Effects of Ammonia on CHO Cell Growth, Erythropoietin Production, and Glycosylation

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Abstract: The effect of ammonium chloride was determined on a culture of CHO cells transfected with the human erythropoietin (EPO) gene. Cell growth was inhibited above a culture concentration of 5 m*M* NH_4Cl with an IC-50 determined to be 33 m*M*. The specific production of EPO increased with the addition of NH_4Cl above 5 m*M*. At 10 m*M* NH_4Cl, the final cell density after 4 days in culture was significantly lower but the final yield of EPO was significantly higher. This appeared to be due to continued protein production after cell growth had ceased. The metabolic effects of added NH_4Cl included higher specific consumption rates of glucose and glutamine and an increased rate of production of alanine, glycine, 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. This molecular heterogeneity was ascribed to variable glycosylation. Complete enzymatic de-glycosylation resulted in a single molecular form with a molecular mass 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 molecular weight and p*I* range. Enzymatic de-sialylation of the EPO from the ammonia-treated and control cultures resulted in identical electrophoretic patterns. This indicated that the effect of ammonia was in the reduction of terminal sialylation of the glycan structures which accounted for the increased p*I*. Selective removal of the N-glycan structures by PNGase F resulted in two bands identified as the O-glycan linked structure (19 kDa) and the completely de-glycosylated structure (18 kDa). The proportion of the O-linked glycan structure was reduced, and its p*I* increased in cultures to which ammonia was added. Thus, the glycosylation pattern altered by the presence of ammonia included a reduction in terminal sialylation of all the glycans and a reduction in the content of the 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. *Biotechnol Bioeng* **68**: 370–380, 2000.

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INTRODUCTION

Mammalian cell cultures are used extensively to produce proteins for therapeutic use because of their ability to perform post-translational modifications, including glycosylation. Recombinant protein production processes depend on high cell yield, steady productivity, and consistent glycosylation. 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 a widely recognized that one of the most important inhibitory substances accumulating in cell cultures is ammonia (NH_3 or NH_4^+). Ammonia is a product of both cellular metabolism and chemical decomposition of glutamine in the medium. 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,b, 1991; Kurano et al., 1990; Ryll et al., 1994; Singh 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).

Some cell lines such as hybridomas are well characterized with respect to the effects of ammonia (McQueen and Bailey, 1991). However, less information is available on the influence of ammonia on the growth and productivity of Chinese hamster ovary (CHO) cells. CHO cells are the most frequently used mammalian host cells for biomedical products and they can express a wide variety of recombinant proteins (Table I). CHO cells have two advantages: (i) high productivity and (ii) ability to synthesize oligosaccharide chain structures resembling those of the natural product.

Erythropoietin (EPO) is a growth factor that stimulates the proliferation and differentiation of erythroid precursor

Table I. Recombinant protein produced by CHO cells.

Recombinant protein	Reference
Granulocyte colony-stimulating factor (G-CSH)	Andersen et al., 1994
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

cells to more mature erythrocytes (Goldwasser and Kung, 1968). Recombinant human EPO has been approved for the clinical treatment of anemia associated with chronic renal failure, as well as nonrenal anemias induced by AIDS, cancer chemotherapy and transfusion-dependent diseases (Ridley et al., 1994).

The synthesis of recombinant human erythropoietin (rHuEPO) from CHO cells has been well characterized (Takeuchi et al., 1988; Sasaki et al., 1987). The sugar chain structures of rHuEPO produced in CHO cells most closely resemble those of urine HuEPO (uHuEPO). In addition, many mutants of CHO cells have been isolated that display altered glycosylation properties. Some CHO cell lines may prove useful hosts for expressing glycoproteins with minimal heterogeneity (Stanley et al., 1989, 1991, 1992). In contrast, other host cell lines have not been studied in sufficient depth to completely define their glycosylation capabilities (Jenkins and Curling, 1994).

Mature human EPO is composed of 165 amino acids (Jelkmann, 1992). The polypeptide chain has two disulfide bonds and four attached polysaccharide chains (three N-linked at Asn 24 Asn 38, Asn 83, and one O-linked at Ser126) (Lai et al., 1986; Recny et al., 1987). The sugar chains comprise 40% of the molecular weight of HuEPO (Sasaki et al., 1987). The major structure of the 3 N-linked oligosaccharides is a tetraantennary complex type which is potentially acidic by virtue of a terminal sialic acid group (Takeuchi et al., 1988). The single O-linked sugar chain in HuEPO is a simple core structure of -Gal-GalNAc containing zero, one, or two sialic acids (Sasaki et al., 1987).

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.

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). Since Lowy et al. (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; Takeuchi et al., 1990; Tsuda et al., 1990). A linear relationship between the in vivo bioactivity and the sialic acid content has been observed for EPO (Morimoto et al., 1996). 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).

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

MATERIALS AND METHODS

Cell Line

A cloned stable transfectant (EPO-81) which expresses human erythropoietin was provided by Cangene Corp. for this work. This was derived from a CHO-K1 cell line transfected with a plasmid containing the gene for huEPO.

Cultures

The transfected CHO cells were maintained in 25 cm^2 T-flasks in humidified incubators at 37°C and 10% carbon dioxide. The medium was a proprietary serum-free formulation designated CHO-SFM2.1. Cells were detached from the growth surface by trypsin (0.05%/3 min). Viable cell concentrations were determined from cell suspensions in an equal volume of trypan blue (0.2%) by haemocytometer counting.

In the experiments described 2×10^5 cells mL^{-1} were inoculated into 7 mL of medium with the addition of NH_4Cl or chloroquine as indicated. For time course experiments, a series of parallel flasks were set up for control cultures and cultures containing 10 m*M* NH_4Cl. Every 24 h cell numbers were determined following trypsinization. After counting, cells were discarded and supernatants were stored until analysis. In order to examine extracellular sialidase activity, 1 or 2 m*M* sialidase inhibitor (2,3-dehydro-2-deoxy-*N*-

acetylneuraminic acid) (Sigma) was added to cultures containing 20 and 40 m*M* NH_4Cl at day 0 or day 3.

Analysis of Glucose, Lactate, Glutamine, and NH_4^+ in the Culture Supernatants

Glucose was determined by an assay based on a kit from Sigma (Glucose Trinder 315-100). Glutamine was measured independently by a specific glutaminase assay based on a method previously described (Lund, 1985). Lactate was determined by a spectrophotometric assay using lactate dehydrogenase (Gutmann and Wahlefeld, 1974). Ammonia was measured by a gas-sensing electrode (Orion, Model 95-12).

Amino Acid Analysis

Amino acids were determined by OPA-derivatization followed by reverse-phase chromatography (LKB/Pharmacia) on a C-18 column (Alltech). The derivatives were monitored by fluorescence detector and the output was recorded and analyzed by computer with EZChrome software (Shimadzu). Peaks were identified by retention time and concentrations evaluated relative to an internal standard (L-α-amino-*n*-butyric acid) added to each sample (Christie and Butler, 1994).

Anti-EPO Monoclonal Antibody

The anti-EPO Mab was obtained from the culture (1 L) of a murine hybridoma (5F12 AD3) obtained from the American Tissue-type Culture Collection and grown for 5 days in a serum-free medium (Barnabé and Butler, 1994). This resulted in 65 mg/L of Mab which was purified by a Protein G Sepharose 4 fast flow column (Pharmacia).

EPO Determination by ELISA

Microtiter plates (Nalge Nunc International) were coated with polyclonal anti-human EPO (4 μg/mL; Sigma) in 0.1 *M* sodium bicarbonate buffer (pH 8.3) at 4°C overnight. The plates were blocked by 3% BSA/PBS for 2 h and then incubated with serial dilutions of an EPO standard (R&D Systems Inc.) or culture supernatant samples for 4 hours at room temperature. After washing, the bound EPO was incubated with monoclonal mouse anti-EPO antibody (1 μg/mL) at 4°C overnight and then alkaline phosphatase (AP) conjugated anti-mouse IgG adsorbed with rat serum protein) (Sigma) for 2 h diluted 1:15,000 in 1% BSA/PBS/0.05% $Tween_{20}$. For the detection of the antigen–antibody reaction, *p*-nitrophenyl phosphate was added as a substrate, and incubated at 4°C overnight. The optical absorbance at 405 nm was measured by an ELISA reader. Each incubation step was followed by washing four times with PBS/0.05% $Tween_{20}$.

De-Salting and Concentration of Culture Supernatants

Culture supernatants were desalted by a Sephadex G-25M column (1.5 × 5 cm; Pharmacia Biotech) and concentrated (10×) by centrifugal filter units (Millipore) prior to analysis by electrophoresis.

Enzymatic Release of Oligosaccharides

Samples (50 μL) of desalted and concentrated culture supernatant containing EPO were denatured by boiling for 3 min with 1 μL SDS (10%) and 1 μL 2-mercaptoethanol. After denaturation 5 μL NP-40 (12.5%) and 40 μL $NaPO_4$ (50 m*M*) pH 7.2 were added to each sample. Enzymes for individual digests were added as follows: 4 U/mL recombinant peptide-*N*-glycosidase F (PNGase F) (Boehringer Mannheim) for removing N-linked glycans; 20 mU/mL *Clostridium perfringens* neuraminidase (sialidase) (Sigma) for removing sialic acids; 4 U/mL PNGase F, 25 mU/mL *Diplococcus pneumoniae* O-glycan-peptide hydrolase (O-glycosidase) (Boehringer Mannheim) plus 20 mU/mL sialidase to remove all oligosaccharides from EPO. The mixtures containing enzymes were incubated at 37°C for 16 h.

SDS–PAGE and Immunoblot Analysis

EPO was analyzed by 14% SDS–PAGE in a discontinuous system according to Laemmli (1970) in a Bio-Rad mini slab gel apparatus. Concentrated supernatant samples were prepared in the sample buffer and boiled for 5 min. Proteins separated by SDS–PAGE were electrophoretically transferred onto nitrocellulose (NC) membrane. The membrane was blocked by incubation with 3% BSA/PBS for 2 h and then incubated with purified mouse monoclonal anti-human EPO (3 μg/mL) at room temperature for at least 3 h. Antibody binding was detected by incubation with 1:30,000 diluted alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma) for 2 h. Nitriblue tetrazolium (0.3 mg/mL; Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (0.15 mg/mL; Sigma) in 5 m*M* $MgCl_2$ plus 100 m*M* Tris Buffer were used as a substrate. The membranes were washed 4 times for 5–10 min with PBS/0.05% $Tween_{20}$ between each step. A standard non-glycosylated EPO sample produced from *Streptomyces* was kindly provided by D. Stewart (Binnie et al, 1997). A prestained protein molecular weight standard (GIBCO-PRL) was used to determine the molecular weight of protein bands.

2-Dimensional Electrophoresis

Isoelectric focusing gels were made in glass capillary tubes. The gel mixture contained 9.2 *M* urea, 4% acrylamide, 2% Triton X-100, 2% ampholyte (preblended pH 4–6), 0.01% ammonium persulfate, and 0.1% TEMED. The mixed solution was drawn into each gel tube and allowed to polymer-

ize. After polymerization, the tubes were connected to a Mini-protein II 2-D Cell (Bio-Rad).

Samples for analysis were prepared by adding an equal volume of sample buffer (9.5 *M* urea, 2% ampholyte (preblended pH 4–6), 5% 2-ME, and 2% Triton X-100). The mixture was incubated at room temperature for 10 min. Following centrifugation, samples (40–60 μL) were loaded into the sample reservoir connected to the capillary tube gel. The sample was overlaid with 30 μL of overlay buffer (9 *M* urea, 1% ampholyte, and 0.0025% bromophenol blue). The cathode reservoir was filled with the cathode electrode solution (100 m*M* NaOH) and the anode reservoir was filled with the anode electrode solution (10 m*M* H_3PO_4). Electrophoresis was performed at 750 V for 3.5 h at room temperature.

After the first dimension, the tube gel was removed by the tube gel ejector and equilibrated in a buffer (0.062 *M* Tris-HCl pH 6.8, 2.3% SDS, 5% 2-ME, and 10% glycerol) for 10 min. The tube gel was then placed on top of the slab gel for electrophoresis in the second dimension. SDS–PAGE and immunoblot detection were performed as previously described. The p*I* was calculated according to the markers for 2-dimensional electrophoresis (Sigma).

Specific Rates and Consumption or Production

Specific EPO productivities and specific rates of glucose, lactate, and amino acid consumption (or production) were calculated from a plot of the substrate or product concentrations against the integral values of the growth curve (Renard et al., 1988). Data were obtained from the supernatants available from cultures during cell growth over 4 days.

RESULTS

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. 1–3). The cells were cultured in a serum-free medium alone or supplemented with different concentrations of ammonium chloride (NH_4Cl). Viable cell yields were determined after 4 days of growth by trypan blue exclusion and EPO was determined in culture supernatant samples by ELISA. All cultures were initiated at an inoculum of 2×10^5 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.1a, cell yields decreased at higher NH_4Cl concentrations. The cell yield was reduced by 25% at 20 m*M* NH_4Cl and 56% at 40 m*M* NH_4Cl. The inhibitory concentration for a 50% decrease in growth (IC-50) was estimated in this system at 33 m*M* NH_4Cl. In order to determine the effect of increased osmolality, control cultures were established in media containing added NaCl up to 40 m*M* (Fig. 1b). Although there was apparently no significant effect of the addition of 20 m*M* NaCl, the cell yield was significantly reduced by 30% in the presence of an added 40 m*M* 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).

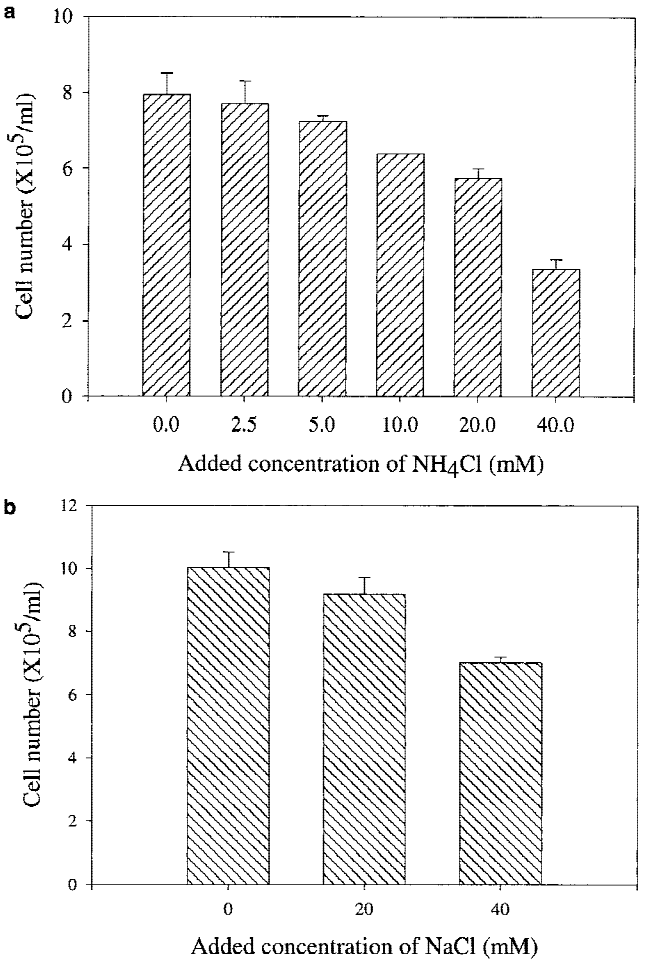

Figure 1. Cell yields under different culture conditions. CHO cells were inoculated at 2×10^5 cells mL^{-1} into 7 mL of CHO-SFM2.1 containing different concentrations of NH_4Cl (a) or NaCl (b) in 25-cm^2 T-flask and cultured for 4 days. Viable cells were determined by haemocytometer counting in a suspension of trypan blue following trypsinization. Values are mean ± SE of duplicate cultures.

The time course of cell growth and EPO production is shown in Fig. 2 for a control culture and one containing 10 m*M* 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 yield was significantly lower (19%) in the culture containing 10 m*M* NH_4Cl compared to the control. Measurement of EPO in the culture supernatant shows that the extracellular concentration increases gradually up to day 6 of culture. The

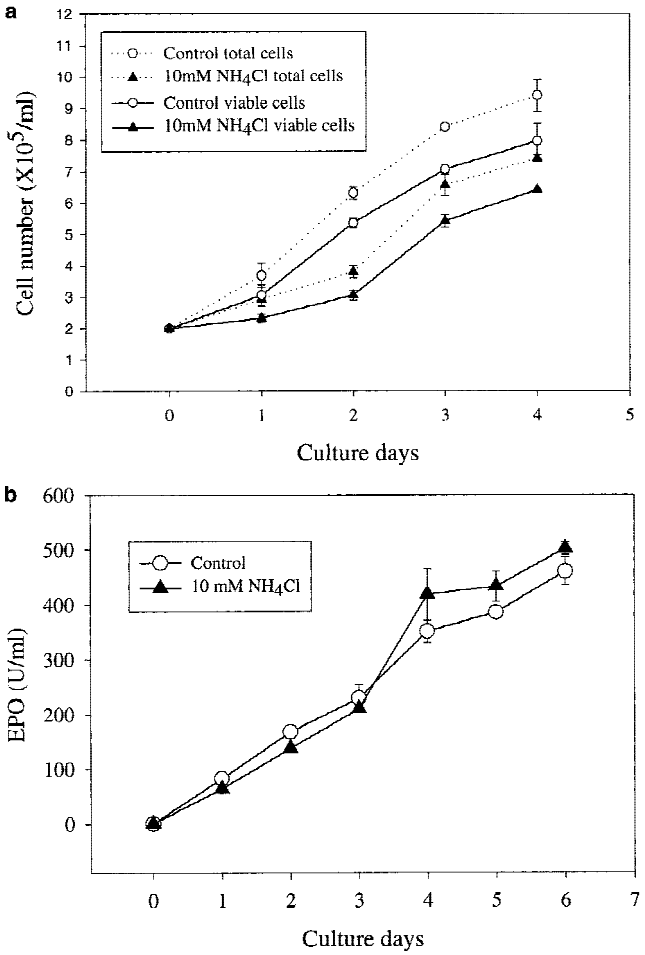

Figure 2. Effect of Ammonium Chloride on CHO cell growth (a) and EPO production (b). CHO cells were cultured in CHO-SFM2.1 alone or containing 10 m*M* NH_4Cl. Total and viable cell concentrations were determined by trypan blue dye-exclusion during exponential growth. EPO concentrations were determined by ELISA from day 0 to day 6. Cell numbers are mean ± SE of duplicate cultures. Values of EPO are mean ± SE of duplicate cultures from two separate experiments ($n = 4$).

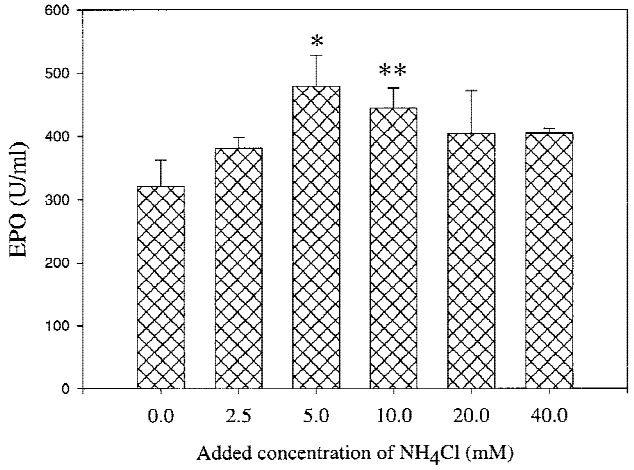

Figure 3. EPO production in cultures containing different concentrations of NH_4Cl. CHO cells were inoculated at 2×10^5 cells mL^{-1} into 7 mL of CHO-SFM2.1 containing different concentrations of NH_4Cl in 25-cm^2 T-flasks. The culture supernatants were collected at day 4 and EPO concentrations were determined by ELISA. Values are mean ± SE of duplicate cultures from two experiment results ($n = 4$, except at 2.5 and 40 m*M* NH_4Cl, $n = 2$). * and ** indicate significant difference from control at $P < 0.05$ and $P < 0.1$ respectively.

yield of EPO was significantly higher in the ammonia-supplemented culture at the end of the culture period (days 4–6).

The final yield of EPO was compared between cultures containing different concentrations of added NH_4Cl (Fig. 3). The EPO concentration was significantly higher in cultures with 5 m*M* ($P < 0.05$) and 10 m*M* NH_4Cl ($P < 0.1$) compared to the control. The specific EPO production of 161 $U/10^6$ cells per day calculated in the control culture increased significantly to 296 $U/10^6$ cells per day in the presence of 5 m*M* NH_4Cl. This higher specific productivity did not change when the ammonium concentration was increased further to 40 m*M*.

Cell Metabolism

Table II 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 m*M*. The fourth column of data in Table II 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 ammonia concentration in the control culture increased to 2.75 m*M* at day 5.

The amino acid content of the medium was analyzed by HPLC during the growth phase (days 0–4) of control and 10 m*M* NH_4Cl-supplemented cultures (Table II). 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 38.2 $\mu mol/10^6$ cell-day in the control culture compared with a net production rate of 14.6 $\mu mol/10^6$

Table II. Specific production (−) or consumption (+) by cells in control and NH_4Cl-treated cultures ($\mu mol/10^6$ cell-day).[a]

	NH_4Cl concentration			
	0 m*M*	10 m*M*	40 m*M*	10 m*M* 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

[a]Each value is a mean based on two independent cultures analyzed over the growth period (0–4 days). nd = not determined.

cell-day in the NH_4Cl-supplemented culture. A major contribution to this net change was the 2-fold increase in the production rate of alanine and glutamate.

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. 4). The data showed that, as the NH_4Cl concentration increased from 5 to 40 m*M*, 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 m*M* NH_4Cl had a much broader band which appeared to extend from 27 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). 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.

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. 6). EPO samples were analyzed from CHO cultures containing 0, 20, or 40 m*M* NH_4Cl. The result showed a significant increase in the p*I* 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 p*I* values of

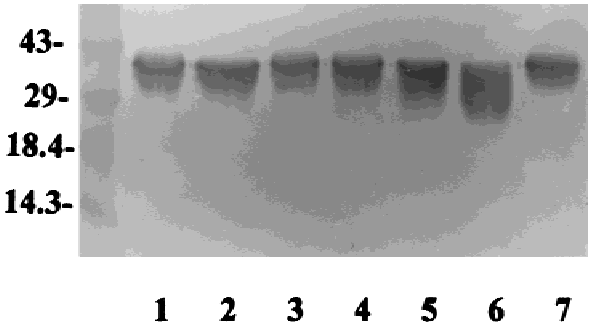

Figure 4. EPO analysis by Western blot. Cell culture supernatants with different concentrations of NH_4Cl were collected at day 4, concentrated and separated by 14% SDS–PAGE. The protein was transferred to an NC membrane and detected by a monoclonal anti-HuEPO antibody. Lane 1–7 are control, cultures containing 2.5, 5, 10, 20, 40 m*M* NH_4Cl, and 10 m*M* NaCl, respectively. Molecular mass standards are indicated ($\times 10^3$ Da).

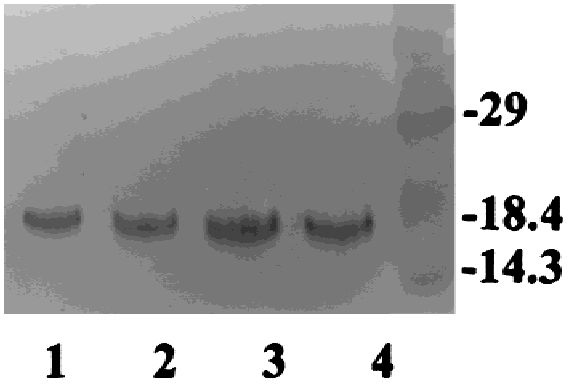

Figure 5. Immunoblot analysis of enzymatically deglycosylated EPO. Cell culture supernatants were collected at day 4. The desalted samples containing EPO were concentrated and treated with PNGase F, O-glycosidase, and sialidase at 37°C for 16 h. The treated samples were separated by 14% SDS–PAGE. The protein was transferred to an NC membrane and detected by anti-HuEPO antibody. Lanes 1–4 are control, and cultures containing 10, 20, and 40 m*M* NH_4Cl, respectively. Molecular mass standards are indicated ($\times 10^3$ Da).

4.06–4.67 (Table III). With the addition of NH_4Cl to the cultures there was a distinct shift of the p*I* range to higher values. At 20 m*M* NH_4Cl nine bands were detected. The band at the lowest p*I* (4.06) in the control sample disappeared and the upper range of p*I* was extended to 5.59. At 40 m*M* NH_4Cl the number of observed bands increased to ten with the appearance of an additional band at a p*I* value of 6.05. EPO from the 40 m*M* NaCl supplemented culture showed the same pattern as that of the control culture. This showed that the altered osmolality of the culture medium did not have effect on protein glycosylation.

In order to determine if the p*I* 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 m*M* NH_4Cl-containing cultures were analyzed by the same 2-dimensional electrophoresis technique (Fig. 6b). The results showed identical profiles for the two samples with two bands at p*I* values between 5.59 and 6.05. These bands were within the p*I* range of the extra bands which appeared in samples from analysis of the untreated samples from NH_4Cl-containing cultures (Fig. 6a). This indicates that the altered heterogeneity of EPO isoforms induced by NH_4Cl is caused by variable sialylation.

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. 7). The lower molecular weight band corresponded to the position of the non-glycosylated EPO band

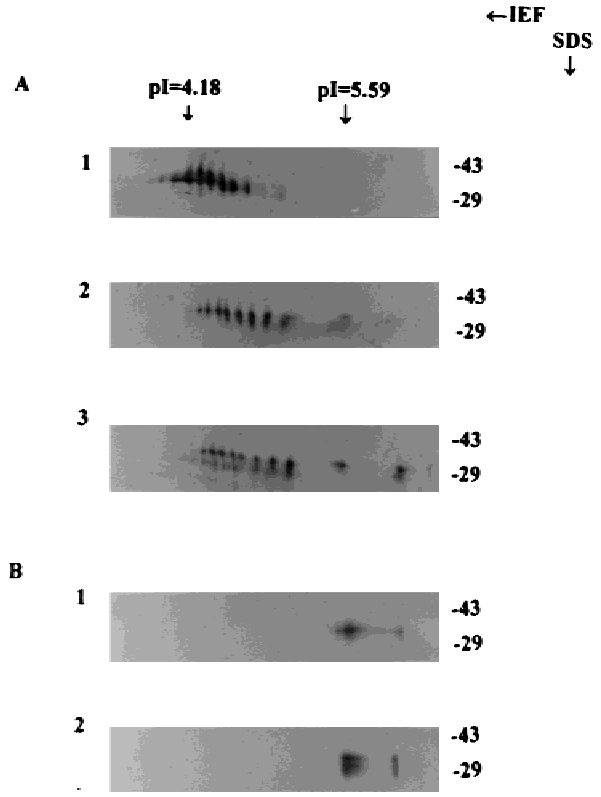

Figure 6. Two-dimensional electrophoresis of EPO samples. (a) Supernatants from CHO cell cultures containing 0 m*M* (1), 20 m*M* (2), and 40 m*M* (3) NH_4Cl were desalted and concentrated. EPO was analyzed by 2-D electrophoresis, separated by p*I* (ampholyte pH 4–6) in the first dimension followed by SDS–PAGE in the second dimension and detected by a Western blot. (b) Culture supernatants from control (1) and cultures containing 20 m*M* NH_4Cl (2) were desalted and concentrated. After treatment with sialidase for 16 h, samples were analyzed by 2-D electrophoresis and detected by Western blot.

resulting from treatment with both PNGase-F and O-glycosidase (Fig. 5). 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 nonglycosylated to the O-linked glycosylated EPO bands increased proportionally to the concentration of NH_4Cl from 2.5 to 40 m*M* (Table IV). This analysis showed that ammonia might prevent O-linked oligosaccharide attachment to the protein.

The PNGase-F treated samples of EPO were also analyzed by 2-D electrophoresis in order to determine any change in the p*I* distribution of the O-linked glycosylation EPO isoforms. The results showed that the p*I* of nonglycosylated EPO did not change for either the control or ammonia containing cultures. However, the 19 kDa molecular mass band from EPO extracted from cultures with 40 m*M* NH_4Cl was shown to have a higher p*I* range than the equivalent sample from the control culture. This indicated that ammonia may inhibit sialylation of the O-linked glycans as well as the N-linked glycans of EPO.

TABLE III. Effect of NH_4Cl on the heterogeneity of EPO isoforms.[a]

NH_4Cl (m*M*) in culture	p*I* range	No. of detectable bands	MW 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

[a]Data were obtained from Fig. 6. The p*I* and molecular weight were calculated based on 2D-electrophoresis markers and pre-stained MW standards.

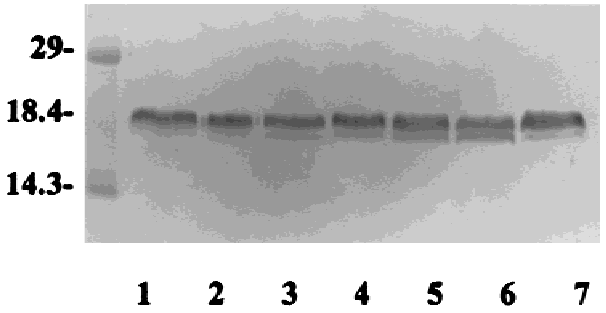

Figure 7. Immunoblot analysis of EPO without N-oligosaccharides. CHO cell culture supernatants were collected at day 4. The desalted samples containing EPO were concentrated and treated with PNGase F at 37°C for 16 h. The treated samples were separated by 14% SDS–PAGE. The protein was transferred to an NC membrane and detected by anti-HuEPO antibody. Lanes 1–7 are control, cultures containing 2.5, 5, 10, 20, 40 m*M* NH_4Cl, and 10 m*M* NaCl, respectively. Molecular mass standards are indicated ($\times 10^3$ Da).

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 (2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid) 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 inhibitor was added at 1 or 2 m*M* on day 0 or 3 of cultures growing in the presence of 20 or 40 m*M* NH_4Cl. In each culture, samples were taken at day 4 and

Table IV. Effect of ammonia on O-linked glycosylation.[a]

	NH_4Cl concentration (mM)						+10 mM
	0	2.5	5	10	20	40	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

[a]Data were obtained from Fig. 7. The relative percentage of each band was determined by Gel Doc analytical Software (Bio-Rad).

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.

Effect of Chloroquine on Cell Growth and EPO Glycosylation

Chloroquine has been shown to increase intracellular pH and effect 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 0.32–40 μM. Over this concentration range the chloroquine reduced the cell yield after 4 days in a dose dependent manner (Fig. 8). 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. 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.

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 erythropoietin synthesis.

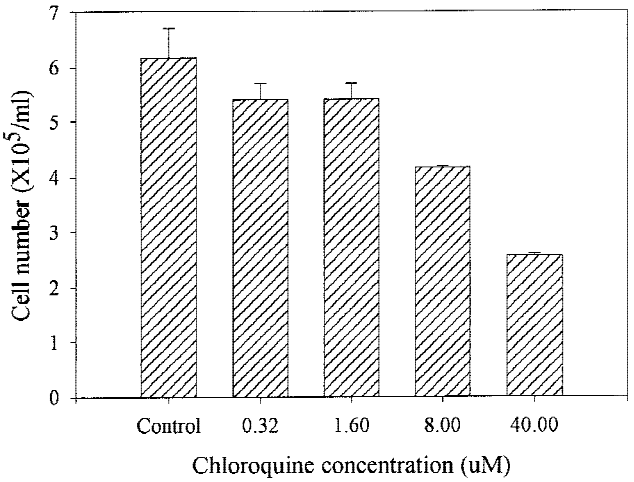

Figure 8. Cell yields in chloroquine cultures. CHO cells were inoculated at 2×10^5 cells mL^{-1} into 7 mL CHO-SFM2.1 alone or containing different concentrations of chloroquine in a 25-cm^2 T-flask and cultured for 4 days. Viable cells were determined by haemocytometer counting in a suspension of trypan blue following trypsinization. Values are mean ± SE of duplicate cultures.

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 to >5 m*M* (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 m*M* 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 m*M* (Hansen and Enborg, 1994). In our study, the IC-50 of the CHO was determined to be 33 m*M* 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 was 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 α-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 to a maximum at 5 m*M* NH_4Cl. This appeared to be correlated with a higher volumetric EPO production at a low ammonia level (5–10 m*M*) and lower cell growth rate at a high ammonia level (20–40 m*M*). This result is consistent with monoclonal antibody and recombinant protein production in other cell lines under the effects of ammonia (Andersen and Goochee, 1995; Hayter et al., 1992; Miller et al., 1988; 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 m*M* 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 m*M*) 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 m*M*) which is typically found at the end of a batch culture. Our data are consistent with previous analysis 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 p*I* 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. 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-*N*-acetylhexosamine (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 of EPO. We therefore attempted to mimic the pH affect 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 p*I* 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 a 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.

In conclusion, 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 m*M* 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.

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