6	46.9	23

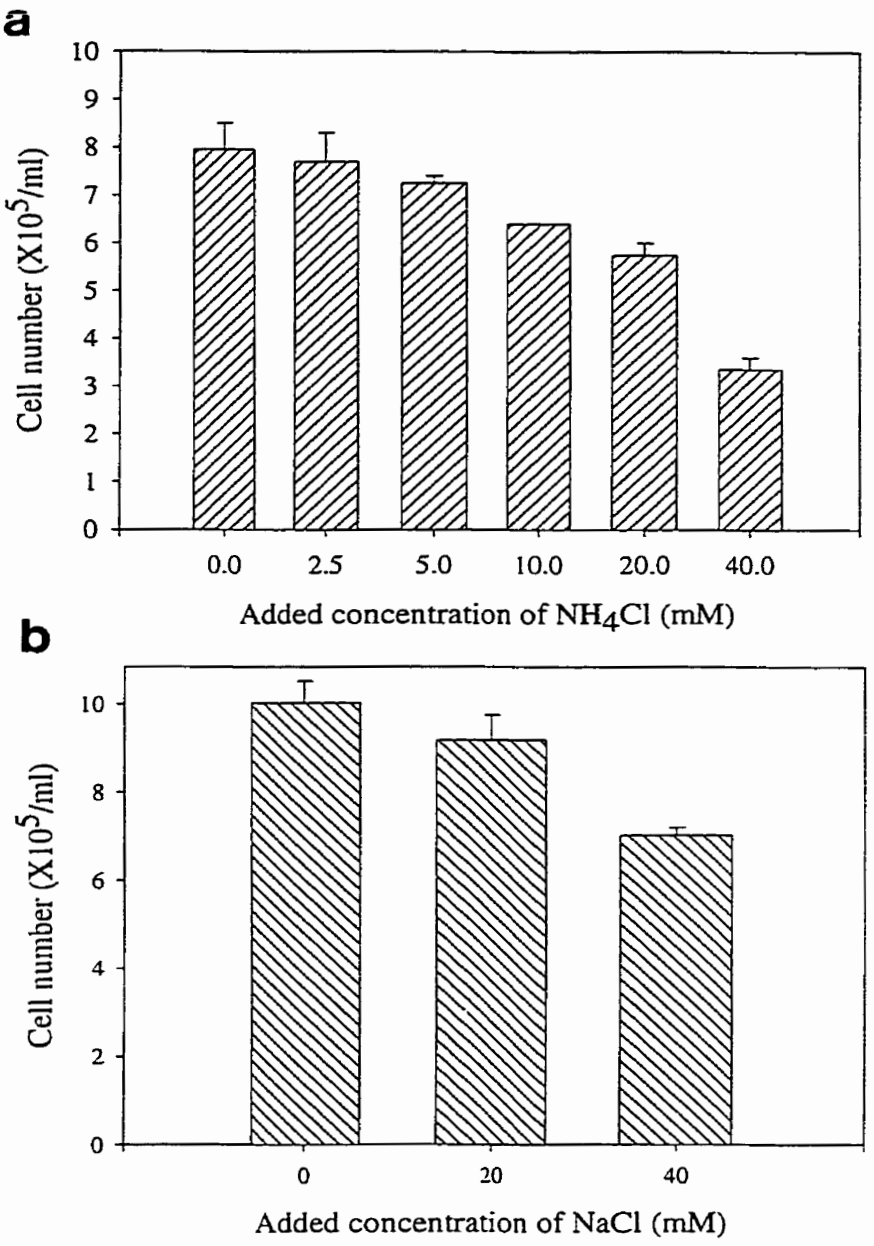


Fig. 5.1 Cell yields under different culture conditions. CHO cells were inoculated at $2x10^5$ cells ml^{-1} into 7 ml in CHO-SFM2.1 containing different concentrations of NH_4Cl (a) or NaCl (b) in $25cm^2$ T-flask and cultured for 4 days. Viable cells were determined by haemocytometer counting in a suspension of trypan blue following trypsinization. Values are Mean ± SEM of duplicate cultures.

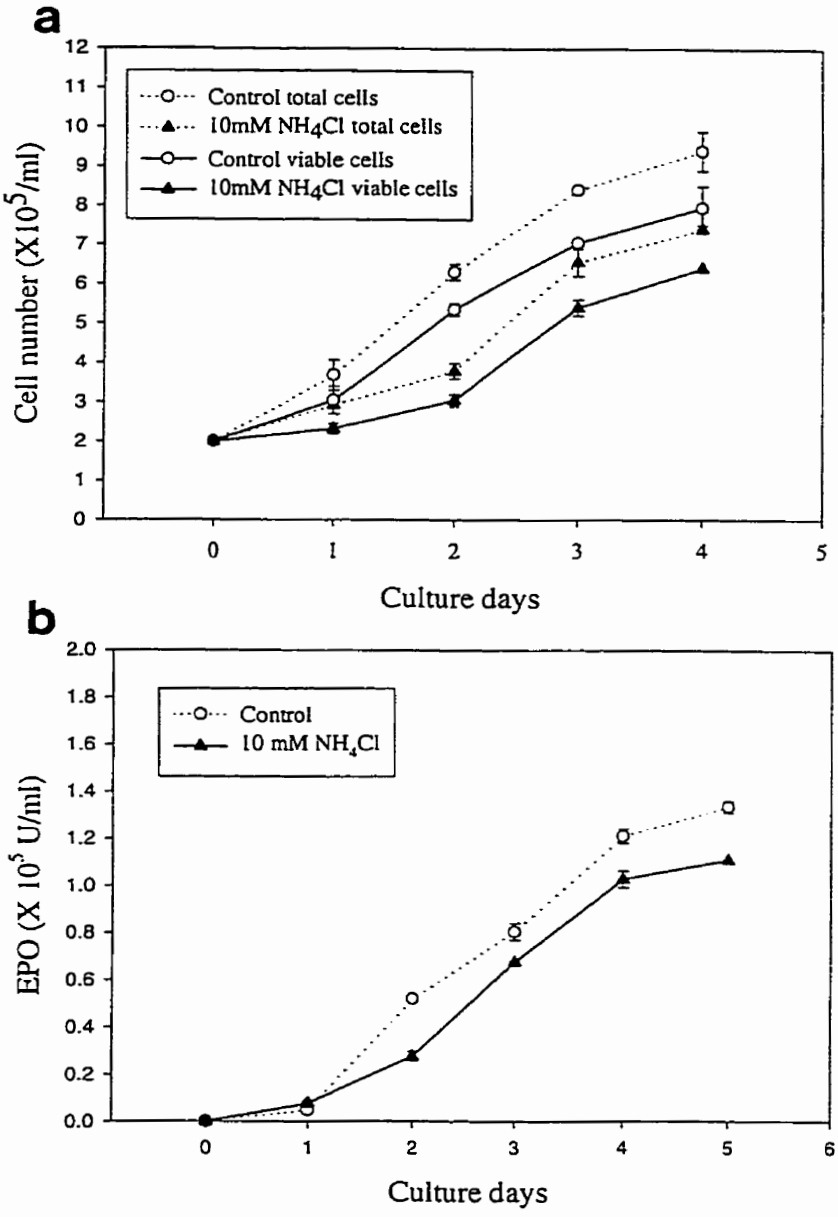


Fig. 5.2 Effect of Ammonium Chloride on CHO cell growth (a) and EPO production (b). CHO cells were cultured in CHO-SFM2.1 alone or containing 10mM NH_4Cl. Total and viable cell concentrations were determined by trypan blue exclusion during exponential growth. Deglycosylated EPO concentrations were determined by ELISA from day 0 to day 5. Cell numbers and values of EPO are Mean ± SEM of duplicate cultures.

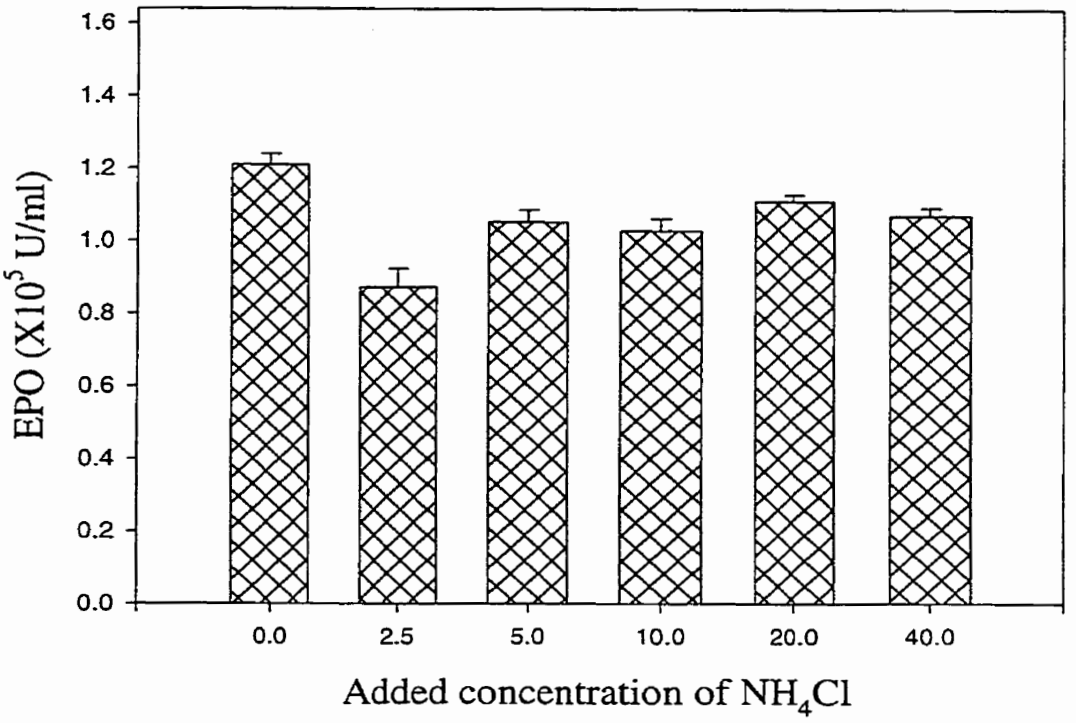


Fig. 5.3 EPO production in cultures containing different concentrations of NH_4Cl. CHO cells were inoculated at $2x10^5$ cells ml^{-1} into 7 ml in CHO-SFM2.1 containing different concentrations of NH_4Cl in $25cm^2$ T-flask. The culture supernatants were collected at day 4 and EPO concentrations after removal of N-linked glycans were determined by ELISA. Values are Mean ± SEM of duplicate cultures.

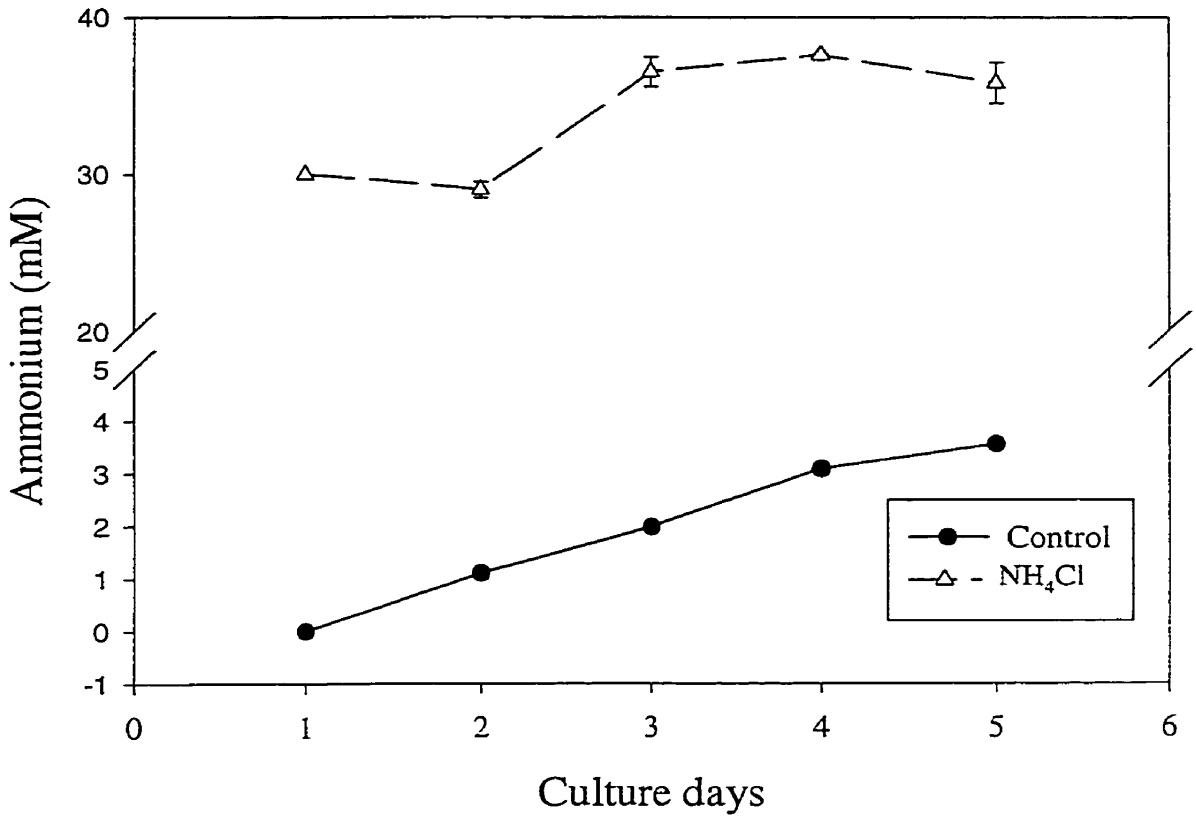


Fig. 5.4 Ammonium concentrations in CHO cell cultures. Ammonium level in the culture supernatants of a control and the culture containing 30 mM NH_4Cl were measured as described in Materials and Methods. Values are Mean ± SEM from two independent cultures.

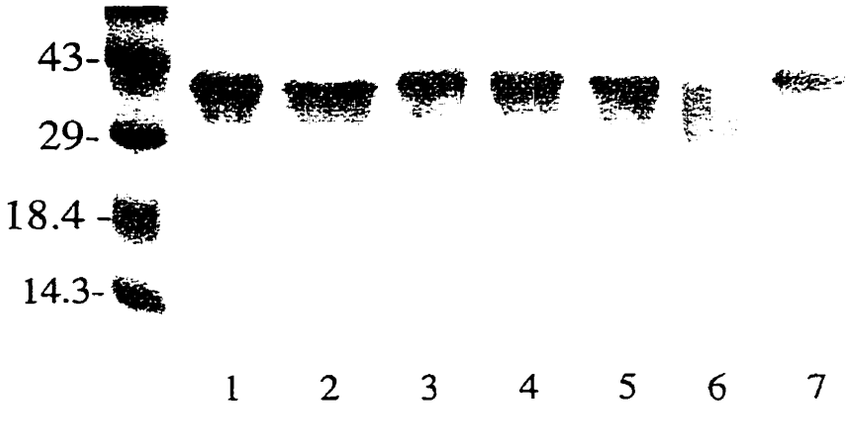


Fig. 5.5 EPO analysis by Western blot. Cell culture supernatants with different concentrations of NH_4Cl were collected at day 4, concentrated and separated by 14% SDS-PAGE. The protein was transferred to an NC membrane and detected by a monoclonal anti-huEPO antibody. Lane 1-7 are control, cultures containing 2.5, 5, 10, 20, 40 mM NH_4Cl and 10 mM NaCl respectively. Molecular weight standards are indicated ($x10^3$ kDa).

Fig. 5.6 Immunoblot analysis of enzymatically deglycosylated EPO. Cell culture supernatants were collected at day 4. The desalted samples containing EPO were concentrated and treated with PNGase F, O-glycosidase and sialidase at 37°C for 16 h. The treated samples were separated by 14% SDS-PAGE. The protein was transferred to an NC membrane and detected by anti-huEPO antibody. Lane 1-4 are control, and cultures containing 10 mM, 20 mM and 40mM NH_4Cl respectively. Molecular weight standards are indicated ($x10^3$ kDa).

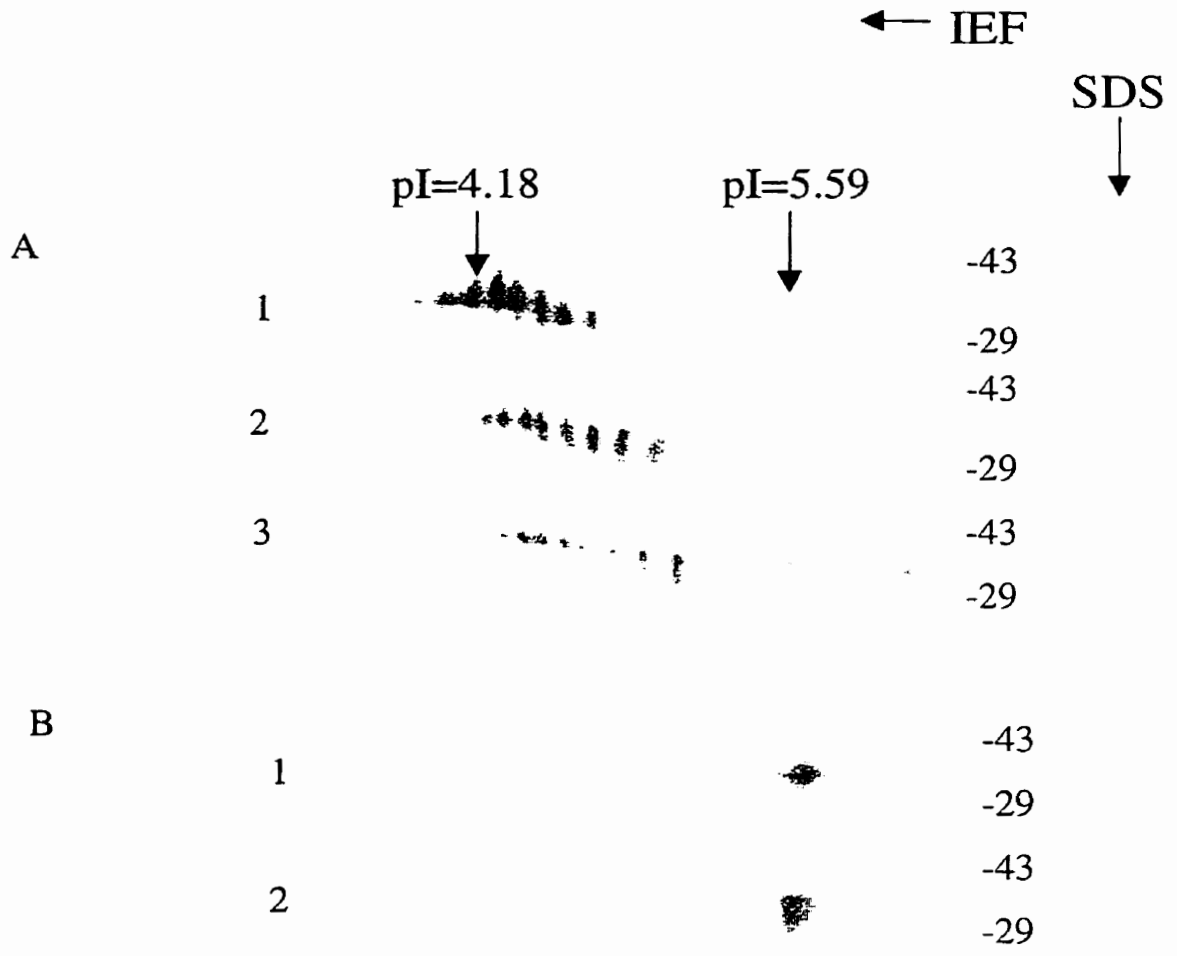


Fig. 5.7 Two-dimensional electrophoresis of EPO samples.

(a) Supernatants from CHO cell cultures containing 0 mM (1), 20 mM (2) and 40 mM (3) NH_4Cl were desalted and concentrated. EPO was analyzed by 2-D electrophoresis, separated by pI (ampholyte pH 4-6) in the first dimension followed by SDS-PAGE in the second dimension and detected by a Western blot.

(b) Culture supernatants from control (1) and cultures containing 20 mM NH_4Cl (2) were desalted and concentrated. After treatment with sialidase for 16 hours, samples were analyzed by 2-D electrophoresis and detected by a Western blot.

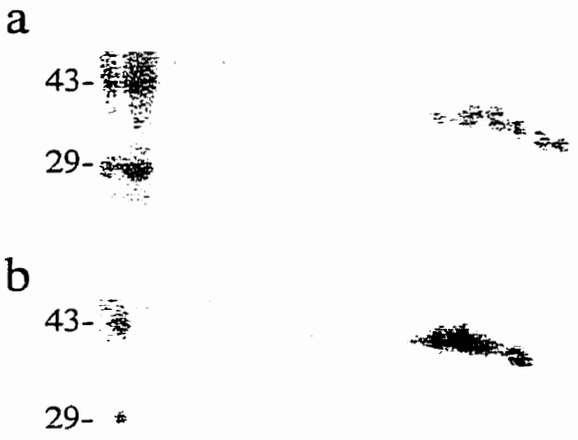


Fig. 5.8 Two-dimensional electrophoresis analysis of control EPO. Culture supernatants from cultures containing 40 mM NaCl (a) and 40 μM chloroquine (b) were concentrated, desalted and analyzed by 2-D electrophoresis. Protein molecular weights are indicated ($X10^3$ kDa).

Fig. 5.9 Immunoblot analysis of EPO without N-oligosaccharides. CHO cell culture supernatants were collected at day 4. The desalted samples containing EPO were concentrated and treated with PNGase F at 37°C for 16 h. The treated samples were separated by 14% SDS-PAGE. The protein was transferred to an NC membrane and detected by anti-huEPO antibody. Lane 1-7 are control, cultures containing 2.5, 5, 10, 20, 40 mM NH_4Cl and 10 mM NaCl respectively. Molecular weight standards are indicated ($x10^3$ kDa).

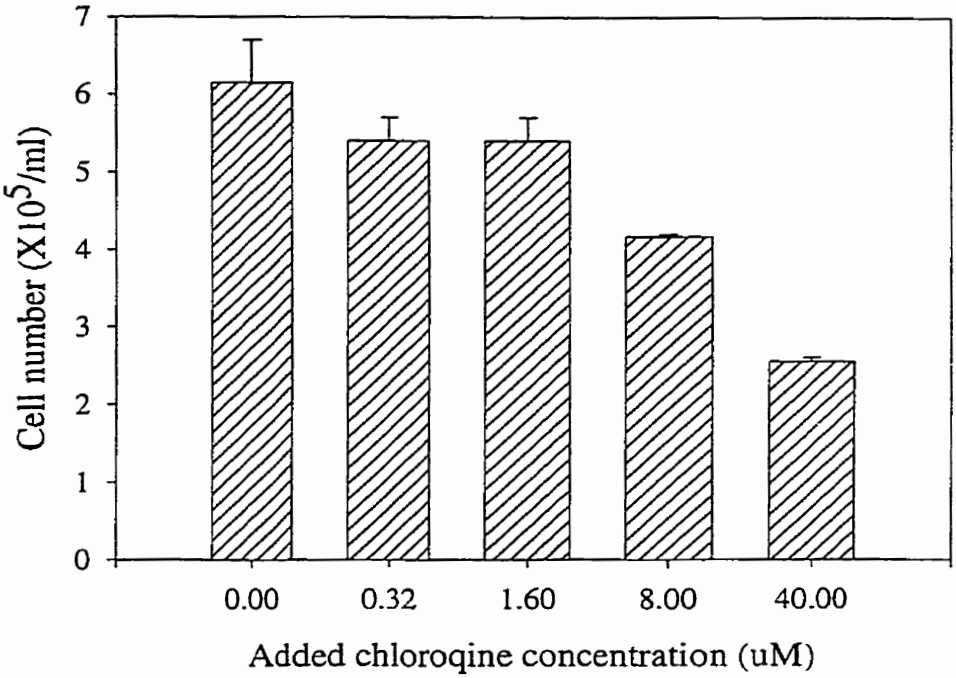


Fig. 5.10 Cell yields in chloroquine cultures. CHO cells were inoculated at $2x10^5$ cells ml^{-1} into 7 ml CHO-SFM2.1 alone or containing different concentrations of chloroquine in a $25cm^2$ T-flask and cultured for 4 days. Viable cells were determined by haemocytometer counting in a suspension of trypan blue following trypsinization. Values are Mean ± SEM of duplicate cultures.

5.3 Discussion

A clear understanding of the effect of cell culture parameters is important in the development of control strategies for mammalian cell culture processes used in the production of recombinant glycoproteins. In the experimental work described here we have studied the effect of ammonia on the growth, metabolism and protein production of a CHO cell line transfected for human EPO synthesis.

The accumulation of ammonia in mammalian cell cultures has been shown to result in a reduction of the specific growth rate and the final cell density (Butler et al., 1983; Butler and Spier, 1984). A quantitative indicator of the growth inhibition of ammonia includes the measurement of the concentration causing a 50% reduction in growth (IC-50). Although the actual value is highly dependent upon the prevailing culture conditions, it can be a useful relative indicator of the sensitivity of cells. A comparative study of the sensitivity of 9 cultured mammalian cell lines to ammonia showed considerable variation with IC-50 values ranging from 0.8 mM to >5 mM (Hassell et al., 1991). Values of IC-50 for hybridomas have been reported within the range of 3-7 mM (Doyle and Butler, 1990; Dodge et al., 1987; Glacken et al., 1986; Ozturk et al., 1992). In contrast, an IC-50 of 8 mM ammonia for CHO cells was observed (Kurano et al., 1990). However, there was no inhibition of CHO growth in continuous cultures by ammonia at a concentration of up to 8 mM (Hansen and Enborg, 1994). In our study, the IC-50 of the CHO was determined to be 33 mM ammonium chloride, which indicates that the cell line is far less sensitive to ammonia than many other cell lines.

The effects of elevated ammonia concentration on cellular energy metabolism that we report for CHO cells are similar to those reported for other cell lines (Ozturk et al., 1992; Schneider et al., 1996). Glucose and glutamine consumption were significantly increased as were the rates of production of lactate and certain amino acids. A plausible explanation for some of these metabolic changes is that the increased extracellular release of glycine, glutamate and alanine is the result of a detoxification mechanism which involves the sequestration of ammonia by alpha-keto acids produced by the catabolism of glucose and

glutamine (Butler et al., 1991).

The specific rate of EPO production (qEPO) increased with the addition of ammonia over 10 mM NH_4Cl. This appeared to be correlated with lower cell growth rate at a high ammonia level (10-40 mM). This result is consistent with monoclonal antibody production in the hybridoma cell line under the effects of ammonia (Miller et al., 1988). They found that the antibody production was not growth-associated and higher specific antibody production rates were obtained at lower specific growth rates. In addition, the specific antibody and recombinant protein productivities remained relatively constant with increasing ammonia concentration (Andersen and Goochee, 1995; Ozturk et al., 1992; Thorens and Vassalli, 1986).

It is important to characterize factors that affect the post-translational modification of recombinant proteins in order to ensure consistency of secreted glycoproteins during a production process. In the present study, ammonia was shown to increase significantly the heterogeneity of EPO isoforms. The addition of NH_4Cl above 2.5 mM to cultures caused a gradual increase in the molecular weight range of secreted EPO. The heterogeneity was analyzed in detail in EPO samples from cultures at a high concentration of added NH_4Cl (>20 mM) when the affect of the ammonia appeared to be maximum. Immunoblot analysis clearly showed that the increased heterogeneity at high concentrations of ammonia was not associated with a change in the EPO peptide but due to differences in the glycosylation of the molecule. Although ammonia produced by cells would not normally reach this high level in batch culture, some effect of ammonia on EPO heterogeneity would still be expected at the lower range (2-5 mM) which is typically found at the end of a batch culture. Our data is consistent with previous analyses of recombinant glycoproteins secreted from mammalian cells where increased heterogeneity of glycoforms was also shown in the presence of ammonia (Andersen and Goochee, 1995; Borys et al., 1994; Gawlitzek et al., 1998; Jenkins and Curling, 1994; Maiorella et al., 1993).

We show that EPO isoforms secreted from cultures containing NH_4Cl have a significantly

higher range of pI values. This is consistent with a decrease in terminal sialylation of the N-glycans of EPO in the presence of ammonia. The extent with which ammonia affects EPO sialylation was correlated with increasing NH_4Cl concentration. These findings are consistent with previous reports that have shown the effect of ammonia in reducing the sialylation of IgM expressed by hybridoma (Thorens and Vassalli, 1986) and G-CSF by CHO cells (Andersen and Goochee, 1995).

EPO contains one O-linked glycosylation site at serine 126. The effect of ammonia on this O-linked oligosaccharide was analyzed by selective removal of the N-linked glycans with PNGase F. In all cases this resulted in two electrophoretic bands with a molecular weight difference of 1 kDa which corresponds to the previously determined size of the O-linked glycan of EPO (Andersen and Goochee, 1995). The effect of NH_4Cl was to decrease the amount of detectable O-linked glycosylation from 77% to 53%. The effect of ammonia on O-linked glycosylation has also been reported for G-CSF synthesis in cell culture (Andersen and Goochee, 1995).

There may be several independent mechanisms for the specific effect of ammonia on protein glycosylation. Grammatikos et al (1998) reported that the ammonia-induced increase in the intracellular pool of UDP-GNAc led to an increased antennarity of a recombinant glycoprotein secreted by BHK cells. The decreased sialylation caused by ammonia was ascribed to an independent mechanism related to an increased intra-Golgi pH (Gawlitzek et al., 1998; McQueen and Bailey, 1990a).

In our culture system we showed that ammonia decreased the sialylation of the glycan structures EPO. We therefore attempted to mimic the pH effect of ammonia by the administration of chloroquine. It has been reported that chloroquine has the same effect as ammonia on recombinant protein glycosylation by increasing intracellular pH (Andersen and Goochee, 1995; Thorens and Vassalli, 1986). These reports showed that 100 μM chloroquine inhibited glycoprotein sialylation in serum-supplemented cultures. However, we were unable to show a similar effect in the synthesis of EPO. The pI range of the EPO isoforms did not

change by the presence of chloroquine up to 50 μM in the cultures. We were unable to grow the CHO cells at higher chloroquine concentrations because of the inhibitory effect on cell growth – this being greater in serum-free medium.

Decreased sialylation may also be associated with extracellular sialidase activity which cleaves terminal sialyl groups. Gramer and Goochee, (1993) identified sialidase activity in CHO cell lysates that was active and stable at pH 7, and was able to release sialic acid from a glycoproteins. Consequently the possible mechanism of reduced sialylation by ammonia could be attributed to extracellular sialidase activity following cell lysis. Gu et al., (1997) found that the loss of sialic acid content coincided with an observed decrease in viable cell density. Gramer et al., (1995) observed a substantial increase in extracellular sialidase activity in CHO cell culture following loss of cell viability. We added a sialidase inhibitor to the cultures in an attempt to reduce this possibility. However, our results showed that there was no increase of EPO sialylation in the presence of the sialidase inhibitor in the cultures containing ammonia.

We have shown in our culture system that the effect of added ammonia on EPO glycosylation was related to a decrease in sialylation of all glycans and a decrease in the proportion of the O-linked glycan. Similar effects may be expected in all cultures in which ammonia is produced, although to a differing extent. In a batch culture the accumulated level of ammonia is dependent upon the initial amino acid concentrations (especially of glutamine) and may be expected to reach up to 5 mM in standard culture medium. The ammonia is generated by both extracellular chemical decomposition of glutamine and intracellular metabolic deamination or deamidation. The relative compartmentation of ammonia resulting from differences in how the ammonia is produced may well give rise to different effects on glycosylation. These different effects were not distinguished in our system. A more detailed understanding and identification the predominant mechanisms of the effect of ammonia on glycosylation in different cell lines would help process control in the large-scale production of recombinant glycoproteins.

5.4 Conclusions

The effect of ammonium chloride was determined on a culture of CHO cells transfected with the human EPO gene. Cell growth was inhibited above a culture concentration of 5 mM NH_4Cl with an IC-50 determined to be 33 mM. The amount of EPO produced decreased in the presence of ammonium. The specific production of EPO increased with the addition of NH_4Cl above 10 mM. The metabolic effects of added NH_4Cl included higher specific consumption rates of glucose and glutamine and an increased rate of production of alanine and glutamate.

At 20 mM NH_4Cl there was a significant increase in the heterogeneity of the glycoforms of EPO. The altered glycosylation pattern included a reduction in terminal sialylation of the three N-liked glycans and a reduction in the content of the one O-linked glycan. The addition of a sialidase inhibitor to the cultures had no effect on the ammonia-induced increase in EPO heterogeneity. Also, the effect of ammonia on glycosylation could not be mimicked using the weak base chloroquine in our system.

CHAPTER 6

The effect of ammonia on the glycosylation of human recombinant erythropoietin in culture*

6.1 Introduction

Since the glycosylation process is a post-translational modification, carbohydrates might be greatly influenced by the culture environment. An understanding of the cell culture parameters that can affect the carbohydrate structure of a recombinant glycoprotein is important for the development of an effective production process for a therapeutic glycoprotein.

In the case of EPO, asialo-EPO has limited therapeutic value because it is rapidly accumulated in the liver as a result of specific binding to a lectin (Fukuda et al., 1989; Spivak and Hogans, 1989). Moreover, the reduction in the antennarity of EPO glycans shows a lower biological activity (Takeushi et al., 1989). Therefore higher branching of the N-linked oligosaccharides with attached sialic acids at the termini is desirable for the effective expression of the *in vivo* biological activity of EPO.

The sequence of oligosaccharide processing for N-linked glycosylation begins with the synthesis of a lipid-linked glycosylation. Oligosaccharides to be attached to asparagine residues are first assembled on a long-chain polyisoprenoid lipid called dolichol phosphate (Carson and Lennarz, 1979; Carson et al., 1987). The lipid-linked oligosaccharides are transferred to the nascent polypeptide chain in the ER. A series of "trimming" reactions are catalyzed by exoglycosidases in the ER. Oligosaccharide processing may continue in the compartments of the Golgi, catalyzed by different exoglycosidases and

* The contents of this chapter were included in a paper: M. Yang and M. Butler. 2000. The effect of ammonia on the glycosylation of human recombinant erythropoietin in culture. Biotechnology Progress. (in press).

glycosyltransferases leading to the complex type oligosaccharide structures (Kornfeld and Kornfeld, 1985). Therefore any culture parameter that can induce changes in the relative activity of these enzymes or intermediates can account for the variations in oligosaccharide structures.

Different effects of ammonia on monoclonal antibody and recombinant protein glycosylation have been reported, e.g. IgM, G-CSF, mPL-I and mutant IL-2. In Chapter 5, it was found that addition of NH_4Cl to the cultures caused a significant increase in the glycoform heterogeneity of EPO as shown by increased molecular weight and pI range. This indicated that the effect of ammonia was in the reduction of terminal sialylation of the glycan structures which accounted for an increased pI (Yang and Butler, 2000a).

It is important to characterize and define carbohydrate structures during a recombinant protein production process in order to ensure consistent glycosylation prior to its *in vivo* administration. In the work presented here, the effect of ammonia on recombinant EPO N-linked oligosaccharide structures, the degree of sialylation and the glycan sequence were analyzed by FACE and HPLC. Our results demonstrate that ammonia inhibits the N-linked oligosaccharide chain complexity and reduces the degree of the sialylation.

6.2 Results

6.2.1 Oligosaccharide analysis by FACE

The effect of ammonia on N-linked oligosaccharide profiles of EPO were analyzed by FACE. The transfected CHO cells were cultured in 150 cm^2 T flasks in serum-free medium with or without the addition of 30 mM ammonium chloride. The culture supernatant (1 liter) from each culture was collected on day 4 and EPO was purified by an immunoaffinity column. The N-linked oligosaccharides were released enzymatically and labeled with ANTS. The ANTS-labeled glycans were separated on polyacrylamide gels. Images were acquired using the Glyco Doc imager and analyzed by associated analytical software.

The results showed that the N-linked oligosaccharides contained at least seven different structures (Fig. 6.1, lane 3). The sialylated glycan sample has one prominent band at a position corresponding to 8 glucose units (GU, relative to the glucose ladder). This major band was identified as the tetraantennary structure with four sialic acids. After removal of the sialic acids, the major band shifted to a position corresponding to about 12 GU, which had a similar migration distance to the asialo tetraantennary standard. Two bands lower than the major band at 9 and 11 GU were positioned at similar migration distances to the asialo bi- and triantennary standards. The three bands higher than the major band were probably tetraantennary structures containing 1 to 3 lactosamine repeats (Morimoto et al., 1999). Our results on N-linked oligosaccharide structures are comparable with previous reports for EPO carbohydrate structures (Tsuda et al., 1988; Morimoto et al., 1999).

The effect of ammonia on the N-linked oligosaccharide profiles of EPO was examined and the result was compared with the control. Each lane was loaded with 20% of the glycan sample obtained from 1 liter of culture. Thus the carbohydrate loaded was proportionally less from the ammonia-supplemented culture because of the lower EPO production. Nevertheless the relative intensities of bands as analyzed by densitometry were significantly different between the cultures. The relative intensity of the major band decreased significantly and overall width of the bands increased significantly in the ammonia-supplemented culture (Fig. 6.2, lane 5). This indicated that the degree of sialylation of the EPO was changed and the proportion of glycan containing four sialic acids was reduced. The asialo-glycans were also analyzed in order to investigate glycan branch variability in the ammonia-supplemented culture. It was found that the proportion of tetra-antennary structures was reduced by about 60%, while the tri- and bi-antennary structures were increased proportionally in the presence of ammonia (Table 6.1). The results indicated that the ammonia reduced the proportion of the tetra-sialylated and the tetra-antennary glycan structures.

6.2.2 N-glycan analysis by HPLC

In order to analyze changes in sialylation induced by ammonia, anion exchange chromatography was performed by HPLC. The degree of sialylation of the oligosaccharides from EPO in the control and ammonia-supplemented cultures was analyzed and compared (Fig. 6.3). The oligosaccharides were separated into groups according to charge. The glycans were identified by comparison with retention times of oligosaccharide standards containing variable numbers of sialic acids. The chromatographs showed a significant degree of variation in the oligosaccharide patterns for control and ammonia-supplemented cultures. The most significant characteristic of the culture containing ammonia was that the relative proportion of EPO with four sialic acids was reduced from 46% to 29% in the culture. The relative proportions of mono- and di-sialylated glycans were increased (Table 6.2). This result is consistent with the analysis by FACE.

The oligosaccharide branch changes induced by ammonia were further analyzed by normal phase chromatography. The N-linked glycans from both the control and ammonia-supplemented culture were treated with sialidase to remove sialic acid. In this procedure, the oligosaccharide structure separation is based on glycan size. Smaller oligosaccharides eluted first followed by larger molecules.

The oligosaccharide peaks were identified by comparison with the retention times of oligosaccharide standards. The major peak has a retention time consistent with the tetra-antennary standard (Fig 6.4). The result showed that the major oligosaccharide structures were of the tetra-antennary type, although these were significantly reduced from 60% to 26% in the presence of ammonia (Table 6.1). This correlated with the analysis by FACE which showed that the tetra-antennary structure comprised 64% of the total glycans. The relative proportions of the bi-and tri-antennary structure were increased in the ammonia-supplemented culture from 13.4% and 25.2% in the control to 32.9% and 40.8% respectively. This result suggested that the presence of ammonia in the culture might inhibit carbohydrate chain antennarity or elongation.

6.2.3 Determination of the N-linked oligosaccharide sequence by FACE

Sequence determination of the oligosaccharides was accomplished by sequential exoglycosidase digestions. Exoglycosidase digestion results in a stepwise release of the monosaccharides from the intact structure eventually to the trisaccharide core structure (Fig. 6.5). The products of each digestion were separated by PAGE. The values of glucose units serve to correlate mobility shifts measured in the sequencing gel with the number of monosaccharides released from a glycan structure. Hence, the oligosaccharide sequence can be deduced from the knowledge of the general structural features of N-linked oligosaccharides.

The charge/ mass ratio of the oligosaccharide structure changed following each digestion. The initial sialidase treatment resulted in reduced migration of the bands because of the loss of the charged sialic acids. However, subsequent enzymic digestions resulted in increased migration as the molecular size of the glycan was sequentially reduced (Table 2.1, 2-4). In this study, we successfully digested down to the N-linked oligosaccharide core structure. The results indicated that EPO contained a typical N-linked complex oligosaccharide structure. Glycans from the ammonia-supplemented cultures showed the same sequence pattern which indicated that ammonia did not affect the N-linked oligosaccharide sequence.

Table 6.1 The relative proportion of oligosaccharide antennarity analyzed by FACE and HPLC. The proportion of each antennary structure was determined by Gel Doc analytical software for FACE analysis.

Analysis Technique	Culture	Relative proportion of different antennary (%) bi-	tri-	tetra-
FACE	Control	14.9	21.0	64
	30 mM NH_4Cl	42.7	33.9	23.3
HPLC	Control	13.4	25.2	59.6
	30 mM NH_4Cl	32.9	40.8	25.9

Table 6.2 Relative proportion of EPO carbohydrates with variable sialic acids as analyzed by anion exchange chromatography

Culture	Relative proportion of sialylated glycans (%) mono-	di-	tri-	tetra-
Control	12.1	14.8	28.2	45.7
30 mM NH_4Cl	25.8	21.5	23.3	29.3

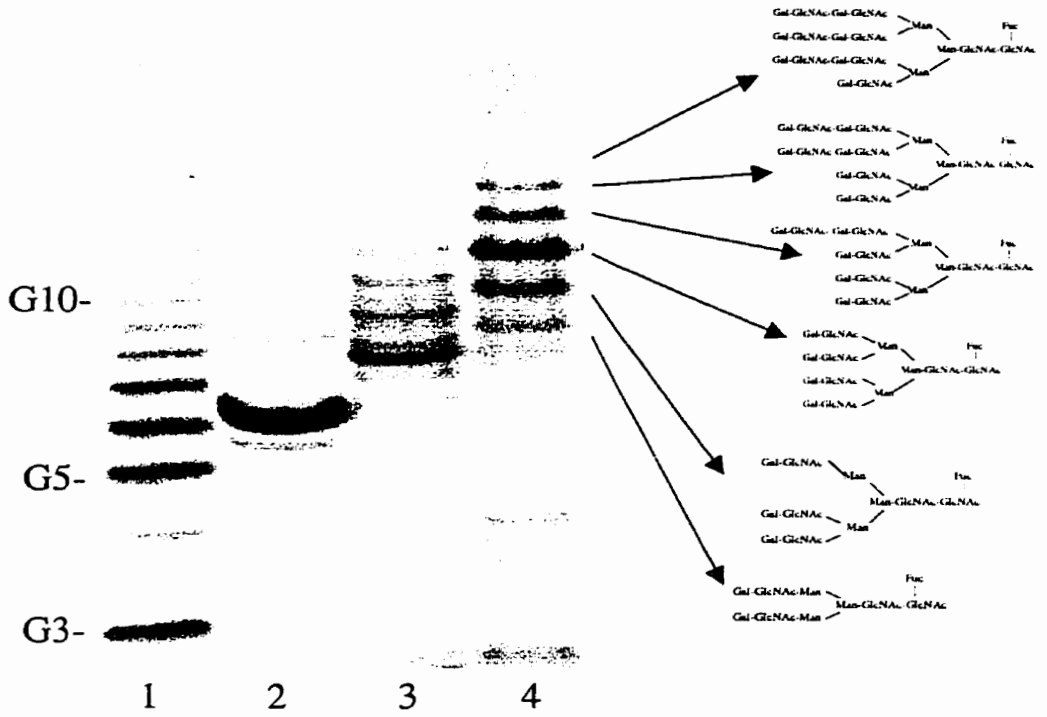


Fig. 6.1 EPO N-linked oligosaccharide profile. EPO N-linked oligosaccharides were analyzed by FACE. Images were acquired using the Glyco Doc imager. Lane 1. Glucose ladder; lane 2. bi-antennary oligosaccharide standards with or without galactose; lane 3. EPO N-linked oligosaccharide profiles and lane 4. asialo-oligosaccharides of EPO.

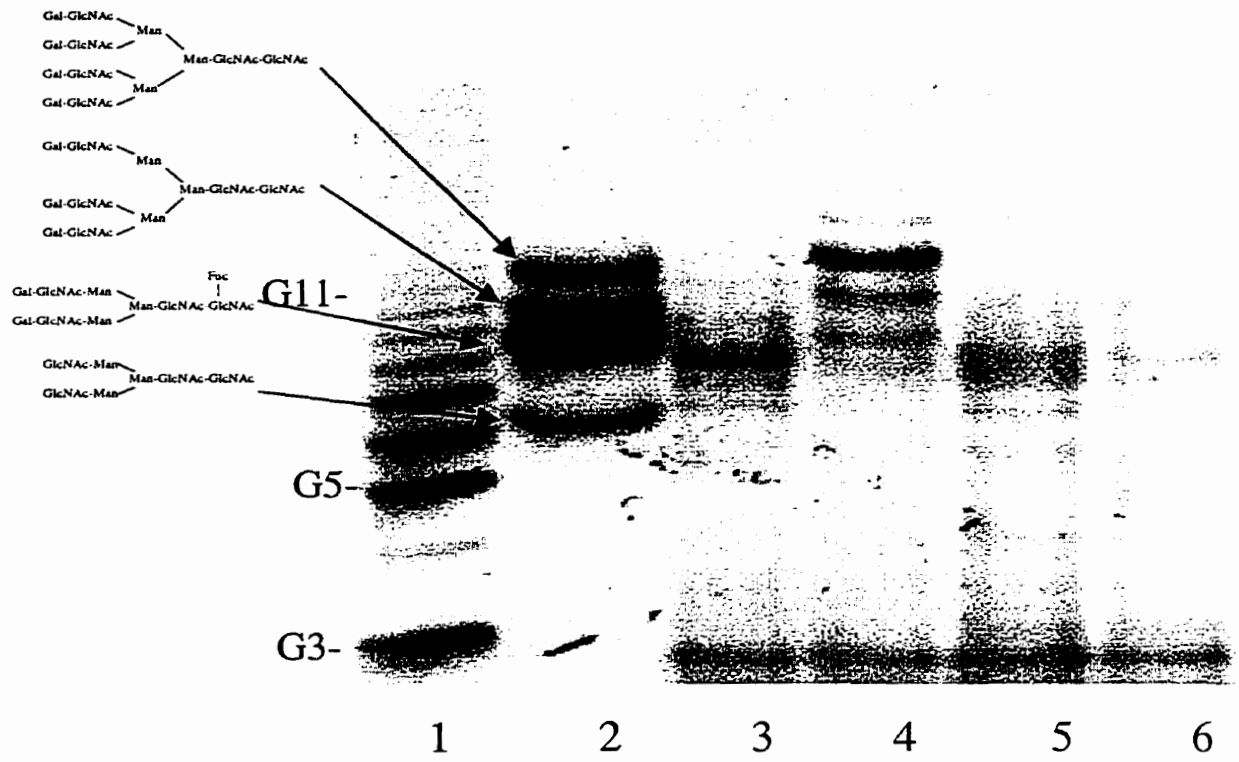


Fig. 6.2 Effects of ammonia on EPO N-linked oligosaccharides. Culture supernatants from cultures with or without added ammonia chloride (30 mM) were collected on day 4. The carbohydrates released from EPO were analyzed by FACE. Lane 1: glucose ladder; lane 2: carbohydrate standards as indicated; lane 3: N-glycans from control; lane 4: asialo N-glycans from control; lane 5: N-glycans from ammonia culture and lane 6: asialo N-glycans from ammonia culture.

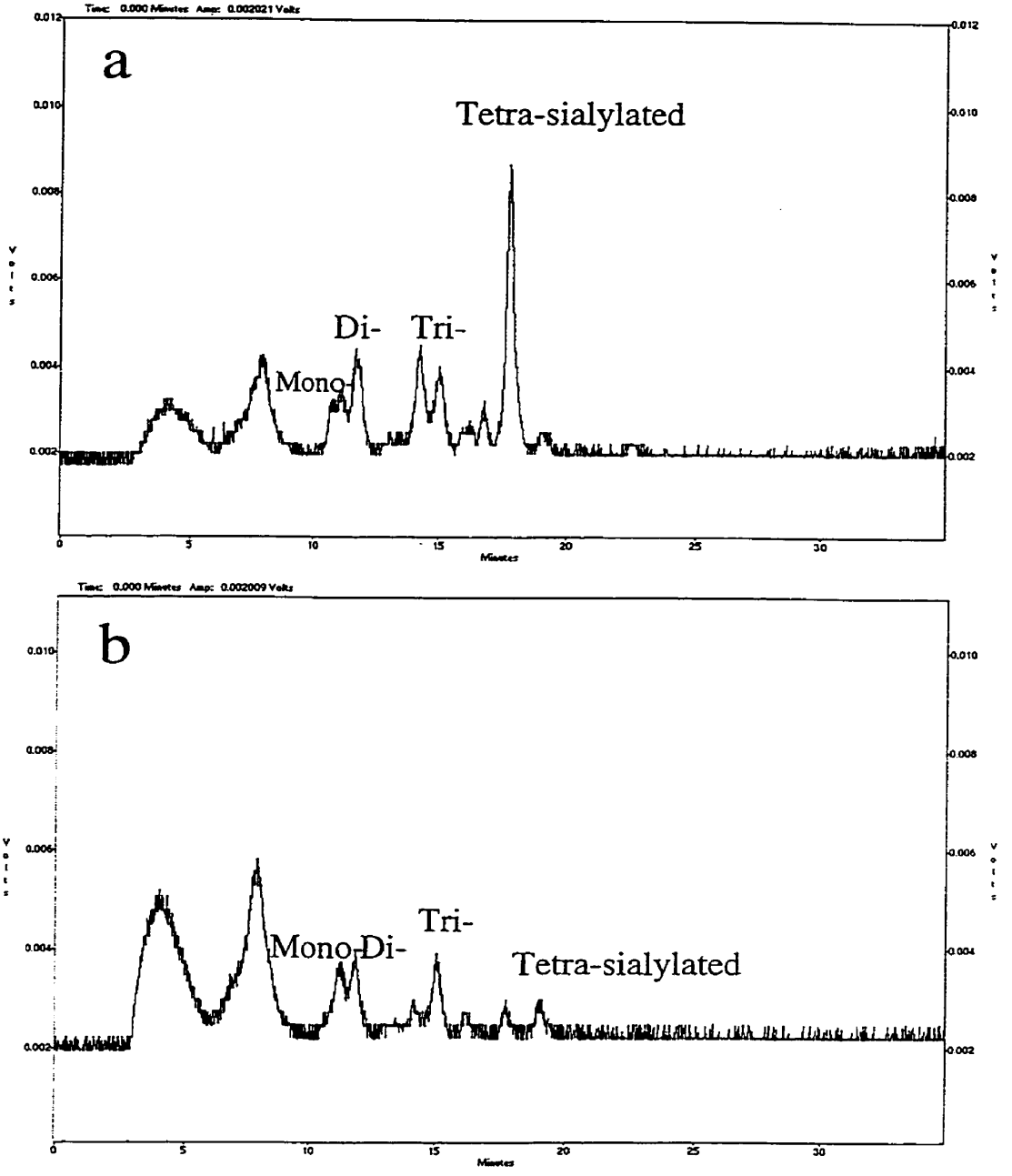


Fig. 6.3 EPO glycans analyzed by anion exchange chromatography. Glycans released from purified EPO were labeled with 2-AB followed by GlycoClean S cartridge treatment to remove free dye. Samples were analyzed by HPLC using a GlycoSep C column (gradient a). (a) glycans released from the control culture and (b) glycans from the culture containing 30 mM NH_4Cl.

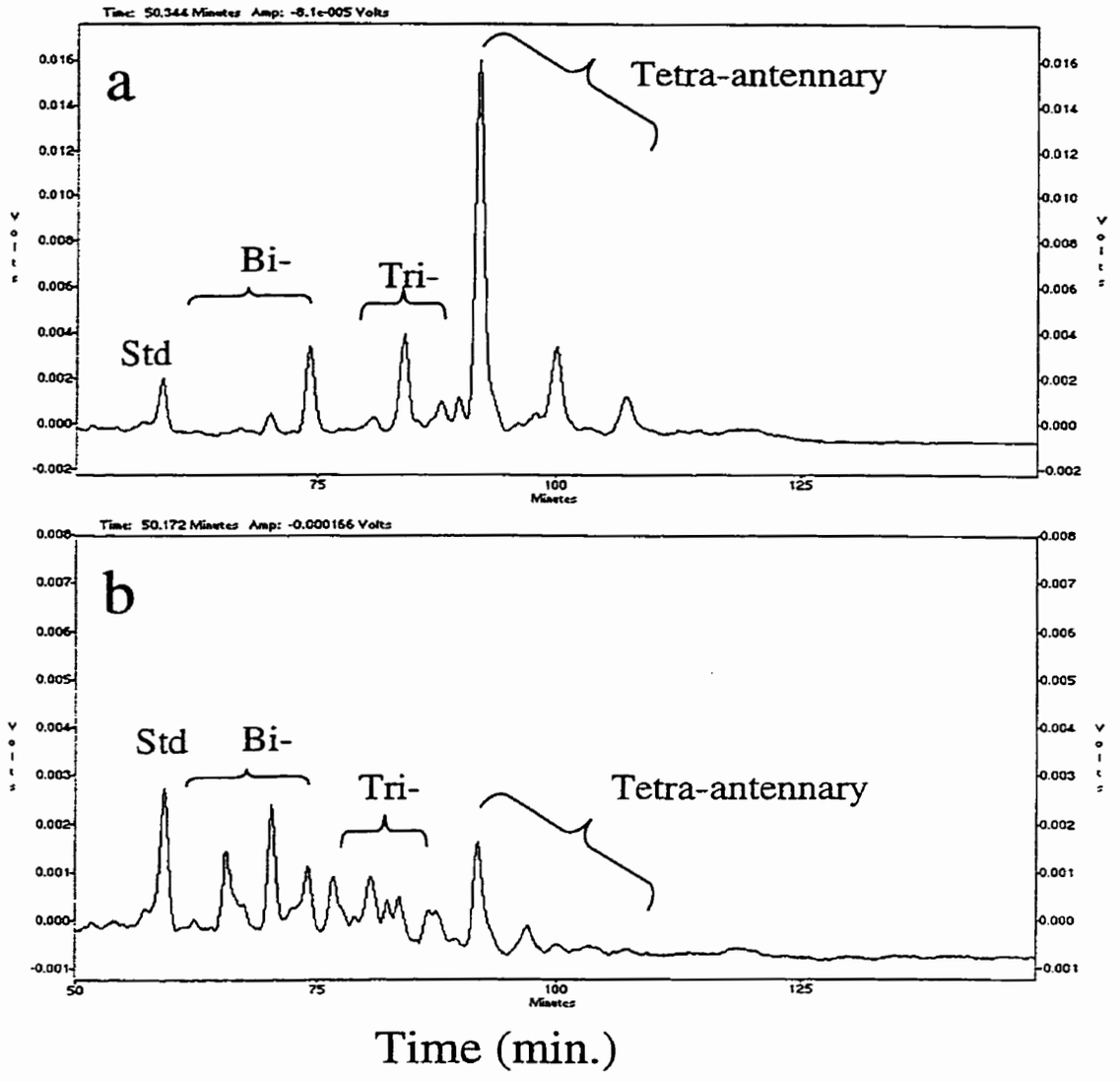


Fig. 6.4 EPO oligosaccharide analysis by normal phase chromatography. Glycans released from EPO in a control culture (a) and a culture containing 30 mM NH_4Cl (b) were labeled with 4-AB and analyzed by HPLC using a GlycoSep N column. The peak "Std" is the asialo-agalacto-binatennary oligosaccharide as the internal standard. The peaks were identified by comparison of retention times with oligosaccharide standards.

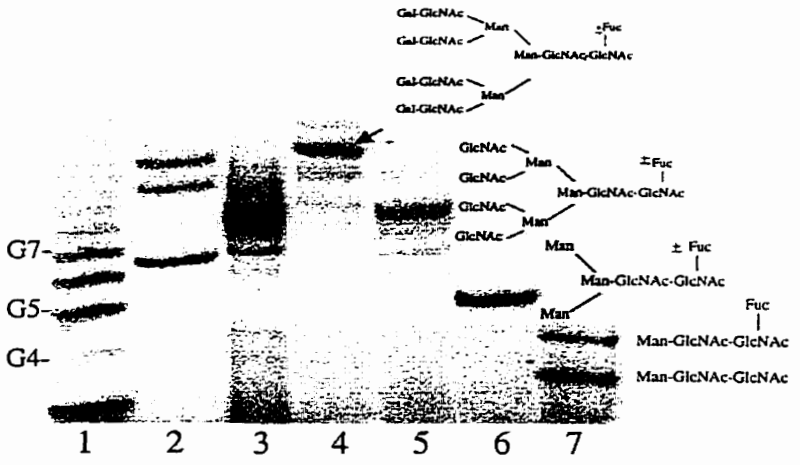


Fig. 6.5 Sequence determination of oligosaccharides by FACE. Oligosaccharides were digested by exoglycosidases using an oligosaccharide Sequencing Kit. Lane (1) glucose ladder; lane (2) agalacto-bi-, tri- and tetra-asialo-antennary standards; lane (3) N-linked glycan profile of EPO; lane (4) +neuraminidase; lane (5) +β-galactosidase; lane (6) +β-N-Acetylhexosaminidase; lane (7) +α-Mannosidase.

6.3 Discussion

The glycosylation of therapeutic proteins synthesized in cell culture processes is essential for the clinical effectiveness of the extracted product. In the case of human EPO it is well established that full sialylation of the three N-linked glycans is important for maintaining a significant residence time in the blood stream. However, the culture parameters that control glycosylation are not well understood. The work presented in this report considers the effect of an elevated ammonia concentration in culture as a parameter that might adversely affect the glycosylation of EPO. The work is important in developing an understanding of how glycan structures may change under different culture conditions.

In Chapter 5, the work showed that CHO cell growth was inhibited above a culture concentration of 5 mM NH_4Cl (Yang and Butler, 2000a). The addition of NH_4Cl to the cultures caused a significant and progressive increase in the heterogeneity of the glycoforms as shown by an increased molecular weight and pI range of the secreted erythropoietin. We concluded that the effect of ammonia was probably in the reduction of terminal sialylation of the glycan structures attached to the protein, which accounted for the increased pI.

In order to confirm these changes to the carbohydrate structures, we decided to perform detailed analysis of erythropoietin produced in culture with a high ammonium content. This treatment maximized the structural difference of EPO compared to that from control cultures. The EPO N-linked oligosaccharides were analyzed by FACE and HPLC using two columns. FACE and HPLC separate glycans according to different principles, providing complementary information. EPO was extracted from control cultures and cultures supplemented with ammonia. The EPO was purified by immunoaffinity chromatography and the N-linked glycans were released by PNGase F. Our results by both FACE and HPLC indicated that the predominant EPO N-linked glycans were tetra-antennary structures with four terminal sialic acids.

The profiles of the N-linked glycans from control and ammonia-supplemented cultures were

compared. The proportion of the major band which contained the four sialic acids was significantly reduced in the ammonia-supplemented cultures. The separation of ANTS-labeled sialylated oligosaccharides on the FACE gel is based on the charge and size (Jackson, 1990; 1994). Structures can be deduced by mobility shifts following sequential enzymatic treatment of the glycans. Hague et al., (1998) demonstrated that the glycan mobility rules for FACE work well for biantennary sialylated oligosaccharides, but may vary for tri- and tetra-sialylated glycans. Quantitative analysis of the sialylation pattern of the glycans was determined by HPLC. The results by anion exchange chromatography indicated that the relative proportion of tri- and tetra-sialylated glycans were reduced in the presence of ammonia-supplemented culture compared to the control culture. The corresponding proportions of mono- and di-sialylated oligosaccharides were elevated. This result combined with the observed change of pI by 2-D electrophoresis suggested that ammonia inhibited the sialylation of EPO.

Our observation is consistent with the reports by Thorens and Vassalli, (1986) and Anderson and Goochee, (1995). They found that the ammonia reduced the sialylation of monoclonal antibody and recombinant granulocyte colony-stimulating factor, although detailed carbohydrate structure changes were not determined. Zanghi et al., (1998a) showed that ammonia causes rapid and dose-dependent inhibition of NCAM polysialylation in CHO and SCLC cells. Recently, it was reported that as ammonia increased from 1 to 15 mM, a concomitant decrease of up to 40% was observed in terminal galactosylation and sialylation of the immunoadhesin tumor necrosis factor-IgG produced by CHO cells (Gawlitzek et al., 2000).

Asialo-oligosaccharide structure analysis indicated that there was a significant reduction of the proportion of tetra-antennary structures in the presence of ammonia, while the relative proportions of the bi- and tri-antennary structures were increased. These results suggested that the ammonia in the culture reduced the degree of oligosaccharide antennarity. Our results are consistent with the report by Borys et al., (1994). They demonstrated that the molecular heterogeneity of mouse placental lactogen-I secreted by CHO cells shifted to a

significantly lower molecular weight range following the addition of ammonia to cultures. Their results indicated that ammonia has the potential for a significant reduction in the overall degree of glycosylation. They concluded that the potential for ammonia to disrupt glycosylation is more than just the disruption of terminal sialylation. Although they did not analyze the oligosaccharide structures, the extensive molecular weight shift they reported could not be explained by reduced sialylation alone. Gawlitzek et al., (1998) observed different carbohydrate structural changes in the presence of ammonia with an increase in the complexity of mutant IL-2 N-glycosylation. In contrast, no effect of ammonia on branching of N-glycans was observed for immunoadhesin glycoprotein (Gawlitzek et al., 1999).

The exact mechanism of the effect of ammonia on recombinant protein glycosylation is unknown. There are several possible mechanisms to explain the observed reduction in the proportion of the tetra-antennary glycan structures and sialylation in EPO. Firstly, the activities of the glycosylation enzymes may be reduced. Secondly, the balance of intracellular nucleotide sugar pools may be perturbed. Thirdly, the availability of oligosaccharide precursors may be reduced and fourthly the glycan chains or sialic acids may be cleaved by the activity of glycosidases.

During the N-linked glycosylation process, the oligosaccharide is assembled in the ER on the lipid carrier dolichol phosphate. The sugars are added in a stepwise fashion derived from the nucleotide sugars and the lipid intermediates (Waetheter and Lennarz, 1976; Snider, 1984). GlcNAc-Man_3-$GlcNAc_2$-Asn is the precursor to complex oligosaccharides having two, three, or four outer branches. The GnT IV and V add GlcNAc in a β-1,4 linkage to the α-1,3 mannose or in a β-1,6 linkage to the α-1,6 mannose to produce structures with three or four outer branches (Kornfeld and Kornfeld, 1985). Ammonia is a weak base and could increase the pH of the micro-environment within the ER or Golgi compartments leading to reduced enzymatic activity. An intracellular pH change could affect the activity of glycosyltransferases or glycosidases, for example the branching enzymes GnT III and IV (Schachter, 1986) and sialyltransferase. Andersen and Goochee (1995) found a twofold decrease in the activity of α-2,6-sialyltransferase in the presence of NH_4Cl in CHO cell

cultures. pH titration of endogenous CHO α-2,3-sialyltransferase and β-1,4 galactosyltransferase revealed a sharp optimum at pH 6.5. Thus at pH 7.0 to 7.2, a likely trans-Golgi pH range in the presence of 10 to 15 mM ammonium, activities for both enzymes are reduced to 50-60% (Gawlitzek et al., 2000). This pH-mediated effect on glycosyltransferase activity is one possible explanation of the reduction of tetra-antennary glycans and sialylation in the presence of ammonia.

In several recent studies, different explanations for ammonia effects on protein glycosylation have been discussed. Changes in the availability of intracellular nucleotide precursors may affect glycosylation. Gawlitzek et al., (1999) and Valley et al., (1999) studied ammonia effects in the synthesis of UDP-GNAc using $^{15}NH_4Cl$ in the culture medium. They found that 60-80% of N-acetylated sugars in N-glycan structures contained ^{15}N indicating that ammonium is used as a building block during synthesis of the carbohydrate structures for IL-2 mutant glycoprotein and immunoadhesin glycoprotein. Pels Rijcken et al., (1995) found that an increase in the intracellular UDP-sugar pool causes a decrease in sialylation which is possibly because the elevated level of UDP-sugar impaired the transport of CMP-acetylneuraminate to the Golgi. Grammatikos et al., (1998) reported that an increased intracellular pool of UDP-GNAc caused by ammonia led to increased antennarity of the glycan structure of a recombinant protein. They also induced an artificial increase in the intracellular UDP-GNAc pool by treating BHK cells expressing an IL-2 mutant with glucosamine and uridine. The oligosaccharide structure showed increased antennarity compared to control conditions. However, the sialylation state remained unaffected. Thus they demonstrated that ammonium ions act on protein glycosylation by at least two independent mechanisms, one of which involves an increase in the UDP-GNAc pool.

Zanghi et al., (1998b) studied the role of UDP-GlcNAc in the ammonia-induced inhibition of NCAM polysialylation by adding glycosamine and uridine to the cultures. By comparison, 20 mM NH_4Cl decreased polysialic acid content by 45% and increased UDP-GlcNAc in SCLC cells by 2 fold. They suggested that accumulation of UDP-GlcNAc is only partially responsible for decreased polysialic acids in response to NH_4Cl. However, no differences in

N-glycosylation were found in the immunoadhesin tumor necrosis factor-IgG synthesized in the presence of a concentration of glucosamine that could increase the intracellular UDP-sugar pool concentration (Gawlitzek et al., 2000). Our data indicated that ammonia reduced the proportion of tetra-antennary glycan structures. This combined evidence suggests that there may not be a direct relationship between changes in intracellular nucleotide sugar pool and changes in glycan structures in our system.

It has been reported that a shortage of oligosaccharide precursors caused the addition of abnormally small oligosaccharides at the initial step of glycosylation from dolichol precursors (Davidson and Hunt, 1985; Elbein, 1987; Rearick et al., 1981). Several studies have shown that glucose limitation results in incomplete protein glycosylation. The low glucose concentration affects the degree of glycosylation of monoclonal antibodies produced by human hybridomas in batch culture (Tachibana et al., 1994). Although our previous study showed that the specific consumption of glucose in the ammonia culture was higher compared to the control, glucose depletion does not occur even at the end of the culture period (Yang and Butler, 2000b).

Another possible explanation for reduced glycosylation by ammonia is that the carbohydrate chains are reduced by the action of glycosidases. Villers et al., (1994) demonstrated that newly synthesized glycoproteins could be degraded at the rough ER level in a mutant CHO cell line. Gawlitzek et al., (1999) observed that the cell culture supernatants contained measurable β-galactosidase and sialidase activity, which increased throughout the culture. A correlation between intracellular β-galactosidase activity and ammonium concentration was found in the culture. However, no loss of N-glycans was observed in incubation studies using β-galactosidase and sialidase containing cell culture supernatants. In addition, another study found several exoglycosidases in CHO cell culture supernatant, including sialidase, β-galactosidase and fucosidase (Gramer and Goochee, 1993). However, we have not been able to detect extracellular sialidase activity in our cultures suggesting that the ammonium effect was biosynthetic and not degradative (Yang and Butler 2000a).

6.4 Conclusion

In the work presented here, the effects of ammonia on EPO N-linked oligosaccharides were analyzed. EPO was purified from culture supernatants by immunoaffinity chromatography. The N-linked oligosaccharides were released enzymatically and analyzed by FACE and HPLC.

The FACE N-linked oligosaccharide profile showed that the sialylated glycans contain one prominent band at a position corresponding to 8 glucose units. The density of the major band was greatly diminished and the width was significantly increased in cultures containing added ammonia. The proportion of tetra-antennary structures was reduced by 60%, while the tri- and bi-antennary structures were increased proportionally in the presence of ammonia. Glycan analysis by HPLC using a weak anion exchange column showed that the most significant characteristic effect of ammonia was a reduction of the proportion of glycans with four sialic acids from 46% in control cultures to 29% in ammonia-treated cultures. Analysis of the de-sialylated glycans by normal phase chromatography indicated a distribution of tetra-, tri-, and bi-antennary structures similar to that shown by FACE. The N-linked glycan sequence was determined by sequential exoglycosidase digestion followed by FACE. The results indicated a typical N-linked complex oligosaccharide structure. Glycans from ammonia-containing cultures showed the same sequence pattern.

The effect of added ammonia in our CHO cultures was to reduce the extent of glycosylation of synthesized EPO. This was observed as a reduced proportion of tri-, tetra-sialylated and tetra-antennary oligosaccharide structures. Of the possible explanations suggested for this the most likely is that ammonia inhibits N-linked oligosaccharide chain complexity and sialylation by inhibiting the activity of specific glycosylating enzymes in the trans-Golgi system.

CHAPTER 7

Enhanced erythropoietin heterogeneity in a CHO culture is caused by proteolytic degradation and can be eliminated by a high glutamine level*

7.1 Introduction

An important criterion for the development of a suitable production processes is to ensure the synthesis of a biologically active product that shows a consistent profile of heterogeneity. The heterogeneity of cell-secreted recombinant glycoproteins arises from two sources - variable peptide size and variable oligosaccharide structures. The heterogeneity of oligosaccharide structures can arise from variable intracellular processing as a result of various culture parameters, only some of which have been characterised. The culture parameters that may affect the variability of glycosylation include glucose depletion (Gershman and Robbins, 1981; Rearick et al., 1981), ammonia accumulation (Borys et al., 1994; Thorens and Vassalli, 1986), lipid composition (Castro et al., 1995; Jenkins et al., 1994), protein content (Castro et al., 1995) and pH (Borys et al., 1993).

Further heterogeneity is caused by proteases and glycosidases released from cells into the culture medium. These enzymes may degrade peptides and oligosaccharides in the culture medium. Thus a prolonged residence time of a secreted glycoprotein may result in some degradation which gives rise to further molecular heterogeneity (Gramer and Goochee, 1993; Gramer et al., 1995; Lind et al., 1991). It has been shown that the level of glycosylation can decrease over time in a batch culture as the chemical environment of the cell changes (Curling et al., 1990). Proteolytic degradation of the N-terminus of interferon-γ was shown to occur during its production in culture and this reduced its anti-viral activity (Hogrefe et al., 1989; Ichimori et al., 1987).

* The contents of this chapter were included in a paper: M. Yang and M. Butler. 2000. Enhanced erythropoietin heterogeneity in a CHO culture is caused by proteolytic degradation and can be eliminated by a high glutamine level. Cytotechnology (in press).

Serum contains specific protease inhibitors which can reduce product degradation in serum-supplemented cultures. However, the disadvantages in using serum in production processes, have led to the widespread use of low protein serum-free formulations for the growth of producer cell lines such as those transfected for synthesis of specific glycoproteins. In these cultures a reduction in proteolytic activity during cultivation of the cells may be achieved by addition of a protease inhibitor. However, these inhibitors are expensive and cause difficulty in product recovery (Schlaeger et al., 1987; Teige et al., 1994).

Other culture parameters may affect protease activity in the medium. It was reported that amino acid starvation (glutamine, asparagine, aspartate and serine) induced protease activity (Cartwright, 1994b).

In this chapter the heterogeneity of human EPO is determined during the batch culture of a transfected CHO cell line. The effect of glutamine concentration on this heterogeneity is also determined. Glutamine is an essential amino acid, a major energy source in proliferating mammalian cells and it serves as both carbon and nitrogen source (McKeehan, 1982; Reitzer et al., 1979). Glutamine in culture medium can prevent hybridoma and murine plasmacytoma cell apoptosis (Singh et al., 1994). Moreover, glutamine is involved in the biosynthesis of purines, pyrimidines and amino sugars, and the efficiency of recombinant protein glycosylation (Castro et al., 1995; Nyberg et al., 1999).

7.2 Results

7.2.1 Effect of glutamine on CHO cell growth and EPO production

The effect of a high (20 mM) or a low (2 mM) glutamine concentration on the growth and productivity of the transfected CHO cells was compared to that of the standard serum-free medium containing 4 mM glutamine. The cells were inoculated at 1 X 10^5 cells / ml into 7 ml culture medium in a 25 cm^2 T-flask. Viable cell number and EPO

concentration was determined daily in each culture from day 1 to day 7 (Fig. 7.1). Under standard conditions (4 mM gln) cells grew with a maximum specific growth rate of (μ= 0.042 h^{-1}) for 5 days after which there was a decline in the viable cell concentration. There was no significant difference in the growth profile in the culture at 2 mM glutamine. However, the maximum specific growth rate of cells in the culture with a high glutamine concentration was 0.032 h^{-1} and the final yield of cells in this culture was about 50% lower.

The highest EPO concentration was attained in the low glutamine culture at 250 U/ml followed by the standard and high glutamine cultures (Fig. 7.1b). Although the EPO concentration in the high glutamine culture was relatively low, the cell specific productivity of EPO was 161 U/10^6cell-day which was higher than the values calculated from the other cultures (Table 7.1). This indicated that the EPO production was not correlated with cell yield.

From this experiment the high glutamine in the culture resulted in a decrease of cell growth and an increase of EPO specific production. This is similar to previously reported results obtained for monoclonal antibody production in high glutamine condition (Flickinger et al., 1992).

7.2.2 The effect of glutamine on cell metabolism

Substrate consumption and by-product formation was measured in each of the cultures over 7 days (Fig. 7.2). Glutamine was almost completely depleted in the low gln culture after 4 days and in the standard culture after 5 days. The rate of ammonia production is reflected by the initial glutamine level in the cultures, with the concentration reaching 6.2 mM in the high gln culture and 2.15 mM in the standard culture after 7 days. Specific utilization and production rates were calculated as mentioned in Materials and Methods. Table. 7.1 shows the increased specific rates of glutamine utilization and ammonia production with increasing glutamine level in culture medium. The rate of glutamine consumption and ammonia production in the high glutamine culture (6.29 μmole/10^6 cells

per day) was X3 that of the standard culture. The rate of glucose consumption and lactate production decreased with increased glutamine concentrations in the culture. The metabolic coefficient, Qammonia / Qgln, progressively decreased whereas the coefficient Qlactate / Qglucose increased with higher initial glutamine levels. This metabolic data suggests an reciprocal relationship between the use of glucose and glutamine for energy metabolism as has been reported previously (Zielke et al., 1978).

7.2.3 EPO heterogeneity during culture progression

The heterogeneity of EPO during the course of a standard batch culture was examined from culture samples taken at regular intervals over a period of 10 days. The samples were concentrated and analyzed by SDS-PAGE and Western blotting detection with an anti-EPO Mab. This analysis showed a gradual increase in the width of the EPO band from day 3 to day 7. By reference to marker proteins, the molecular weight range of EPO at day 3 was 33-39 kDa, whereas on day 7 and day 10, the range extended from 31 kDa to 39 kDa. This suggested a greater heterogeneity of the recombinant EPO at the end of the batch culture (Fig. 7.3), possibly resulting from molecular degradation.

The altered pattern of EPO heterogeneity was confirmed by 2D-electrophoresis combined with Western blot analysis (Fig. 7.4). Protein patterns are shown at day 4 and day 7 of a standard culture (Fig. 7.4a and b) as well as for day 7 samples taken from a high glutamine (Fig. 7.4c) or low glutamine culture (Fig. 7.4d). Seven protein spots were observed from the 4 day culture supernatant extending over a pI range of 4.06-4.67 and a molecular weight range of 33-39 kDa. The pattern of spots from the day 7 culture supernatant under standard conditions was different. The last three protein bands (with high pI) disappeared and some new spots with lower molecular weight and lower pI were observed (Fig. 7.4b). The patterns observed in Figs. 4c and 4d were taken from day 7 samples of cultures grown under different glutamine concentrations. At low glutamine (2.5 mM; Fig. 7.4d)) the pattern appeared to be the same as under standard conditions. However, at high glutamine (16 mM; Fig. 7.4c), the pattern was identical to that at day 4 for a standard culture. In particular, the low molecular weight bands characteristic of a

degraded product observed at day 7 in standard and low glutamine cultures (Fig. 7.4b & d) were absent in the sample from the high glutamine culture (Fig. 7.4c). This appeared to indicate less breakdown of EPO under high glutamine conditions.

In order to determine if the degradation of EPO from day 4 to day 7 occurred as a result of extracellular components, a cell-free supernatant taken from the 4 day culture was incubated for 3 days at 37°C. Further analysis by 2-D electrophoresis showed an identical pattern to the original 4 day sample. This indicated that the observed altered pattern of EPO from day 4 to day 7 required the presence of cells. This suggests that any degradative enzymes causing these changes would be produced by the cells over this time period.

7.2.4 Analysis of the N-linked glycan profile of EPO by FACE

The N-linked oligosaccharide profile of EPO was analyzed by FACE from samples taken from a batch culture. EPO was purified from culture samples taken at days 4 and 7. The N-linked glycans were removed by incubation with PNGase F with or without the presence of sialidase. Separation of these samples by electrophoresis (Fig. 7.5) indicated that there was no difference in the resulting patterns between day 4 or day 7 samples for either the PNGase F treated (lanes 3 and 4) or the PNGase F/ sialidase treated (lanes 5 and 6) samples. The sialylated glycans (lanes 3 and 4) from purified EPO contained at least seven different structures with one prominent band at a position corresponding to 8 GU (glucose units) as related the glucose ladder in lane 1.

The result of the analysis by FACE indicated that the profiles of sialylated and asialylated N-linked glycans structures of EPO did not change over the culture period between days 4 and 7. This indicated that any EPO heterogeneity would have to be explained by changes in the polypeptide structure.

7.2.5 Analysis of the N-glycan profile of EPO by HPLC

In order to confirm the FACE analysis by an independent technique, the sialylated N-glycans released from purified EPO were also analyzed by HPLC using the anion exchange column, GlycoSep C. The oligosaccharides were detected by a 2-AB label. A typical chromatogram of EPO oligosaccharides is shown in Fig 7.6. There are 7 peaks which corresponds to the number of bands identified by electrophoresis.

EPO oligosaccharide peaks were identified by comparison with the retention times of oligosaccharide standards containing variable number of sialic acids. The relative peak areas of the mono- (peak 1), di- (peak 2), tri- (peaks 3-4) and tetra- (peaks 5-7) sialylated structures were calculated as 12.1%, 14.8%, 28.2% and 49.7% respectively (Fig 7.6a). The data showed that the major portion of the EPO glycans were tetra-sialylated with the most prominent peak occurring at a retention time of 24 min. The multiple peaks corresponding to each state of sialylation probably reflect different antennarity or variable lactosamine repeats.

Analysis of samples for days 4 and 7 by HPLC indicated identical glycan patterns. This result confirmed that the sialylation of EPO at the end of the batch culture was not reduced which is consistent with the results obtained by FACE.

7.2.6 Protein degradation during culture

We attempted to determine whether the observed time-dependent increase in EPO heterogeneity during batch culture was due to changes in polypeptide structure. The N-linked and O-linked oligosaccharides were removed from EPO by enzymatic deglycosylation and the resulting peptides were analyzed by SDS-PAGE and Western blotting (Fig. 7.7). A 4-day sample from a culture in standard medium (containing 4 mM glutamine) resulted in a protein band with a molecular weight of 16.5-19.1 kDa (lane 1). This corresponded to the position of the band from non-glycosylated EPO from *Streptomyces* (not shown). However, the 7-day sample of the same CHO culture resulted

in a significantly broader band with molecular weight range of 15.8–19.9 kDa (lane 2). This suggested that a proportion of the EPO peptide may have been degraded toward the later stages of the batch culture. This evidence combined with the observed consistent glycosylation pattern suggests that the enhanced EPO heterogeneity during batch culture was likely to be due to proteolytic break down.

7.2.7 Effect of glutamine on EPO heterogeneity

Evidence from the data presented from the 2-dimensional electrophoresis in Fig. 7.4 suggested that glutamine levels in the culture had an effect on EPO heterogeneity, with an apparent decrease in degradation at high glutamine. This finding was confirmed by the appearance of the deglycosylated EPO bands in lanes 3 and 4 of Fig. 7.7. These were taken from samples of culture at day 7 at low and high glutamine respectively. The narrow band width for the sample from the high glutamine culture is similar in appearance to the day 4 band under standard conditions (lane 1), whereas the low and standard glutamine culture samples at day 7 (lanes 2 and 3) are wider and are consistent with the occurrence of degradation.

The effect of excess glutamine on EPO degradation was confirmed by analysis of these samples by simple one dimensional SDS-PAGE (Fig. 7.8). The results showed that the increased breadth of the protein band from day 4 to day 7 observed under low or standard glutamine conditions was not observed at high glutamine. Thus, considering that there was no observed change in the glycosylation pattern, glutamine appeared to have an effect in reducing EPO peptide degradation during the course of the batch culture.

The effect of glutamine was also determined in a fed-batch culture in which it was attempted to maintain a standard concentration of glutamine throughout the growth period. Cells were inoculated into standard medium (4 mM glutamine) and fed with glutamine (4 mM) on day 3 and day 5. Thus, a total of 12 mM glutamine was added to the culture. However, the characteristics of the isolated EPO from this culture were

identical to those from a batch culture in standard medium. This showed that under these conditions of glutamine feeding the extent of EPO degradation was not reduced.

Table 7.1 Specific consumption and production under different glutamine concentration (U or μmole/ 10^6 cells/day).

Initial Gln (mM)	QEPO/ (U/10^6cell/d)	qGln (μmole/ 10^6 cell/d)	qNH_3 (μmole/ 10^6 cell/d)	qGlc (μmole/ 10^6 cell/d)	QLactate (μmole/ 10^6 cell/d)	qAmm/ qGln	qLac/ qGlc
2	130	1.13	0.74	13.0	15.4	0.65	1.18
4	132	1.85	1.18	11.0	14.5	0.64	1.32
20	161	6.29	3.55	6.7	13.0	0.56	1.94

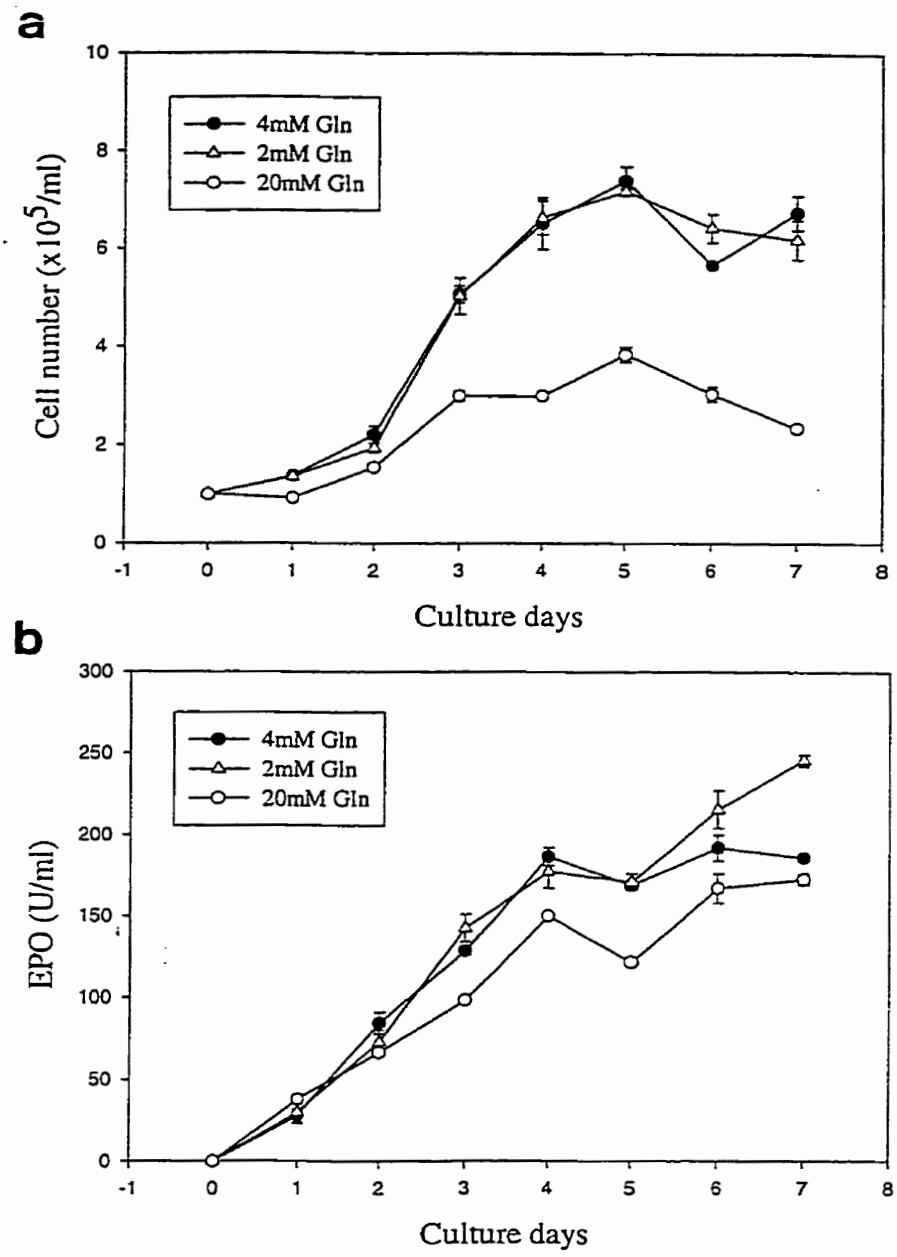


Fig. 7.1 Cell yield and EPO concentration in cultures with different glutamine levels. CHO cells were inoculated at $1X10^5$ cells ml^{-1} into 7 ml in standard (4 mM gln), low gln (2 mM) or high gln (20 mM) media in $25cm^2$ T-flask and cultured for 7 days. Viable cells and EPO concentrations were determined from day 1 to day 7. Values are means ± SEM of duplicate cultures.

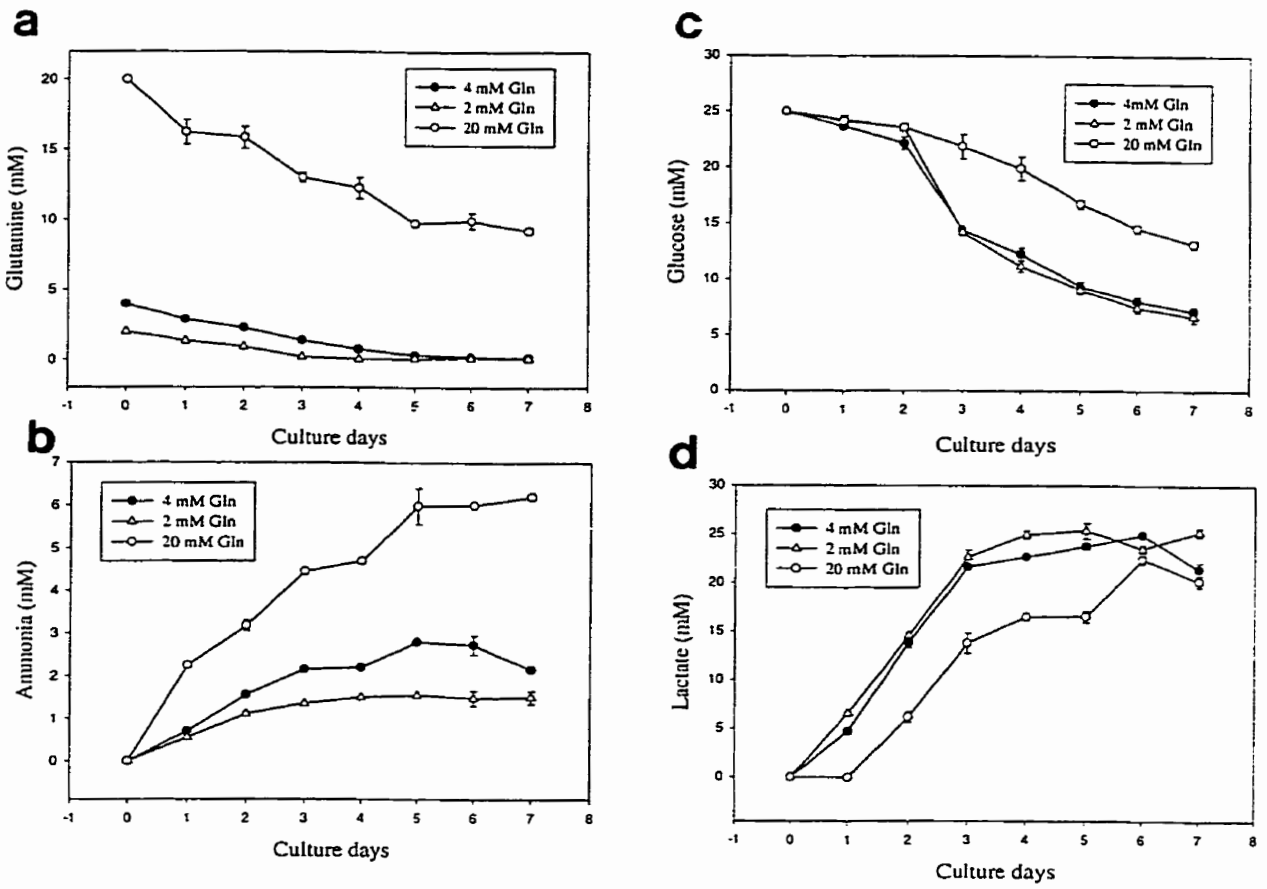


Fig. 7.2 Substrate utilization and by-product formation. CHO cells were inoculated at $1X10^5$ cells ml^{-1} into 7 ml media containing different concentrations of glutamine in $25cm^2$ T-flask and cultured for 7 days. Supernatants were harvested from day 1 to day 7. Glutamine (a), ammonia (b), glucose (c) and lactate (d) concentrations were measured. Values are means ± SEM of duplicate cultures.

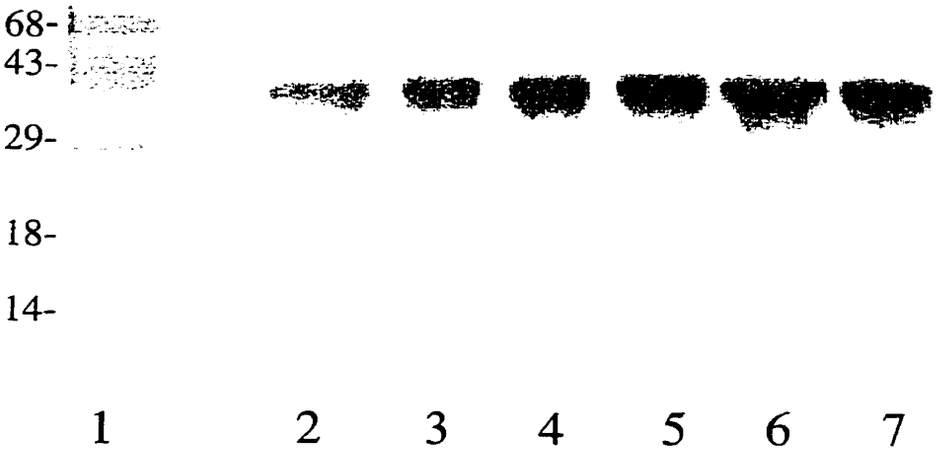


Fig. 7.3 Gel electrophoresis of EPO during the course of a culture. Supernatants were harvested at time intervals from standard cultures, concentrated and separated by 14% SDS-PAGE. The proteins were transferred to an NC membrane and detected by mouse monoclonal anti-EPO antibody. Lane 1 shows the separation of protein molecular weight marker (X 10^3 kDa). Lane 2-7 are culture supernatants harvested at days 3, 4, 5, 6, 7, and10.

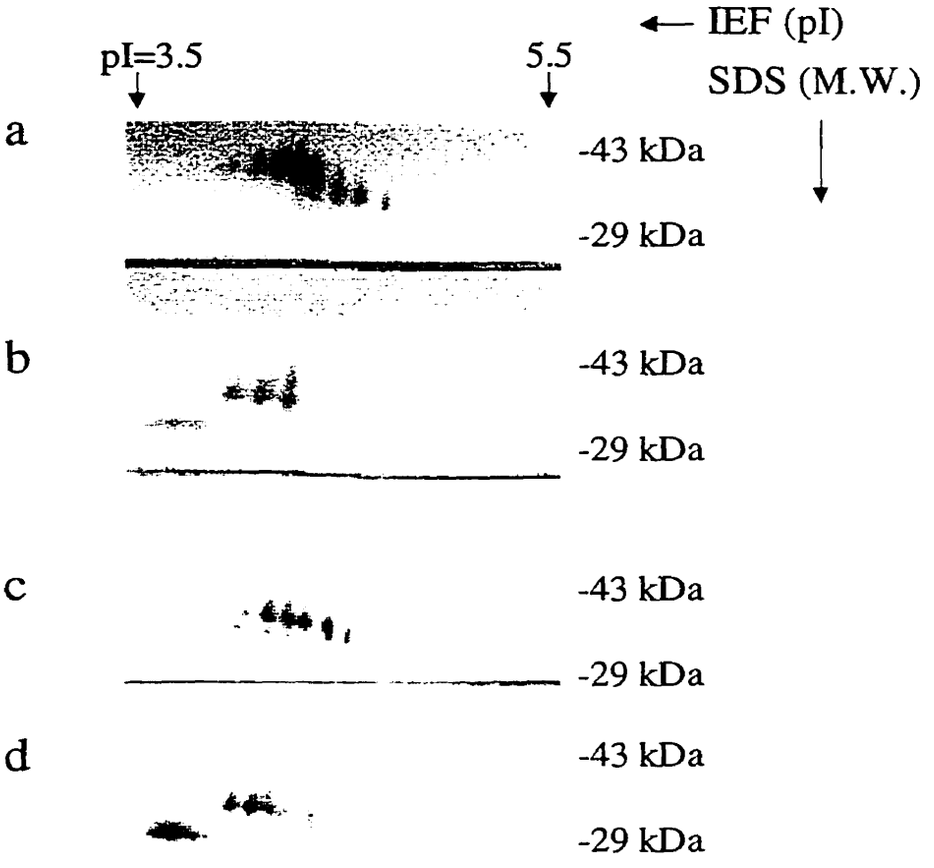


Fig. 7.4 Two-dimensional electrophoresis and Western blot analysis of EPO. Supernatants from CHO cell cultures were desalted and concentrated. EPO were separated by pI in the first dimension followed by SDS-PAGE in the second dimension and analysed by a Western blot. (a) Standard culture at day 4. (b) Standard culture at day 7. (c) High gln (16 mM) culture at day 7 and (d) low gln (2.5 mM) culture at day 7.

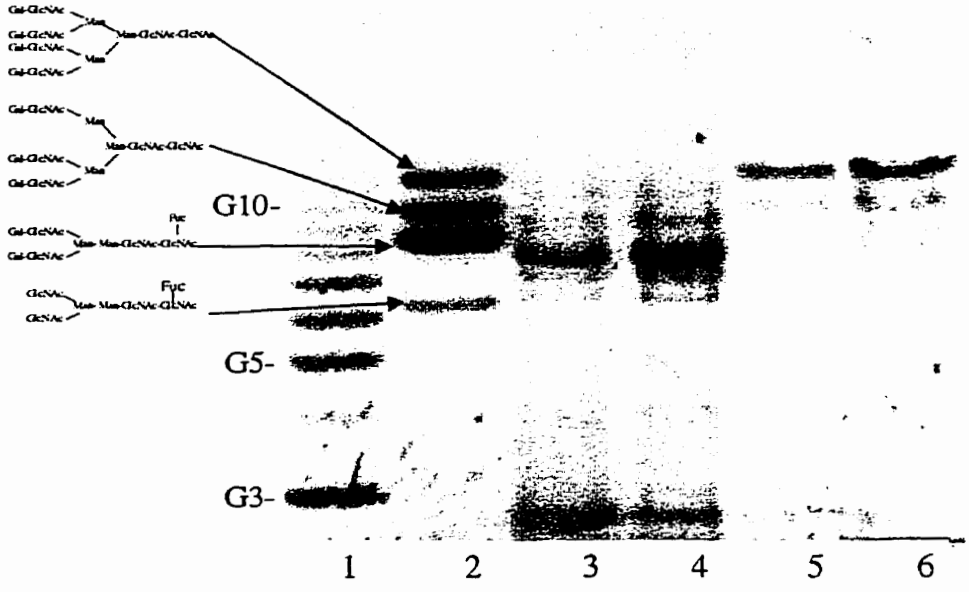


Fig. 7.5 EPO N-linked oligosaccharide profiles analyzed by FACE. Culture supernatants harvested on day 4 and day 7 were purified by an immunoaffinity column. The N-linked oligosaccharides were released by PNGase F or plus sialidase at 37 °C for 16 hours. The ANTS labeled carbohydrates were separated by polyacrylamide gel and visualized by detection of the fluorescence under UV illumination. Lane 1: glucose ladder; lane 2: carbohydrate standards - asialo-agalacto-biantennary with core fucose; asialo biantennary with core fucose; asialo triantennary and asialo tetraantennary. Lane 3 and 4 are sialylated N-linked glycans from culture supernatants collected on day 4 and day 7. Lane 5 and 6 are asialo N-linked glycans from culture supernatants collected on day 4 and day 7.

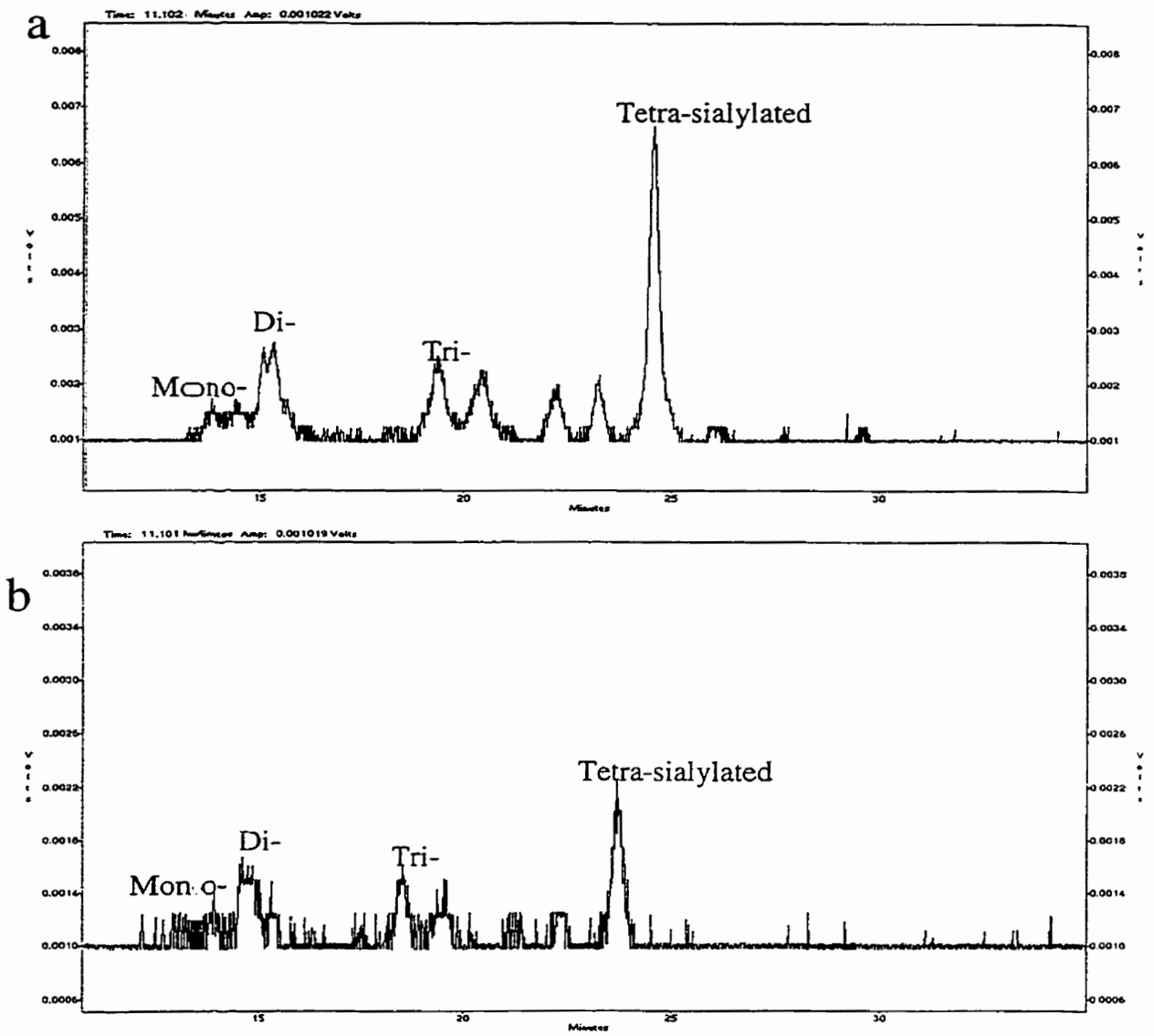


Fig. 7.6 Analysis of sialylated N-glycans of EPO by HPLC. N-linked glycans released from 4- (a) and 7–day (b) culture samples were labeled with 2-AB and analyzed by ion exchange chromatography with a GlycoSep C column using gradient (b). The peaks were identified by comparison with the retention times of oligosaccharide standards.

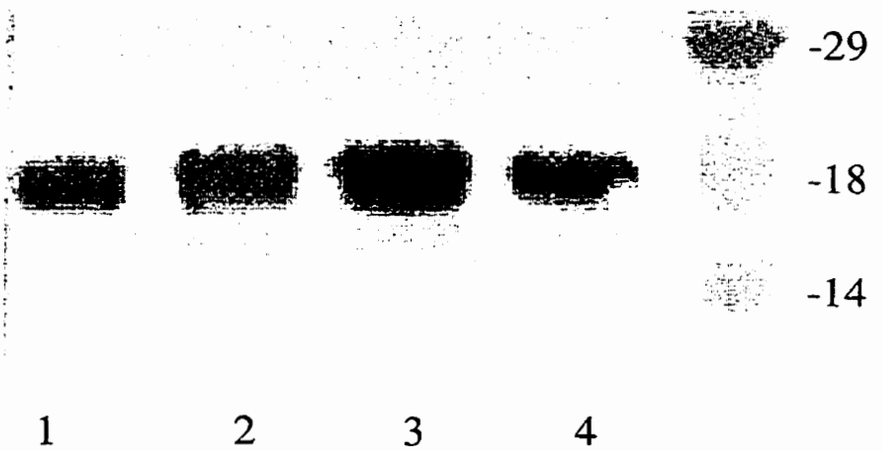


Fig. 7.7 Immunoblot analysis of enzymatically deglycosylated EPO. Culture supernatants were collected on day 4 or day 7. The desalted samples containing EPO were concentrated and treated with PNGase F, O-glycosidase and sialidase at 37°C for 16 h. The treated samples were separated by 14% SDS-PAGE and transferred to NC membrane. Lane 1 is supernatant containing 4 mM glutamine collected on day 4, Lane 2-4 are supernatants containing 4 mM, 2.5 mM and 16 mM glutamine respectively harvested on day 7. Molecular weight markers are indicated (X 10^3 kDa).

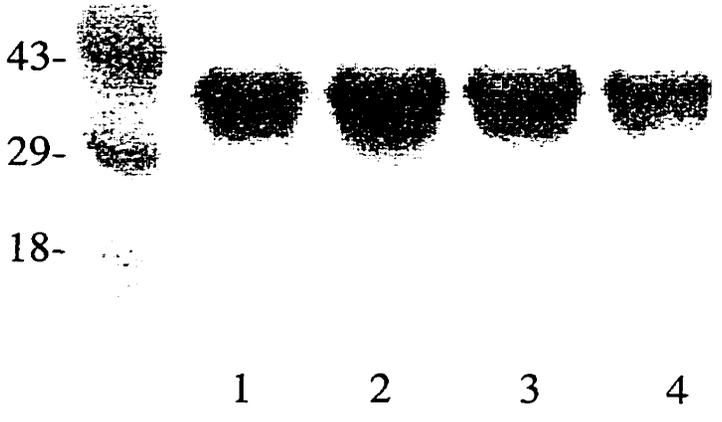


Fig. 7.8 Gel electrophoreis of EPO from cultures with different glutamine levels. Culture supernatants with different concentrations of glutamine were collected on day 4 or day 7. Samples were desalted, concentrated and separated by 14% SDS-PAGE. The protein was transferred to NC membrane and detected by Western blot analysis with a monoclonal anti-huEPO antibody. Lane 1 is the supernatant from a standard containing 4 mM glutamine harvested on day 4. Lane 2-4 are supernatants containing 4 mM, 2.5 mM and 16 mM glutamine respectively harvested on day 7. Molecular weight markers are indicated (X 10^3 kDa).

7.3 Discussion

The structural heterogeneity of glycoproteins produced from cells in culture is due to a number of factors including variable intracellular glycosylation, variable extracellular glycosidase and proteolytic degradation. The cell culture parameters known to affect glycosylation include glucose depletion (Gershman and Robbins, 1981; Hooker et al., 1995), ammonia accumulation (Andersen and Goochee, 1995; Borys et al., 1994; Gawlitzek et al., 1998; Jenkins and Curling, 1994; Yang and Butler, 2000a), growth rate (Hayter et al., 1993), lipid availability (Jenkins et al., 1994) and dissolved oxygen (Kunkel et al., 1998). These parameters may change during the course of a batch culture and this may well result in an increased heterogeneity over time. Such changes are exacerbated by the release of variable glycosidases and proteases secreted from viable cells or from lysis of dead cells.

Changes in the glycan structure have been analysed during the course of many culture systems (Ferrari et al., 1998; Gawlitzek et al., 1995; Hooker et al., 1995; Munzert et al., 1996). The presence of extracellular sialidase has been reported responsible for the loss of terminal sialic acid during the culture of anti-thrombin III (Munzert et al., 1996) and DNase (Ferrari et al., 1998) in batch culture of CHO cells. A progressive loss of sialic acid caused by an increased level of sialidase in the culture fluid can also be shown (Gramer et al., 1995). For EPO production from CHO cells we report the presence of at least 7 different glycoforms as analysed by both FACE and HPLC anion exchange, showing that the predominant structure was a tetra-antennary sialylated structure with other structures having lower antennarity and sialylation. However, the glycan patterns were identical throughout the culture, which indicated that extracellular sialidase activity was not evident in our culture system.

The stability of the EPO oligosaccharide pattern in our culture system may be due to a number of reasons. Firstly, glucose was not completely consumed and was maintained at a relatively high level (7 mM) even on at the end of the culture period. Secondly, the level of ammonia at the end of culture may not have been high enough to induce the

carbohydrate heterogeneity of EPO (Yang and Butler, 2000a). Thirdly, the susceptibility of a glycoprotein to glycosidase degradation may vary between proteins and may depend upon the protein structure. It is possible that a fully glycosylated and non-denatured EPO is resistant to any glycosidase that may be present in the cultures.

The enhanced structural heterogeneity of EPO during the course of our CHO batch culture was found to be entirely due to peptide degradation. The evidence for this was the increased width of the electrophoretic band of de-glycosylated EPO and an altered pI range at the end of the culture. The degradation occurred during the latter part of the culture (day 4 to 7) and did not occur as result of incubation in conditioned cell-free medium. This evidence suggests that the effect was due to a protease activity released from the cells. The high viability of the cell population throughout the culture, including the period in which peptide degradation occurred, argues for the secretion of protease from metabolically active cells.

Although proteases may be secreted from many cell lines in culture, the enzymatic activity may be minimised in cultures supplemented with serum. Serum contains a large quantity of protease inhibitors, estimated at up to 15% of the total protein (Travis and Salvesen, 1983). However, despite the many advantages of serum-free media, the potential proteolytic degradation of secreted proteins is enhanced in media not supplemented with serum (Curling et al., 1990; Schlaeger et al., 1987; Sugimoto et al., 1992; Teige et al., 1994). The CHO cultures described here were grown in a serum-free medium formulation developed in our laboratory and did not include components likely to inhibit any protease activity.

The characteristics of the proteases released by various mammalian cell lines have been studied with respect to the type of protein cleavage and susceptibility to inhibition. Proteases of hybridoma cultures have been characterised as similar to lysosomal cathepsin D (Schlaeger et al., 1987) or as a serine protease (Kratje et al., 1994). For BHK cell cultures, a dipeptidyl aminopeptidase has been described (Gawlitzek et al., 1995). Satoh et al., (1990) described the production of two types of proteases from

CHO cultures - exopeptidases and endopeptidases. The exopeptidase had an aminopeptidase activity which increased linearly with the time and correlated with an increase in the non-viable cell count, suggesting release from lysed cells. The endopeptidase was described as a cysteine protease which was secreted continuously by viable cells.

Wang et al., (1985) analysed the susceptibility of human erythropoietin to proteolytic cleavage. They showed that there is a small region of the peptide that is sensitive to digestion by trypsin, chymotrypsin, V-8 protease and Lys C. Limited proteolysis with trypsin leads to the fragmentation of EPO into two domains of 16 kDa. However, the proteolytic cleavage we show for EPO in the CHO culture is clearly quite different with an estimated reduction in size of the native peptide chain of 700 Da which approximates to the cleavage of about 6 amino acid residues. This phenomenon of limited fragmentation has also been shown in other systems. Gawlitzek et al., (1995) reported the removal of up to 8 amino acids following proteolytic degradation of interleukin-2 secreted from BHK cells. A fragmentation of up to 10 amino acids was reported from interferon-γ secreted from CHO cells (Goldman et al., 1997).

The observed molecular size of EPO analyzed from the early phase of the CHO cultures suggests that this consists of an intact peptide structure, corresponding to authentic human EPO (34-38.5 kDa) (Sasakiet al., 1987). Peptide heterogeneity is observed only during the later stages of culture with the appearance of a truncated peptide. This differs from previous work on recombinant IFN-γ produced by CHO cells, in which Curling et al., (1990) and Goldman et al., (1997) reported that no full length peptide was detected at any stage of cultivation.

The effects of changes of carbon substrate levels in culture have been studied for many cell lines. High concentrations of glutamine caused reduced growth rates but enhanced antibody production of hybridomas (Omasa et al., 1992; Flickinger et al., 1992). Similarly our results showed that a high glutamine concentration caused a decreased specific cell growth rate of the CHO cells but an enhanced specific EPO productivity.

The high glutamine also showed the characteristic metabolic changes of a decreased Qamm / Qgln coefficient but an increased Qlac / Qglc coefficient as has been noted previously for other mammalian cell lines (Hassell and Butler, 1990;. Butler et al., 1991). However, the net ammonia production in cultures is increased at high glutamine. In a previous publication we showed that the IC-50 of ammonia added to a CHO culture was 33 mM and that cell yield was reduced by 19% at 10 mM NH_4Cl (Yang and Butler, 2000a). Although this suggests that CHO cells may be less sensitive to ammonia than many cell lines (Hassell et al, 1991), the generation of ammonia at high glutamine was likely to be the inhibitory factor to cause the observed reduction in the cell growth rate of the CHO cells.

A surprising effect of a high glutamine was that the apparent peptide degradation observed in our standard CHO cultures was eliminated. This result is consistent with several studies which have shown that the induction of protease may be a direct result of amino acid starvation (Cartwright, 1994b). Froud et al., (1991) showed that production of a truncated form of the HIV envelope protein, gp120 from CHO cells was due to extracellular protease activity and this coincided with the depletion of several amino acids in the culture medium. A reduction of this proteolytic activity was achieved in a fed-batch culture in which the amino acid concentration was maintained at a critical level. Gawlitzek et al., (1995) showed that several truncated variants of IL-2 lacking up to 8 N-terminal amino acids were observed when cells were grown under limiting serine or aspartate concentrations. The fact that there was significant peptide degradation in our fed-batch culture which maintained glutamine at around the standard concentration (4 mM) suggests that this was below the critical glutamine concentration for reduced protease induction.

There are several possibilities for the mechanism of reduced protease activity in cultures with a high level of glutamine. It is possible that a low level of amino acids in the standard CHO cultures at day 4 cause the induction of a protease to be secreted from viable cells. This relates to previous work from our laboratory in which we showed that cultures supplemented with glutamine dipeptides but with a low level of amino acids

induced the secretion of an extracellular protease from hybridoma cells (Christie and Butler, 1994). However, we cannot exclude the possibilities of a specific effect of glutamine in inhibiting protease function or of protecting EPO from protease attack in the medium. Further work is required to determine the precise mechanism behind the influence of glutamine on the proteolytic enzyme activity.

7. 4 Conclusion

It was shown that in a batch culture of stably transfected CHO cells EPO was produced with a consistent glycosylation pattern throughout but with a truncated peptide in the later stage of the culture. Supplementation of the culture with a high level of glutamine eliminated the observed reduction in peptide size. It is possible that a regime of continuous feeding of nutrients, particularly amino acids may be important to ensure the synthesis of a recombinant glycoprotein with a consistent molecular structure.

CHAPTER 8

The effect of the intracellular nucleotide sugar-pool on the glycosylation of recombinant erythropoietin in a mammalian cell culture*

8.1 Introduction

Intracellular nucleotide sugars are activated sugars that are precursors for oligosaccharide synthesis. UDP-GlcNAc and UDP-GalNAc are the two forms of UDP-GNAc and can be interconverted by an isomerase enzyme. The metabolic pathway for the synthesis of UDP-GNac involves fructose-6-P as an intermediate that forms GlcN-6-P in the presence of a nitrogen source. This metabolite is then transformed to UDP-GlcNAc which is in equilibrium with UDP-GalNAc. These nucleotide sugars are important precursors for the glycosylation of proteins, a process that is mediated by a series of specific glycosyltransferase enzymes present in the Golgi apparatus of mammalian cells.

Several factors are known to perturb the intracellular UDP-GNAc pool concentration. The addition of glucosamine which is the direct precursor of the activated nucleotide sugar has previously been shown to increase the level of UDP-GNAc pool in chicken embryo fibroblasts (Koch et al., 1979). Ammonia and glutamine in the culture medium incorporate into frc-6-P to synthesize GlcN-6-P, which is a precursor of the UDP-GNAc (Ghosh et al., 1960). Culture media supplemented with NH_4Cl has been shown to increase intracellular UDP-GNAc synthesis in hybridoma, BHK and CHO cell lines (Barnabé and Butler, 1994; Ryll et al., 1994). A low glutamine concentration in the culture may limit the available nitrogen source and cause a decrease in intracellular UDP-GNAc.

* The contents of this chapter were included in a manuscript: M. Yang and M. Butler. The effect of the intracellular nucleotide sugar-pool on the glycosylation of recombinant erythropoietin in a mammalian cell culture (in preparation).

Different mechanisms have been suggested to explain the effect of ammonia on protein glycosylation. Ammonia is known to raise the pH of acidic intracellular compartments (Schneider et al., 1996). An intracellular pH change could affect the activity of glycosyltransferases or glycosidase, e.g. branching enzymes GlcNAc-transferases III and IV (Schachter, 1986). Andersen and Goochee (1995) found a twofold decrease in the activity of α-2,6-sialyltransferase in the presence of NH_4Cl in CHO cell cultures. In several recent studies, different explanations for ammonia effects on protein glycosylation have been discussed. Gawlitzek et al. (1998) and Grammatikos et al. (1998) suggested that an increase of the intracellular UDP-GNAc pool caused by elevated concentrations of NH_4^+ or glucosamine in the culture medium contributed to the formation of more complex oligosaccharide structures. Another finding indicated that a high intracellular UDP-GNAc level can impair the transport of CMP-NeuAc, the precursor for sialic acids into the trans Golgi compartment resulting in decreased sialylation (Pel Rijken et al., 1995).

We showed that ammonia in the culture medium inhibited glycan complexity and sialylation of rHuEPO (Yang and Butler, 2000c). The purpose of the chapter presented here was to develop an understanding of the relationship between the intracellular UDP-GNAc pool and the glycosylation of EPO. The UDP-GNAc pool was perturbed by varying the glutamine concentration or supplementing the cultures with glucosamine or ammonium chloride. The effect of these changes were examined with respect to heterogeneity, oligosaccharide structure and sialylation of glycosylated EPO.

8.2 Results

8.2.1 Effect of glucosamine on CHO cell growth

The effects of added glucosamine on CHO cell growth were examined. CHO cells were inoculated at $1x10^5$ cells ml^{-1} into 7 ml CHO-SFM2.1 or medium containing different concentrations of glucosamine up to 40 mM in 25 cm^2 T-flasks. Viable cell concentrations were determined after 4 days growth by trypan blue exclusion. The cell

growth was reduced significantly in cultures containing above 10 mM glucosamine. However, the cell yields were not affected significantly when the glucosamine concentration was below 5 mM in the culture medium (Fig. 8.1).

The culture pH was reduced by the addition of glucosamine. At 40 mM added glucosamine the culture pH was 6.5 which potentially could be inhibitory to cell growth. Figure 8.1 shows the effect of glucosamine on cell yield with or without adjusting culture pH to 7.2. The results showed that the cell growth inhibition induced by glucosamine was greater following pH adjustment. The inhibitory concentration for 50% decrease in growth (IC-50) was estimated from the experiments at 15.8 mM without pH adjustment and 7.4 mM glucosamine for cultures adjusted to pH 7.2 (Fig. 8.1). This result indicated that glucosamine is more toxic to CHO cells under neutral pH conditions.

8.2.2 Cell metabolism in glucosamine containing cultures

The glutamine, ammonia, glucose, and lactate concentrations were measured in cultures supplemented with concentrations of glucosamine up to 40 mM. Table 8.1. shows that the specific rates of substrate utilization and by-product formation. The specific glucose utilization as well as lactate production decreased progressively as the added glucosamine concentration up to 40 mM. The specific rates of glutamine utilization and ammonia production were significantly increased as the elevated glucosamine level in the medium. The specific ammonium production rates were double and 15 fold higher in 10 mM and 40 mM glucosamine containing cultures compared with a control culture. The ammonia level was 13 mM in the culture containing 40 mM glucosamine, whereas it was only 3.5 mM in the control culture. This higher ammonia concentration in glucosamine-containing cultures may be caused by an increased glutamine utilization, glutamine degradation or by glucosamine metabolism.

8.2.3 EPO molecular heterogeneity

In order to determine the molecular heterogeneity of EPO induced by glucosamine, samples from cultures containing glucosamine were subjected to SDS-PAGE and Western blot analysis. The data showed that, as the glucosamine concentration increased in the medium up to 10mM, there was a gradual increase in the width of the EPO band, suggesting a greater heterogeneity of molecular forms. The molecular weight range of the EPO from the control culture was 33-39 kDa. Samples analyzed from the cultures containing above 10 mM glucosamine had a much broader band which appeared to extend from 18 kDa to 39 kDa (Fig. 8.2a). There was no further increase for EPO heterogeneity above 10 mM glucosamine.

In order to analyze further this heterogeneity, EPO peptides were prepared for electrophoresis by deglycosylation. Samples of EPO were enzymatically deglycosylated by PNGase F and O-glycosidase to remove both N-linked and O-linked carbohydrates. The immunoblot analysis of all the enzyme-treated samples showed a single protein band with molecular weight of 18 kDa which corresponded to a standard non-glycosylated EPO (Fig. 8.2b). This indicated that the effect of glucosamine was likely to be on the heterogeneity of the oligosaccharide side chains and not the EPO peptide.

The heterogeneity of EPO was analyzed by an alternative technique, 2D-electrophoresis combined with Western blot analysis (Fig. 8.3). Seven protein spots were observed from the control culture supernatant extending over a pI range of 4.06-4.67. The pattern of the EPO from the 10 mM glucosamine-containing culture was significantly different. The pI range was increased and extended from 3.5 to 7.5 and a total of more than twenty protein spots were determined. This result confirmed that glucosamine induced a significant increase in the molecular heterogeneity of EPO.

8.2.4 EPO production

The effect of glucosamine on EPO production was determined by a sandwich ELISA. The EPO samples were assayed following deglycosylation using PNGase F and O-

glycosidase. The EPO concentration in the control culture was significantly higher than cultures supplemented with glucosamine (Fig. 8.4), indicating reduced EPO production in response to the presence of glucosamine.

8.2.5 Oligosaccharide analysis

(a) Anion exchange chromatography using HPLC

EPO sialylation was examined by anion exchange chromatography using HPLC. The N-linked oligosaccharides were released enzymatically and labeled with 2-AB. The degree of glycan sialylation of EPO isolated from control (4mM gln), low glutamine (0.4mM gln), glucosamine and ammonia supplemented cultures were analyzed and compared (Fig. 8.5). The chromatographs showed a degree of variation in the oligosaccharide patterns from different cultures. The relative proportion of mono-, di-, tri- and tetra-sialylated glycans were calculated according to the relative peak areas as shown in Table 8.2. The data shows that a high proportion of tetra-sialylated oligosaccharides was present in the standard culture. The low glutamine sample showed a similar chromatography pattern. However, the relative proportion of EPO with four sialic acids was significantly reduced in the samples from the ammonia-supplemented and the glucosamine-supplemented cultures. The relative proportion of tetra-sialylated glycans decreased by 73 % in the ammonia-supplemented culture and by 40 % in the glucosamine-supplemented culture. This indicated that both ammonium and glucosamine inhibited glycan sialylation and / or antennarity.

(b) Analysis N-linked asialo-oligosaccharides

The oligosaccharide branching (antennarity) was analyzed by normal phase chromatography. The N-linked glycans derived from EPO isolated from control, low glutamine, ammonia and glucosamine containing cultures were treated with sialidase to remove sialic acid. The resulting N-linked asialo-oligosaccharides were derivatized with 4-AB and analyzed by HPLC.

An internal glycan standard, asialo-agalacto-biantennary was added to each sample before deglycosylation in order to standardize the analysis. The peaks could be grouped into bi-, tri- and tetra-antennary structures (Fig. 8.6). The chromatograph showed that the major oligosaccharide structures were of the tetrantannary type under standard culture conditions (Fig. 8.6b). However, significant differences in the oligosaccharide profiles were apparent in samples from the other cultures. The proportion of tetra-antennary structures was significantly reduced in the glucosamine-supplemented and ammonia-supplemented cultures (Fig. 8.6c,d). The proportion of the tetra-antennary structures from the low glutamine culture was slightly higher than the control sample. The relative proportion of each structure was analyzed by reference to the internal standard (Table 8.3). In the samples from the glucosamine-supplemented and ammonia-supplemented cultures there was a significant shift in the glycan profile with a lower proportion of tetra-antennary structures and a higher proportion of bi-antennary structures. The proportion of the tetra-antennary structure was reduced by 40% in the glucosamine-supplemented culture sample and by 60% in the ammonia-supplemented culture sample as compared to the control.

(c) N-linked oligosaccharide profile determination by FACE

The N-linked oligosaccharide profiles of EPO under different culture conditions were analyzed by FACE. The N-linked glycans derived from EPO isolated from control, low glutamine, ammonia and glucosamine containing cultures were derivatized with ANTS and the resulting glycans separated on a polyacrylamide gel. The fluorescent images of the bands were acquired using the Glyco Doc imager.

The results showed that the N-linked oligosaccharides contained one prominent band which was identified as the tetra-antennary structure with four sialic acids by reference to standards run under the same conditions (Fig. 8.7). The intensity of the major band decreased significantly in the sample from ammonia-supplemented and glucosamine-supplemented cultures compared with control and low glutamine cultures (Fig. 8.7a). However, the reduction of this band was more noticeable in the sample from the

ammonia-supplemented culture than the glucosamine-supplemented culture. Even though each lane was loaded with glycan samples from approximately the same quantity of original EPO it is noticeable that there was poor definition in the lanes corresponding to the ammonia-supplemented culture and the glucosamine-supplemented culture. This is likely to be due to greater molecular heterogeneity in these samples that leads to poor focusing on the gel. This data confirms the previous conclusion from the anion exchange chromatography that the degree of sialylation was reduced in the EPO from ammonia and glucosamine-supplemented cultures.

The asialo-glycans were also analyzed in order to confirm the variability of glycan antennarity under the different culture conditions. Each glycan sample was treated with sialidase to remove the terminal sialic acids from the structures. The gel profiles showed that the major band shifted to a position corresponding to about 12 GU, which had a similar migration distance to an asialo tetra-antennary standard (Fig. 8.7b). Densitometry of the digitized images from the gels allowed a determination of the proportion of each antennary group of structures (Table 8.3). The data showed close although not identical correlation with the HPLC data. These results confirmed the conclusion that ammonia and glucosamine reduced the proportion of tetra-antennary glycan structures. The proportion of tetra-antennary structures was reduced in the presence of glucosamine and ammonia by 26 % and 60 %, respectively while the tri- and bi-antennary structures were increased proportionally (Table 8.3).

8.2.6 Nucleotide sugar-pool analysis

The effects of culture conditions on intracellular nucleotide pools were examined. The intracellular nucleotides were extracted from cell lysates obtained from control, low glutamine, ammonia-supplemented and glucosamine-supplemented cultures. The extracts were analyzed by reverses phase HPLC.

The glucosamine-dependent UDP-GNAc formation was determined in the cells in a series of cultures supplemented with glucosamine up to 40 mM (Fig 8.8). The addition of

2.5 mM glucosamine resulted in a significant and substantial increase in the UDP-GNAc concentration as well as the proportion of UDP-GNAc as a percentage of the total nucleotides analyzed. The maximum concentration of UDP-GNAc was in cells derived from 10 mM glucosamine-supplemented cultures and was 22.5 fold greater than control cultures. However, the intracellular UDP-GNAc concentration did not increase further in cultures containing higher glucosamine levels up to 40 mM. This is consistent with the previous observation of feedback inhibition of hexosamine synthesis by nucleotide-sugars (Kornfield et al., 1964)

The UDP-GNAc pool of ammonium and glucosamine-supplemented cultures accounted for 60% of total nucleotides compared to 9.2% for control cultures. No significant increases in the percentage of UDP-GNAc pool were found in the cultures treated with concentrations of glucosamine higher than 2.5 mM. The total intracellular nucleotide pool was significantly higher in cells containing a high level of UDP-GNAc.

A summary of various parameters associated with the four culture conditions are presented in Table 8.4. Mean values of intracellular UDP-GNAc, ammonium concentration, the relative percentage of carbohydrate sialylation and antennarity under different culture conditions are shown. Cultures supplemented with NH_4Cl and glucosamine resulted in a pronounced increase in the UDP-GNAc pool. The cells obtained from cultures supplemented with 10 mM glucosamine or 30 mM ammonium chloride appeared to have similar intracellular UDP-GNAc concentrations. This was accompanied by a decrease in the percentage of tetra-antennary and tetra-sialylated glycan structures determined from the isolated EPO. However, these alterations in structure were far more pronounced in the ammonia-supplemented cultures.

Table 8.1 Specific lactate, ammonium production and glucose, glutamine consumption by cells in the culture containing different concentrations of glucosamine. Each value is a mean based on two independent cultures calculated using the equation described in the Materials and Methods.

Glucosamine (mM)	Glucose	Lactate	Glutamine	Ammonium
			(μmol / 10^6 cells-day)	
			Mean ± SEM	
0	7.44±0.45	22.3±2.22	2.25±0.23	2.47±0.19
2.5	6.59±0.01	19.4±1.14	2.37±0.06	2.54±0.17
5	4.80±0.11	16.8±0.18	2.39±0.13	3.09±0.18
10	2.67±0.11	18.8±2.69	3.00±0.23	4.48±0.19
20	3.72±0.62	13.7±1.57	3.92±0.03	12.0±0.30
40	4.25±1.35	12.5±1.30	5.32±0.07	37.0±2.90

Table 8.2 Relative peak area of EPO N-linked oligosaccharides with variable number of sialic acids as analyzed by HPLC.

	% sialylated glycan			
	Mono-	Di-	Tri-	Tetra-
Low gln	12.7	10.4	32.8	44.2
Control	5.4	15.2	25.5	53.9
10mM Glucosamine	11.6	12.0	44.8	31.7
30 mM NH4Cl	13.7	11	60.8	14.6

Table 8.3 Relative proportion of the asialo-glycan structures under different culture conditions analyzed by a normal phase HPLC and FACE.

	%		
	Bi-	Tri-	Tetra-antennary
HPLC			
Low glutamine	10.3	27.7	62
Control	13.4	25.2	59.6
10 mM glucosamine	20.0	42.1	37.9
30 mM NH_4Cl	32.9	40.8	25.9
FACE			
Low glutamine	20.2	22.5	57.3
Control	20	21.9	57.9
10 mM glucosamine	26.4	31	42.8
30 mM NH_4Cl	42.7	33.9	23.3

Table 8.4 Summary of EPO heterogeneity, ammonium concentration, intracellular UDP-sugar level and glycosylation under different culture conditions.

	EPO				Glycan (HPLC)	
Culture conditions	M.W. (kDa)	pI range	NH_4+ (mM)	UDP-GNAc (nmol/10^6 cells)	% tetra-sialylated	% tetra-structure
Low glutamine	33-39	ND	0.98	0.14 ± 0.03	44.2	62
Control	33-39	4.06-4.67	3.95	0.52 ± 0.005	53.9	59.6
Glucosamine (10mM)	18-39	3.5-7.5	4.78	9.60 ± 0.54	31.7	37.9
NH_4Cl (40mM)	27-37	4.18-6.05				
(30mM)			32.9	10.48 ± 0.23	14.6	25.9

EPO molecular weights were determined by Western blot. pI was determined by 2-D electrophoresis. Ammonium concentrations in 4 days culture supernatants were determined by a gas-sensing electrode. Intracellular UDP-GNAc concentrations were determined by HPLC. Oligosaccharides were analyzed by HPLC using the anion exchange and normal phase columns. ND = not determined

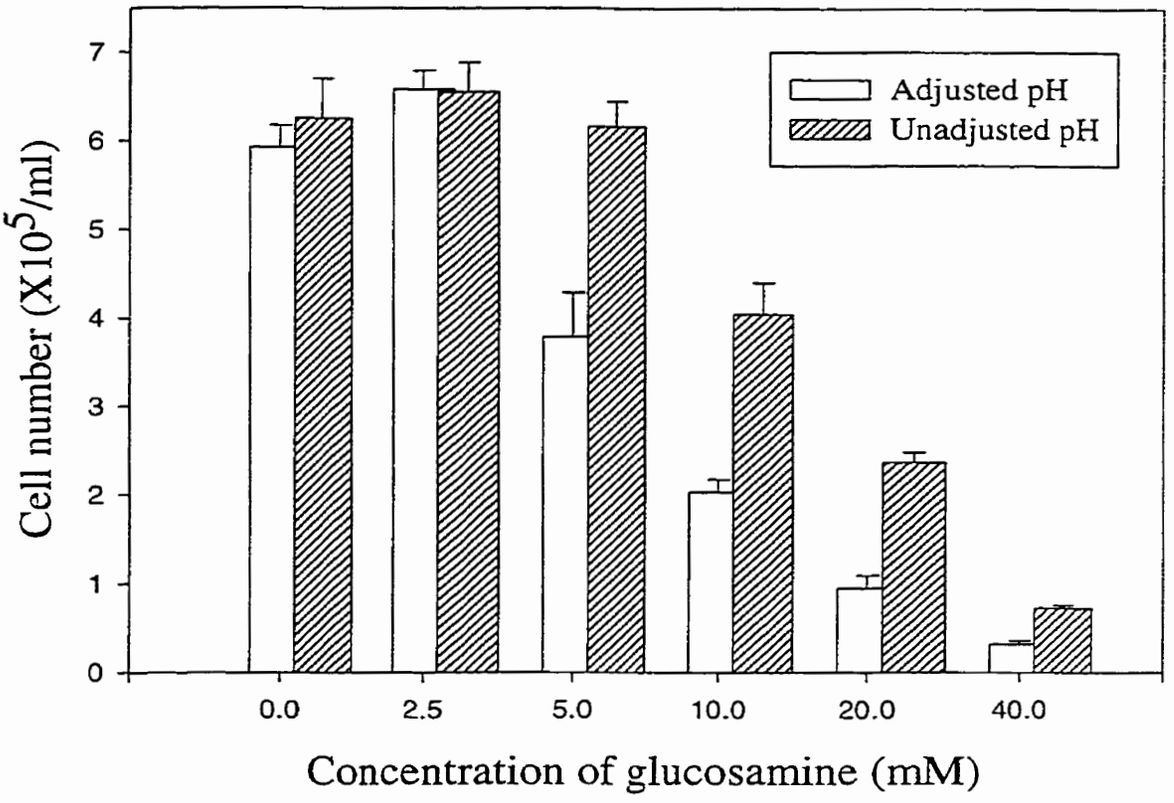


Fig. 8.1 Cell yields in cultures containing different concentrations of glucosamine. CHO cells were inoculated at $1x10^5$ cells ml^{-1} into 7 ml CHO-SFM2.1 containing different concentrations of glucosamine in $25cm^2$ T-flask. The media pH was either adjusted to 7.2 or unadjusted. The viable cell numbers were determined at day 4. Values are Mean ± SEM of duplicate cultures.

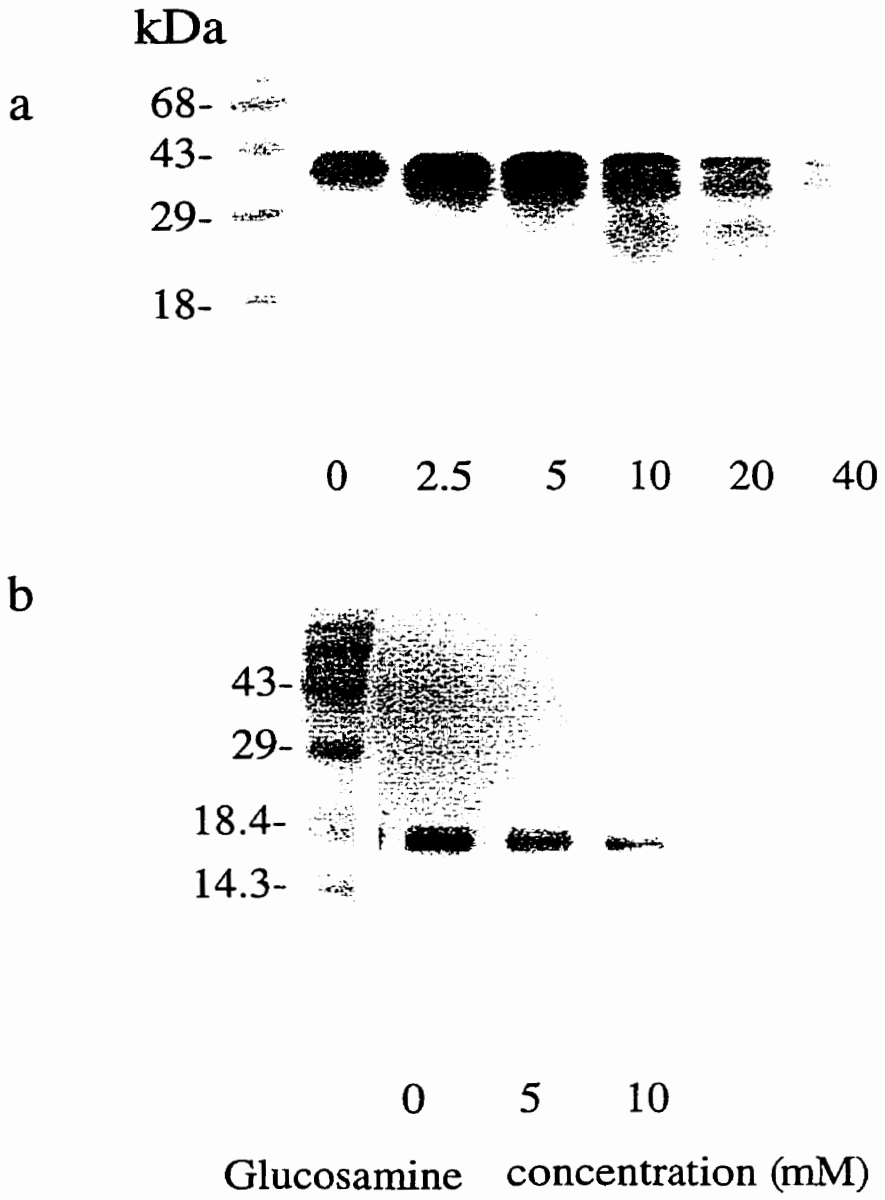


Fig. 8.2 Effect of glucosamine on EPO glycosylation. Cell culture supernatants with different concentrations of glucosamine were collected on day 4, concentrated and separated by 14% SDS-PAGE. The protein was transferred to a NC membrane and detected by a monoclonal anti-huEPO antibody. The samples from cultures containing different glucosamine concentrations are indicated. (a) Glycosalated EPO; (b) Deglycosylated samples. Molecular weight standards are indicated ($x10^3$ kDa).

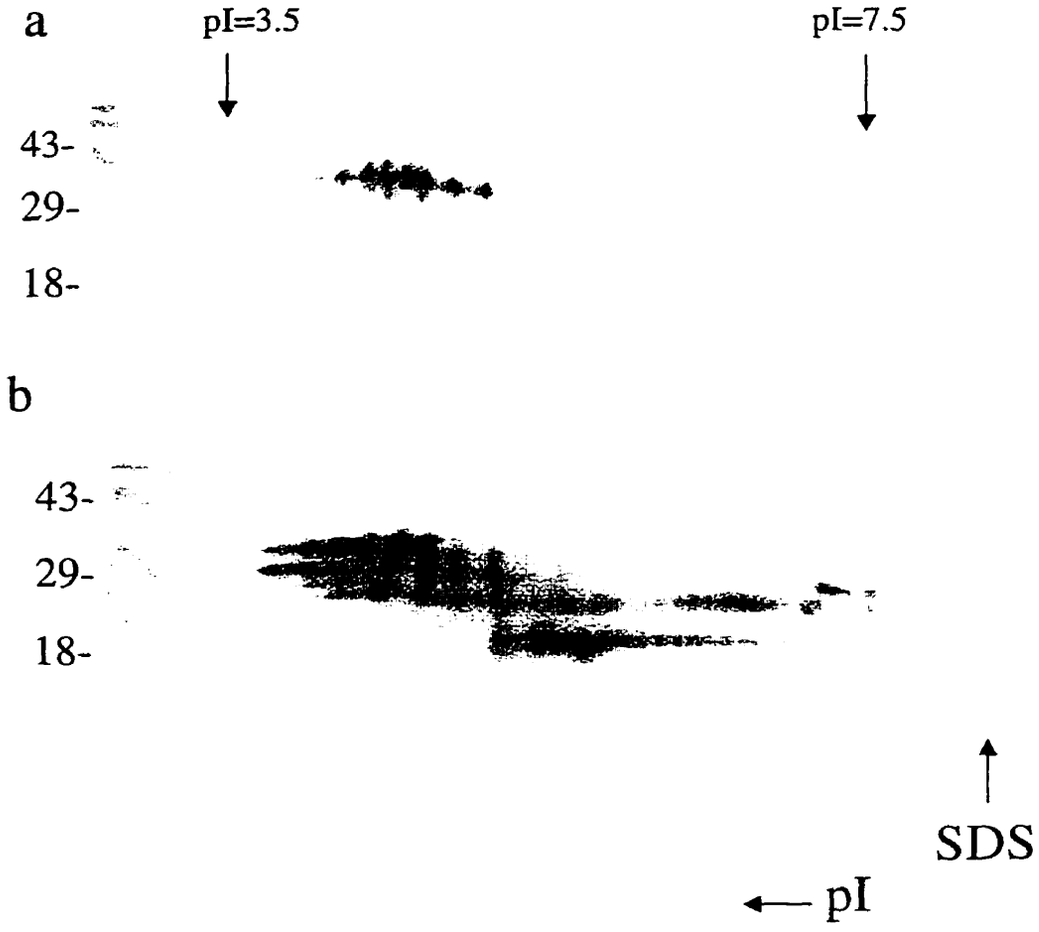


Fig. 8.3 Two-dimensional electrophoresis of EPO samples. Supernatants containing 0 mM (a) and 10 mM (b) glucosamine were desalted and concentrated. EPO was analyzed by 2-D electrophoresis, separated by pI (ampholyte pH 4-6) in the first dimension followed by SDS-PAGE in the second dimension and Western blot. The pI range and molecular weight standards are indicated ($x10^3$ kDa).

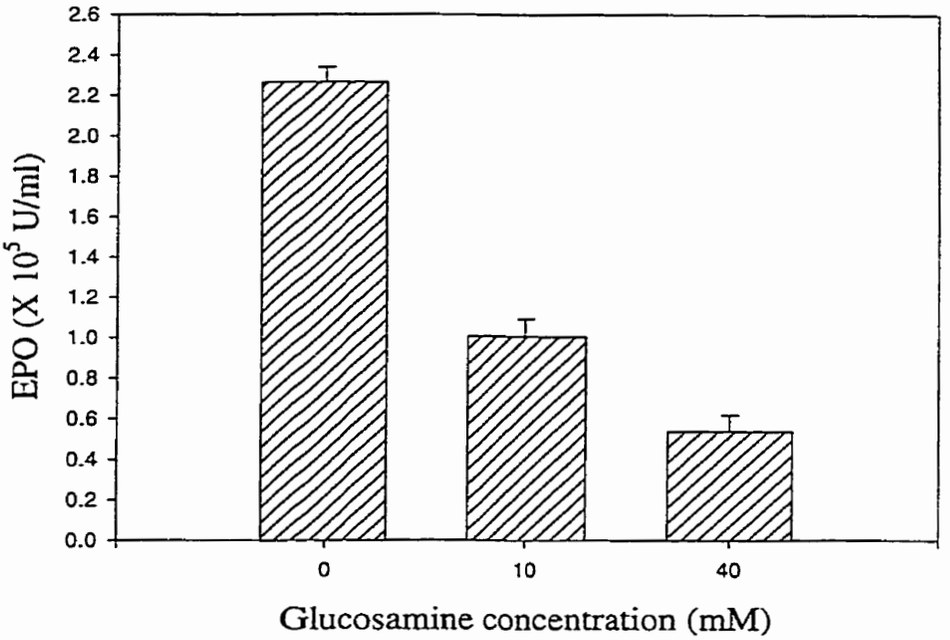


Fig. 8.4 EPO production in cultures containing different concentrations of glucosamine. CHO cells were inoculated at $1x10^5$ cells ml^{-1} into 7 ml CHO-SFM2.1 containing different concentrations of glucosamine in $25cm^2$ T-flask. Culture supernatants were collected on day 4 and EPO concentrations were determined by ELISA after deglycosylation. Values are Mean ± SEM of duplicate cultures.

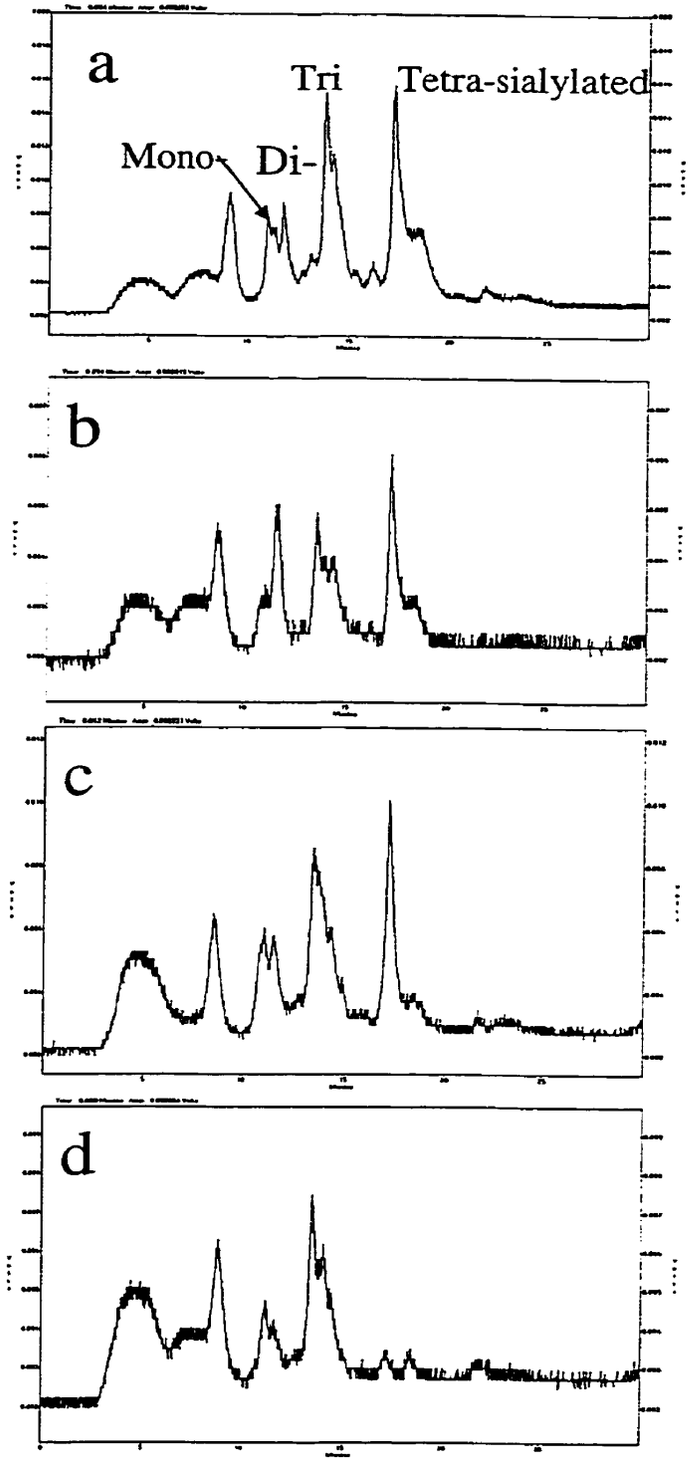


Fig. 8.5 EPO glycans analyzed by HPLC using anion exchange column. Glycans released from purified EPO were labeled with 2-AB and analyzed by GlycoSep C column using gradient a. (a) Low glutamine culture; (b) Control culture; (c) Culture containing 10 mM glucosamine and 30 mM NH_4Cl (d).

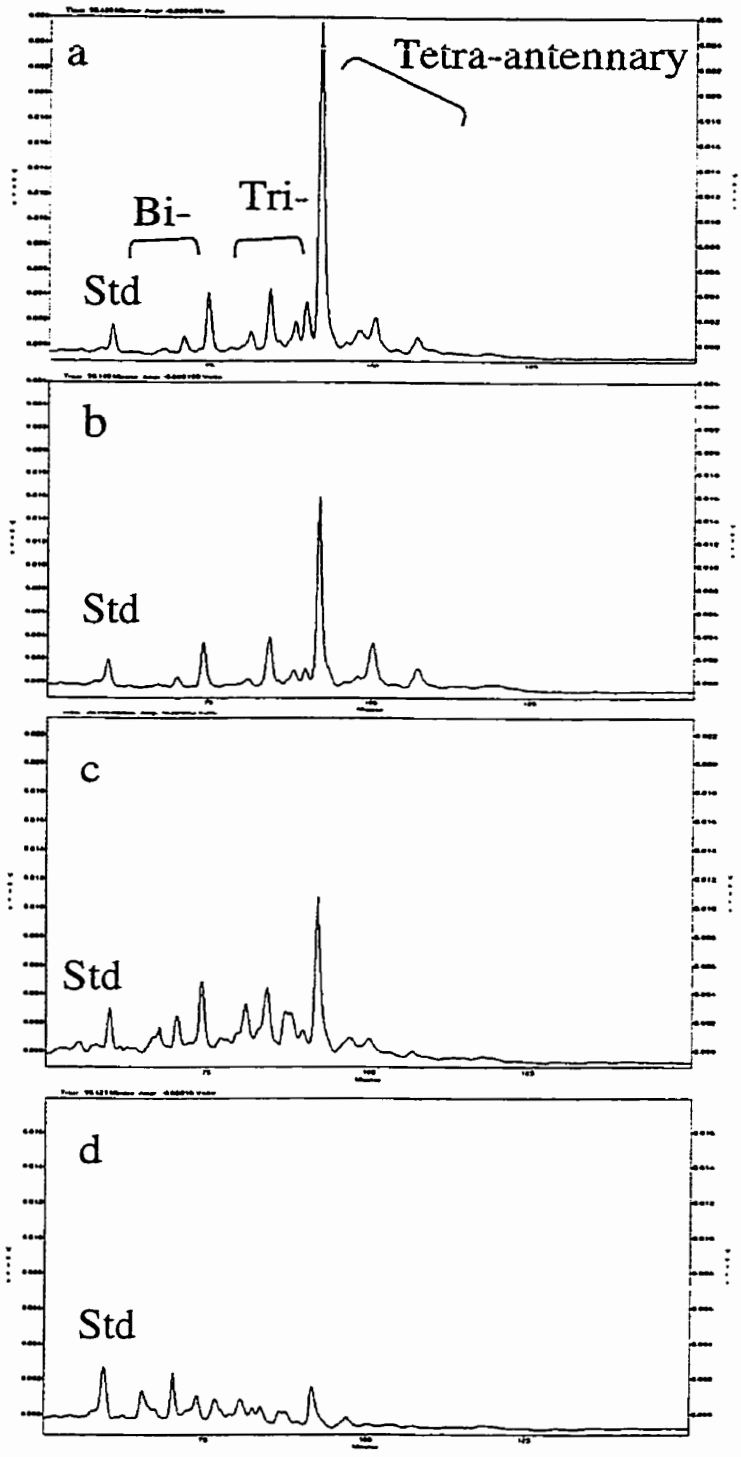


Fig. 8.6 EPO oligosaccharide analysis by normal phase HPLC. Glycans released from purified EPO were labeled with 4-AB. Samples were analyzed by GlycoSep N column. (a) Low glutamine culture; (b) Control culture; (c) Culture containing 10 mM glucosamine and 30 mM NH_4Cl (d).

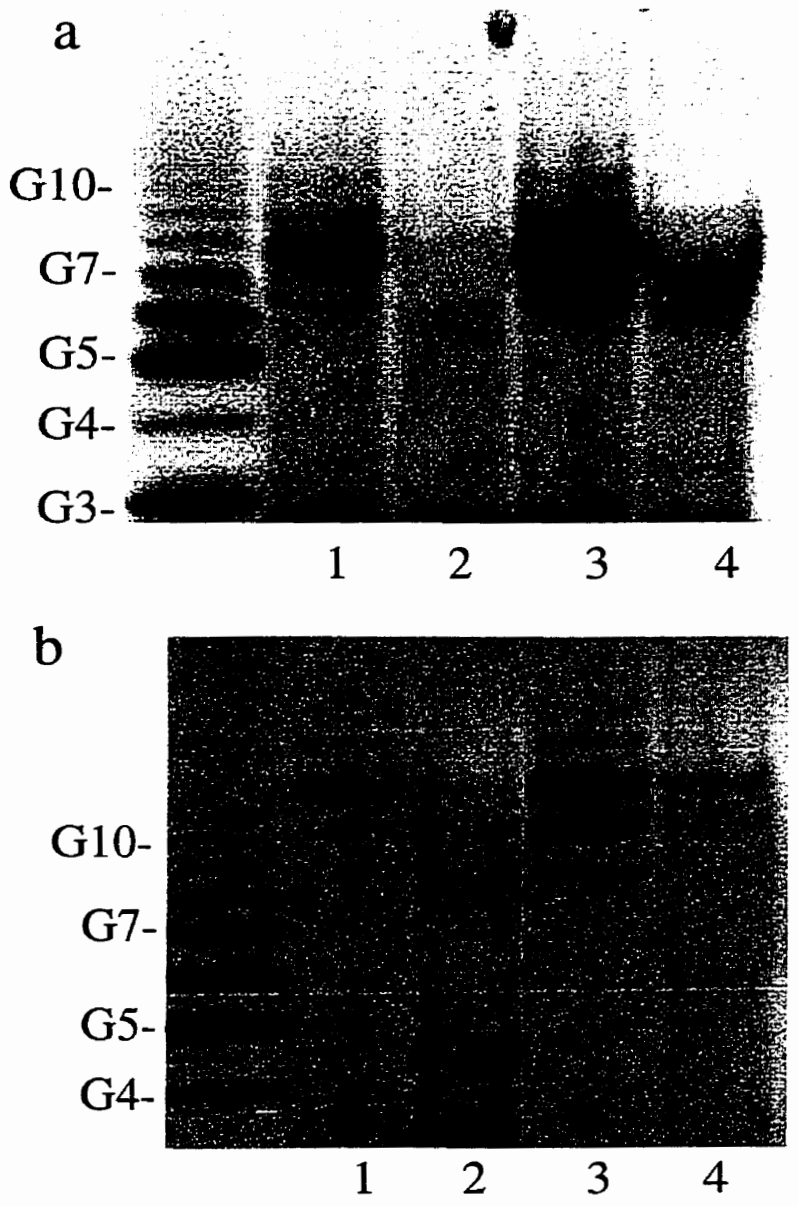


Fig. 8.7 EPO N-linked oligosaccharides analyzed by FACE. Culture supernatants from control (lane 1), culture containing 30 mM ammonia chloride (lane 2); low glutamine (0.4 mM gln) (lane 3); and 10 mM glucosamine (lane 4) were collected on day 4 and purified by an immunoaffinity column. The carbohydrates released from EPO were analyzed by FACE. (a) N-linked oligosaccharide profiles. (b) Asialo-oligosaccharide profiles. Glucose ladder as indicated.

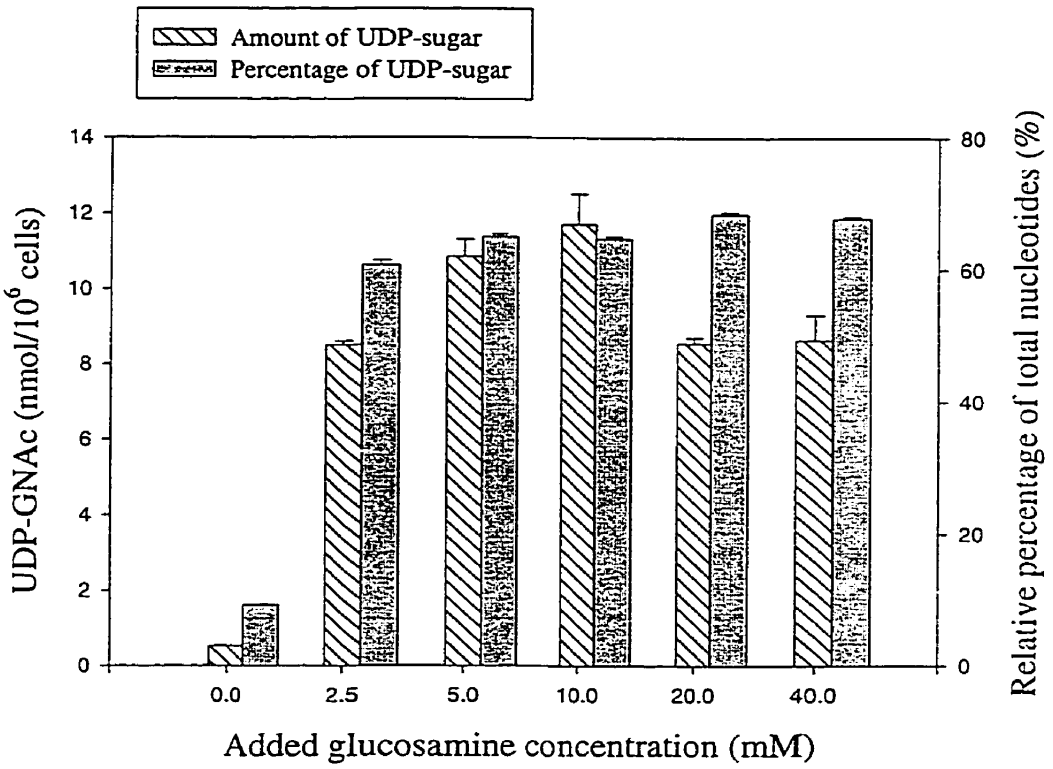


Fig. 8.8 Glucosamine effects on CHO intracellular UDP-sugar pool levels. Cultures were established in $25cm^2$ T-flask by inoculating cells at $1x10^5$ cells ml^{-1} into CHO-SFM2.1 containing different concentrations of glucosamine. After 4 days culture, the intracellular nucleotides were extracted and analyzed by HPLC using a reversed phase column.

Discussion

The purpose of the work presented here was to characterise the metabolic changes in CHO cells that were associated with culture conditions that caused alterations to the glycan structures of EPO. There are three N-glycan structures of EPO, each with a potential for variable branching (antennarity) from 2 to 4. Each of the antennae has a potential sialic acid terminal group. Variations in these glycan structures cause variability in the isolelectric point and molecular weight of the whole glycoprotein molecule.

The mammalian cell culture conditions that affect the extent of glycosylation of a producer cell line are not well understood. We have previously shown that the media concentration of ammonia can give rise to changes in the glycosylation process associated with the secretion of human EPO from a transfected CHO cell line (Yang and Butler, 2000a; Yang and Butler, 2000c). These changes in intracellular glycosylation gave rise to altered states of antennarity and sialylation of the glycans of the secreted EPO.

The mechanism by which ammonia interferes with glycosylation is not clear. It has been reported previously that amongst the various metabolic effects of ammonia is the increase in the intracellular pool of UDP-GNAc (Ryll et al., 1994). This may occur by amination of frc-6-P to GlcN-6-P, which is a direct precursor for the formation of UDP-GNAc. The nucleotide sugar, UDP-GNAc is an important carrier molecule in the glycosylation process. It is involved in the transfer of GNAc to dolichol phosphate which leads to an activated core oligosaccharide for initial attachment to an N-glycan site on the growing peptide chain in the endoplasmic reticulum. UDP-GNAc is also a substrate for GlcNAc transferase enzymes involved in the elongation and the branching of the glycan chain in the Golgi apparatus.

It is possible that an altered UDP-GNAc pool may change the formation of the oligosaccharide structure in recombinant proteins. Culture conditions chosen for CHO

cells in our experiments included low glutamine, addition of glucosamine and NH_4Cl, all of which have the potential to change the intracellular UDP-GNAc pool level and/or protein glycosylation.

Glucosamine is an intermediate in the pathway of UDP-GNAc synthesis. Therefore cells grown in cultures supplemented with glucosamine have the potential for enhanced intracellular levels of UDP-GNAc. Glucosamine is also an inhibitor of cell growth in tumor cells and viruses (Bekesi et al., 1969; Friedman et al., 1977) In our CHO cultures concentrations of glucosamine up to 5 mM did not alter cell growth but significant inhibition occurred at concentrations higher than 10 mM. Growth inhibition by glucosamine was greater at neutral pH which may be a reflection of ammonia accumulation in the culture and is consistent with the pH-dependent growth inhibition of ammonia (Doyle and Butler, 1990). The ammonia is formed following metabolic deamination of glucosamine (Comb and Roseman, 1958). However our experiments showed that glucosamine is more toxic to CHO cells than ammonia. The IC-50 for glucosamine was determined as 16 mM, as compared to a previously determined value of 33 mM for ammonium chloride (Yang and Butler, 2000a).

Our results show that increased concentrations of a glucosamine supplement in cultures led to a progressive increase of the EPO molecular weight range (18-39 kDa) that is associated with a decrease in the extent of glycosylation. Glucosamine also induced a wider range of pI values (3.5 - 7.5) of secreted EPO that was associated with decreased sialylation. Similar observations have been reported for glycosylation in virus-infected cells treated with glucosamine (Datema and Schwarz, 1979; Klenk et al., 1972; Scholtissek et al., 1975). Furthermore, hemagglutinin produced by Fowl plague virus infected chicken embryo fibroblast cells were found to be completely lacking in glycosylation under high levels of glucosamine (Schwarz and Klenk, 1974).

Glucosamine caused an increase (x8) in the intracellular UDP-GNAc pool of CHO cells on addition of 2.5 mM glucosamine. However, the subsequent increase in UDP-GNAc at higher levels of glucosamine was minimal. Supplementation of CHO cells with NH_4Cl

(30 mM) also resulted in an increase in intracellular UDP-GNAc to a level comparable with that observed in the glucosamine-supplemented culture. However, the increased range of EPO molecular weight and pI values induced by glucosamine and NH_4Cl were not comparable (Yang and Butler, 2000a). The EPO M.W. and pI ranges were 27-37 kDa and 4.18-6.06 in 40 mM ammonium supplied culture, whereas were 18-39 kDa and 3.5-7.5 from 10 mM glucosamine containing culture. The number of protein bands analyzed by 2D-electrophoresis were 10 and over 20 from 40 mM ammonium and 10 mM glucosamine supplemented cultures respectively.

Since both glucosamine and ammonia serve as precursors for the synthesis of the UDP-sugars, the increased intracellular nucleotide sugar pool may be responsible for the observed effects on glycosylation. Several studies have indicated a relationship between intracellular UDP-sugar pool concentration and protein glycosylation (Gawlitzek et al, 1998; Grammatikos et al., 1998; Pel Rijken et al., 1995). However, there is evidence to indicate that glucosamine itself, rather than its metabolic products may inhibit glycosylation. Firstly, Koch et al., (1979) reported that removal of glucosamine from a chicken embryo fibroblasts culture in which glycosylation was inhibited resulted in the re-establishment of the normal rate of glycosylation of an influenza virus glycoprotein within 10 min. The onset of glycosylation was associated with a rapid reduction of the intracellular level of glucosamine without a significant change in the concentrations of its metabolites for at least 30 min after reversal of the block. Similar observation was reported by Datema and Schwarz, (1979). Secondly, the inhibition of $Glc_3Man_9(GlcNAc)_2$-PP-dolichol synthesis by MDCK cells in the presence of glucosamine could not be mimicked by other amino sugars such as galactosamine, mannosamine, or N-acetylglucosamine (Elbein, 1987) This suggests that glucosamine exerts its effect directly on glycosylation, rather than via one of its metabolites.

The mechanism of inhibition of glycosylation by glucosamine has been investigated by several researchers. Plagemann and Erbe, (1973) have shown that glucosamine is transported into cells by the glucose transport system. Thus, glucosamine can competitively inhibit the transport of glucose (Spiro, 1958). At a low concentration

glucosamine enters N1S1-67 cells by facilitated diffusion via the glucose transport system whereas, at high concentrations, simple diffusion may occur (Plagemann and Erbe, 1973). The metabolic effects of glucosamine are reversible. After removal of this amino sugar, the cell could renew its synthesis of $Glc_3Man_9(GlcNAc)_2$ instead of $Man_3(GlcNAc)_2$ and $Man_7(GlcNAc)_2$ (Pan and Elbein, 1982). The explanation for this is that the glucose transport assumed a normal rate after removal of glucosamine that acted as a transport inhibitor.

Some reports indicate that glucosamine inhibits the formation of lipid-linked oligosaccharides at an early stage of assembly. When influenza-virus-infected chicken embryo cells were treated with glucosamine, incorporation of [^{3}H]mannose into lipid-linked oligosaccharide and into glycoprotein was decreased. This led to the elimination of whole oligosaccharide chains in influenza virus hemagglutinin (Datema and Schwarz, 1979). Similarly, Pan and Elbein, (1982) found that glucosamine inhibited the incorporation of radioactively-labeled mannose into lipid-linked oligosaccharides and into glucoproteins of influenza virus-infected MDCK cells. When MDCK cells were incubated with glucosamine, there was a complete shift in the size of the lipid-linked oligosaccharide from the normal, $Glc_3Man_9(GlcNAc)_2$ to $Man_7(GlcNAc)_2$ and $Man_3(GlcNAc)_2$ (Pan and Elbein, 1982). It was observed that the shortened oligosaccharides were not suitable precursors for the enzymic transfer to proteins. In CHO, NIL, 3T3 cells (Gershman and Robbins, 1981; Stark and Heath, 1979), vesicular stomatitis virus-infected BHK cells (Turco, 1980), and mouse myeloma cells (Stark and Heath, 1979), there was a similar alteration in lipid-linked oligosaccharide profiles in glucose starved cultures.

In our study, the rate of glucose consumption as well as the concomitant rate of production of lactate was significantly lower in a glucosamine-supplemented culture. This indicates that glucose utilization was inhibited by the presence of glucosamine. This suggests the possibility that glycosylation inhibition by glucosamine could occur by competitive inhibition of glucose transport, thus causing a depletion of intracellular glucose.

This suggested that the increased UDP-sugar pool level was not the factor that caused abnormal glycosylation. Because of the shortage or abnormal precursors, the oligosaccharide attachment might alter. One EPO molecule could have zero to four attached oligosaccharides and zero to fourteen sialic acids. This may explain why glucosamine induced so much EPO heterogeneity whereas ammonium did not.

The N-linked oligosaccharide analysis showed that the relative proportion of EPO with four sialic acids and tetraantennary structure were significantly reduced in the ammonium containing culture. The N-linked oligosaccharides from the culture supplemented with 10 mM glucosamine showed a similar pattern to the ammonium sample. This indicated that both ammonium and glucosamine inhibited the oligosaccharide sialylation and complexity but glucosamine has less effect than ammonia. A similar observation that glucosamine inhibits sialylation of recombinant interleukin-2 mutant has been reported (Gawlitzek et al., 1998). However, our finding is opposite to the report by Gawlitzek et al., (1998) and Grammatikos et al., (1998). They found that the ammonium ion and glucosamine increased of oligosaccharide complexity in a recombinant protein secreted from cultivated BHK-21 cells.

Although glucosamine altered oligosaccharide sialylation and structures in a similar manner as ammonium, the degree was not to the same extent as ammonia. If glucosamine effects on glycosylation was through the inhibition of glucose transport, all of the glycan structures should proportionally reduce, not only the proportions of tetra-antennary and tetra-sialylated glycans. It has been reported that glucosamine was converted to ammonia and the usual products of glycolysis catalyzed by glucosamine-6-phosphate deaminase which was found in *E. coli* and hog kidney cells (Comb and Roseman, 1958). We proposed that the increased level of ammonium produced by glucosamine metabolism was responsible for the oligosaccharide changes. Although the ammonium level in glucosamine culture (10 mM) was not significantly higher than control (X2), the ammonia level we measured was extracellular ammonia concentration. Previous study indicated that the physiological consequences of adding ammonia extracellularly to the medium are very different to those resulting from ammonia produced intracellularly

(Schneider et al., 1996). Ammonia is known to raise the pH of acidic intracellular compartments, e.g. ER and Golgi compartments where glycosylation processing occurs (Schneider et al., 1996). An intracellular pH change could reduce the activity of glycosyltransferases or glycosidase, e.g. branching enzymes (Schachter, 1986) and sialyltransferase (Andersen and Goochee, 1995). This may be the mechanism by which ammonia effects on protein glycosylation.

Since glucosamine did not increase EPO heterogeneity and decrease the oligosaccharide sialylation as well as complexity to the same extent as ammonia, the increased pool size of UDP-GNAc caused by elevated levels of glucosamine and ammonium could not explain the glycosylation effects. In addition, the maximum EPO heterogeneity was observed at 10 mM glucosamine by Western blot, so the reduced oligosaccharide changes induced by glucosamine was not related to the concentration. Although the intracellular UDP-sugar concentration was lower in low gln culture than the control, the oligosaccharide structure did not show significant differences compared to the sample from the control culture. A similar result was observed for monoclonal antibody synthesis in hybridomas that led to the conclusion that UDP-sugar pool concentration did not directly affect protein glycosylation (Barnabé and Butler, 1998). It can be assumed that the elevated UDP-sugar pool is not the only factor involved.

Conclusion

In conclusion, the ammonia and glucosamine added to CHO cell culture had different effects on EPO heterogeneity although both of them significantly increased intracellular UDP-sugar pool concentrations. EPO molecular weight and pI range were 18-39 kDa and 3.5-7.5 in 10 mM glucosamine containing culture, whereas were 27-37 kDa and 4.18-6.05 in high concentration ammonium supplied culture. The reduced proportion of tetra-antennary and tetra-sialylated glycans by 10 mM glucosamine were 40% and 60-70% by the ammonium supplemented culture. Glucosamine affected EPO glycosylation possibly by two separated mechanisms. Glucosamine might inhibit glucose transport into cells and affect lipid-linked oligosaccharide synthesis which might be responsible for the increased

EPO heterogeneity. Secondly, the oligosaccharide changes induced by glucosamine might be caused by increased ammonia level in glucosamine containing culture from cell metabolism that explained glucosamine effects on glycan structures. Our study indicated that there may not be a direct relationship between intracellular nucleotide sugar pool and glycan structures.

CHAPTER 9

Conclusions and further studies

9.1 The development of an ELISA for glycoproteins

A sandwich ELISA was developed for the detection of EPO in culture supernatants. Good reproducibility of the ELISA is evidenced by the small coefficients of variations between wells, dilutions, and assays. However, it was found that the ELISA was more sensitive to denatured and deglycosylated EPO. This was a problem for the determination of EPO concentrations in culture supernatants, since non- or partially glycosylated EPO showed higher values than glycosylated EPO. Since this was found in the later stage of the study. However, EPO concentrations presented in the thesis were re-analyzed for glycosylated and deglycosylated samples by ELISA.

Before establishing an ELISA for a glycoprotein, it is critical to characterize the monoclonal antibody binding site. Ideally an antibody should be selected against the polypeptide which recognizes both glycosylated and deglycosylated proteins. Secondly, it is important to determine how close the epitope is to the attached carbohydrates. If the binding epitope is close to or buried by the attached glycans, the ELISA may be more sensitive to non- or partially glycosylated protein than glycosylated protein. This can be identified by determination of the binding capacity of the Mab to denatured, deglycosylated and glycosylated proteins using ELISA. The ideal choice is to select an antibody that has a binding site located on the polypeptide which is far away from attached oligosaccharide chains.

9.2 Glycoprotein solubility, purification and detection

The solubility of proteins differs considerably. EPO is insoluble in aqueous solutions and tends to form aggregates at high concentrations. Special treatment is required to bring EPO molecule into solution.

When using low pH buffer, EPO is insoluble. Proteins tend to be less soluble near the pI and more soluble farther from it. Many properties of proteins, including charge and solubility, are dependent on the pH of the solution.

Besides pH, another method to increase protein solubility is to add a solubilizing agent. There are three types of solubilizing agents: detergents, chaotropic agents, and reducing agents (Wheelwight, 1991). Detergents are classified by their ionic nature: anionic, cationic, and nonionic. Proper selection of the detergent is important. It is suggested that mild conditions, such as physiological pH and nonionic detergents (Tween or Triton), are required to avoid denaturation. In this study, purified EPO at high concentration was solubilized using PBS (0.1X) containing a detergent, 0.1% Tween $_{20}$.

Immunopurification has been perceived as one of the most popular methods for isolating a glycoprotein from cell culture. Affinity purification is a fast, simple procedure to purify a glycoprotein. EPO produced from CHO cell was isolated from culture supernatant in a relatively high yield with a simple purification procedure using an immunoaffinity column attached with a monoclonal anti-EPO antibody. However, 30% EPO in each sample did not bind to the column, since the monoclonal antibody had a high binding affinity to low or non-glycosylated EPO. Thus highly glycosylated EPO might not bind to the affinity column. Since the EPO from different cultures were went though the same purification procedure, the oligosaccharide structures were considered comparable.

During the development of immunoaffinity purification for a glycoprotein, a key step is to select and identify an antibody against the antigen. An antibody with (i) an intermediate affinity to antigen; (ii) binding site located on the polypeptide and (iii) binding site unrelated to the carbohydrates is more desirable. For purification of a highly

sialylated glycoprotein, the best purification procedure may be ion exchange chromatography followed by gel filtration chromatography. By this technique, the highly sialylated and glycosylated protein can be concentrated.

For detection of glycoproteins on SDS-PAGE, highly sialylated proteins could not be stained by the commonly used protein stain techniques, e.g. Commassie blue. Pepsin, the highly acidic protein stains very poorly using regular protein stain methods (Fairbanks, et al., 1971). Similarly, EPO could not be detected with Commassie blue, but only by a silver stain. The most usual detection method for glycoproteins is the carbohydrate-specific periodic acid-Schiff (PAS) staining methods which offers a good choice for staining of glycoproteins (Fairbanks, et al., 1971). Other methods include selective radiolabelling of the carbohydrate moiety prior to electrophoresis then glycoprotein detection by fluorography/autoradiography after electrophoresis, and the use of radiolabelled, flurorescent, or peroxidase-conjugated lectins (Hames, 1981).

Quantitative determination of glycoproteins is difficult. EPO was not successfully quantified by a non-specific protein assay, since non- or partially glycosylated protein gave higher adsorbance readings using an UV detector. Quantitation of glycoproteins on polyacrylamide gels stained with PAS has been reported. A linear relationship between the amount of protein and the area for fetuin and ovalbumin were observed (Matthieu and Quarles, 1973). This technique could be tested and applied to EPO or other glycoproteins.

9.3 Oligosaccharide analysis

A key feature of the glycosylation process in cells is that many of the reactions are often incomplete, leading to the secretion of a mixture of different glycoforms of a given protein. Common sources of heterogeneity of glycosylation include (1) variation in the initial site of attachment of the oligosaccharide to the protein, (2) variation in the branching reactions of N-linked glycosylation, and (3) variation in the addition of terminal sialic acid residues. Each of these sources of oligosaccharide heterogeneity can have significant effects on protein structure and function. Information as to the exact

structure of the relevant oligosaccharide moieties is important for the development of bioprocesses in ensuring consistency of glycosylation.

FACE is a rapid, simple, sensitive, and cost-effective method for carbohydrate analysis. FACE oligosaccharide profiling is informative and gives the properties of the oligosaccharides such as charge, size and sequence. Using the FACE technique, EPO N-linked oligosaccharide profiles were obtained. Moreover, the EPO N-linked oligosaccharide sequence was successfully determined. This is often a difficult step in the complete structural determination of a glycoprotein. The results indicated that EPO contains a typical N-linked complex oligosaccharide structure. The drawback of FACE method is that it is difficult to identify the sialylated oligosaccharides.

Carbohydrate analysis by HPLC has the advantages of fast separations, high resolution and great sensitivity. The HPLC separations for neutral and charged oligosaccharides have proved to be highly useful for analysis of derivatised oligosaccharides. The EPO glycans were separated into four groups which represent different numbers of sialic acids using anion exchange column. Normal phase column was used for the separation of the uncharged oligosaccharides. The results indicated that the major proportion of the oligosaccharide was tetra-sialylated (46 %) and tetra-antennary type oligosaccharides (60%). This correlated with the analysis by FACE which showed that the tetra-antennary structure comprised 64% of the total glycans.

However, a relative large quantity of material is required for both techniques. For every single analysis, the carbohydrate from 0.5 liter culture supernatant was the minimum requirement. Mass spectrometry (MS) on the other hand has been shown to provide a rapid and sensitive technique for the analysis of underivatised oligosaccharides. MS is also the important method for detailed elucidation of carbohydrate structure. We have been able to analyze samples on a Sciex quadrepole time-of-flight tandem mass spectrometer in collaboration with the Department of Physics, University of Manitoba.

The spectra of a glucose ladder and a glycan standard showed good resolution. However, for analysis of carbohydrates using MS, the deglycosylation procedure needed to be modified. The regularly used detergent, SDS and buffer concentration have to be reduced, since those would significantly affect the analysis. Fetuin was chosen as a standard glycoprotein because of its similarity with EPO in so far as both contain 3 N-linked glycans. Unfortunately, the spectrum was difficult to interpret because of the degree of heterogeneity inherent in these structures. Because of this, the best approach for MS analysis of glycans would be to isolate individual glycan-linked peptides prior to carbohydrate analysis. This would not only simplify the interpretation of the mass spectra it would give added information as to the factors that affect individual N-glycan sites on EPO.

Glycoproteins usually contain more than one glycosylation site. The information on each glycosylation site is also important. However, from the analytical techniques used in this program no information was obtained on (1) glycosylation site occupancy and (2) oligosaccharide structure heterogeneity on each glycosylation site. Analysis of glycopeptides derived from glycoproteins is ideal to obtain this information. Proteolysis can be used and optimized for each glycoprotein to produce glycopeptides of suitable size and homogeneity for separation by a reverse phase column using HPLC. By comparison the peptide profile before and after deglycosylation, site-specific glycopeptides would be resolved from non-glycosylated peptides. In addition, glycopeptides would be isolated and the oligosaccharide structure on this specific glycosylation site could be further analyzed.

9.4 Ammonium effects on EPO glycosylation

This study indicated that the addition of NH_4Cl to the cultures caused a significant increase in the heterogeneity of the glycoforms as shown by an increased molecular weight and pI range of the secreted EPO. Glycan analysis showed that the most significant characteristic effect of ammonia was a reduction of the proportion of glycans with four sialic acids and tetra-antennary structures. This indicated that ammonium

inhibited sialylation and the complexity of the oligosaccharide structures in the CHO cell culture.

However, in this study, oligosaccharide changes induced by ammonium below 30 mM were not investigated. A high level of ammonia was chosen to obtain an EPO molecule with maximal structural difference from the control. A further examination of the effects of different ammonia concentrations on carbohydrate changes would be worthwhile. In general, the typical ammonia concentrations are 2-5 mM in batch cultures (Miller et al., 1988). They can be higher in fed-batch reactors where glutamine is added into the reactor, leading to continuous accumulation of ammonia. It is important to find the ammonia concentration that affects protein glycosylation in mammalian cell cultures.

In addition, the results obtained from this study were from the CHO cell batch culture and not extended to other mammalian cell culture process. Detailed information of how ammonia affects glycosylation is necessary for therapeutic protein production in large scale-cultures. Ammonium concentration may be controlled to ensure the products are highly glycosylated and less heterogeneous.

9.5 Mechanisms of ammonium effect on protein glycosylation

It was shown that the added ammonia and glucosamine to CHO cell culture had different effects on EPO heterogeneity although both significantly increased intracellular UDP-sugar pool concentrations. The oligosaccharide changes induced by glucosamine is possibly caused by an increased ammonia level from cell metabolism. The conclusion is that there may not be a direct relationship between intracellular nucleotide sugar pool and glycan structures from the present study.

We hypothesized that the glucosamine affected EPO glycosylation through inhibition of glucose transport into cells and altered lipid-linked oligosaccharide synthesis. In order to confirm this hypothesis, further experimentation should be done to compare the glycosylation changes induced by both glucose starvation and glucosamine. If similar

effects on EPO glycosylation were observed, it would prove that our speculation is correct.

Ammonia is known to raise the pH of acidic intracellular compartments, e.g. ER and Golgi compartments, where the glycosylation processing occurs (Schneider et al., 1996). An intracellular pH change could reduce the activity of glycosyltransferases or glycosidase. This is one possible mechanism of the reduction of EPO tetra-si alylated and tetra-antennary type glycans in the presence of ammonia. In order to confirm this, we attempted to mimic the pH affect of ammonia by the administration of chloroquine which is known to increase intracellular pH. The results from Andersen and Goochee, (1995); Thorens and Vassalli (1986) showed that 100 μM chloroquine inhibited glycoprotein sialylation in serum-supplemented cultures. However, in this study a similar effect of chloroquine on EPO was not observed. The difficulty was that the CHO cells were unable to grow at a chloroquine concentration over 50 μM because of the inhibitory effects in a serum-free medium. In order to elucidate the mechanism for the ammonia effect on glycosylation, further studies could be performed using higher chloroquine concentration in serum containing medium. The oligosaccharide structures could be analyzed under these culture conditions. Understanding the mechanism of ammonia effects on protein glycosylation would enhance our knowledge and ability to control recombinant protein production in large-scale bioprocesses.

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