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Effect of Ammonia on the Glycosylation of Human Recombinant Erythropoietin in Culture

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Recombinant human erythropoietin (EPO) was produced by a stable transfected CHO-K1 cell clone (EPO-81) grown in serum-free medium. Our previous work showed that there was a significant increase in the heterogeneity of the glycoforms of EPO and a reduction of the sialylation at 20 mM NH_4Cl. 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 fluorophore-assisted carbohydrate electrophoresis (FACE) and HPLC. The FACE N-linked oligosaccharide profile showed that the sialylated glycans contain one prominent band at a position corresponding to eight 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 tetraantennary structures was reduced by 60%, while the tri- and biantennary 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 desialylated glycans by normal phase chromatography indicated a distribution of tetra-, tri-, and biantennary 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. In conclusion, we showed that ammonia in the culture medium affected EPO glycosylation, which was observed as a reduction of the tetraantennary and tetrasialylated oligosaccharide structures. However, the presence of ammonia in the cultures did not change the oligosaccharide sequence.

Introduction

Mammalian cell cultures are used to produce recombinant biological proteins. Since the glycosylation process is a posttranslational 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. The culture parameters that may affect the variability of glycosylation include glucose depletion, ammonia accumulation, lipid composition, protein content, and culture period.

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 endoplasmic reticulum (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 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 can account for the variations in oligosaccharide structures.

Ammonia is an accumulated waste product of both cellular metabolism and chemical decomposition of glutamine, which is a major nitrogen and energy source. Different effects of ammonia on monoclonal antibody and recombinant protein glycosylation have been reported. Thorens and Vassalli (1986) reported that addition of NH_4Cl to cultured plasma cells resulted in the inhibition of secreted IgM sialylation. Increased concentration of ammonia in the culture medium resulted in reduced O-linked sialylation of granulocyte colony-stimulating factor produced by CHO cells (Andersen and Goochee, 1995). Borys et al. (1994) indicated that ammonia has the potential for affecting the entire glycosylation process of recombinant placental lactogen-I (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 proteins produced 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

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immunoadhesin tumor necrosis factor-IgG (TNFR-IgG) produced by CHO cells (Gawlitzek et al., 2000).

Human EPO is a 39 kDa molecule containing two disulfide bonds and three N-linked and one O-linked oligosaccharide chains. The carbohydrates comprise 40% of its molecular weight (Tsuda et al., 1988). The structure of the carbohydrate moiety of a glycoprotein is a critical determinant of the biosynthesis, secretion, solubility, and in vivo bioactivity of the molecule. Desialylated rHuEPO has low activity in vivo (Lowy et al., 1960; Dordal et al., 1985; Takeuchi et al., 1990; Tsuda et al., 1990). 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) produced an unusual form of HuEPO (EPO-biantennary) in addition to the usual form (EPO-tetra). Despite an in vitro activity 3-fold higher than that of standard HuEPO, EPO-biantennary showed very little in vivo activity, even though it had sufficient sialic acid to cover most of its galactose residues. This finding suggests that glycan antennarity in addition to terminal sialylation might be important for maintaining EPO activity in vivo. A good positive correlation between the in vivo activity of HuEPO and the ratio of tetraantennary to biantennary oligosaccharides was observed. They suggested that the branching structure of sugar chains of HuEPO has a role in decreasing a nonspecific clearance mechanism, such as filtration by the kidney, or in enhancing homing to bone marrow (Takeuchi et al., 1989). Therefore higher branching of the N-linked oligosaccharides with attached sialic acids at the terminal is desirable for the effective expression of the in vivo biological activity of EPO.

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. We found previously that addition of NH_4Cl to the cultures caused a significant increase in the glycoform heterogeneity of EPO, as shown by an 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, 2000). 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.

Materials and Methods

1. Cell Line and Cultures. A stable CHO-K1 cell clone (EPO-81) transfected with the human EPO gene was provided by Cangene Corp. The transfected CHO cells were maintained in culture flasks in humidified incubators at 37 °C and 10% carbon dioxide. The serum-free medium designated CHO-SFM2.1 was used.

CHO cells at 1×10^5/mL were inoculated into 100 mL of CHO-SFM2.1 and cultured in 150 cm^2 T flasks. In the experiments, cultures were supplemented with ammonia (as NH_4Cl) up to a concentration of 30 mM. Culture supernatants were collected from day 1 to day 5. The EPO production and ammonium concentration were determined. Supernatant (1 L) was collected from multiple cultures on day 4 and stored at −20 °C until required for purification.

2. EPO Concentration Determination by ELISA. EPO concentration in culture supernatant was determined by a sandwich ELISA as described previously with modifications (Yang and Butler, 2000). Briefly, polyclonal antihuman EPO (Sigma) was coated onto microtiter plates and then incubated with serial dilutions of EPO standard or culture supernatant samples, which were deglycosylated by PNGase F and O-glycosidase following denaturation. EPO was detected with a monoclonal mouse antihuman EPO antibody (5F12 AD3) (produced and purified in our laboratory), followed by an alkaline phosphatase (AP) conjugated antimouse IgG (adsorbed with rat serum protein) (Sigma). For detection of the antigen−antibody reaction, the SIGMA FAST *p*-nitrophenyl phosphate tablet was added as a substrate. The optical absorbance at 405 nm was measured by the THERMOmax plate reader (Molecular Devices).

3. Ammonia measurement. The ammonium ion concentration was measured by an Orion 9512 ammonia probe connected to a Fisher Scientific pH meter 25. 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 M 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. Briefly, standards from 10^{-3} to 10^{-5} M were prepared by 10-fold serial dilutions. Samples were diluted 1:5 in water for a total volume of 1 mL, and 10 μL of 10 M NaOH was added to 1 mL 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. The sample concentration was determined from a calibration curve of the electrode potentials for a series of standard ammonium solutions.

4. Immunoaffinity Purification of Human EPO. The anti-EPO Mab was obtained from the culture of a murine hybridoma (5F12 AD3) obtained from the American Tissue-type Culture Collection and grown for 5 days in a serum-free medium. This resulting Mab was purified by a Protein G Sepharose 4 fast flow column (Pharmacia).

Culture supernatant (1 L) was loaded on to a column consisting of purified monoclonal anti-EPO antibody bound on to the Affi-prep 10 matrix (Bio-Rad). The column was extensively washed with PBS and 10 mM sodium phosphate/0.5 M NaCl (pH 7.4). The bound EPO was eluted with 3 M KSCN, 20 mM Tris, 3 mM EDTA, pH 7.0. The eluant fraction was dialyzed against (0.1×) PBS/0.1% $Tween_{20}$ and concentrated by a centrifugal filter unit (Millipore).

5. Glycan Analysis by FACE. ***5.1. N-Linked Oligosaccharide Profile Analysis.*** Glycan release and separation from EPO were performed with a Bio-Rad N-linked oligosaccharide profile analysis kit used according to the manufacturer's instructions. The released N-linked glycans were labeled with the fluorophore 8-aminonaphthalen-1,3,6-trisulfonic acid (ANTS) at the reducing terminus by reductive amination (Jackson, 1994). Labeling was performed by incubation at 37 °C for 16 h. After labeling, samples were dried in a Speed-Vac, and one-fifth of the sample was loaded onto the polyacrylamide gel. Fluorescently labeled oligosaccharides were electrophoretically separated in an N-linked oligosaccharide gel using the Mini-Protein II electrophoresis cell. The electrophoresis buffer was obtained from Bio-Rad. Polyacrylamide gels were subjected to an electric field of 15 mA/gel at 4 °C. Images were acquired with the Glyco Doc imager and analyzed by Glyco Doc analytical Software (Bio-Rad).

Table 1. Exoglycosidases Used in N-Linked Glycan Sequence Determination

entry	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

Glycan standards, asialo-agalacto-biantennary with core fucose, asialo-biantennary with core fucose, asialo-triantennary, and asialo-tetraantennary were purchased from Oxford Glyco-Systems.

5.2. N-Linked Oligosaccharide Sequence Determination. The N-linked oligosaccharide sequencing kit (Bio-Rad) was used to determine the oligosaccharide sequence. Sequence analysis was accomplished by following a sequential series of exoglycosidase digestions. Four separate enzymic digests were established. Into each of four tubes was added an equal amount of ANTS labeled glycan (2 μL), 5× reaction buffer (4 μL), and one to four exoglycosidases (2 μL) supplied in the kit (Table 1). The samples were incubated at 37 °C for 16 h and then for an additional 4−6 h after supplementing half the original amount of reaction buffer and enzymes. After incubation, samples were dried by a Speed-Vac. The dried samples were dissolved in water and separated by electrophoresis. The sequence of monosaccharides in each glycan was determined from the mobility shift of bands following electrophoresis of these enzyme digests.

6. Oligosaccharide Analysis by HPLC. ***6.1. Oligosaccharides Release and Cleanup.*** Purified EPO (100 μg in 50 μL) was denatured by boiling for 3 min in a mixture containing 1 μL of 10% SDS, 1 μL 2-mercaptoethanol, 5 μL of 12.5% NP-40, and 40 μL of 50 mM $NaPO_4$ pH 7.2. Each denatured sample was then incubated at 37 °C for 16 h with recombinant peptide-*N*-glycosidase F (PNGase F from Boehringer Mannheim; 4 U/mL) plus *Clostridium perfringens* neuraminidase (sialidase from Sigma; 20 mU/mL).

Released N-glycans were separated from the EPO polypeptide by ethanol precipitation. Three volumes of cold 100% ethanol was added to samples, which were then incubated on ice for 10 min. Samples were centrifuged for 5 min to pellet the protein. The supernatant containing glycans was then dried with a Speed-Vac. A clean up procedure was followed by passage through a GlycoClean S cartridge (Oxford Glyco-Systems). Briefly, the dried glycan was resuspended in 10 μL of H_2O and applied to a GlycoClean S cartridge. Nonglycan material was removed with 96% acetonitrile (5 × 1 mL) and glycans were eluted using water (3 × 0.5 mL). The glycan eluant was dried and treated with 2-aminobenzamide (2-AB) or 4-aminobenzonitrile (4-AB) for labeling.

6.2. Fluorescent Labeling of Glycans with 2-Aminobenzamide (2-AB). The labeling reagent was prepared by dissolving 10 mg of 2-AB in 200 μL of 70% DMSO/30% glacial acetic acid (v/v). This mixture (100 μL) was added to a tube containing 6.2 mg of sodium cyanoborohydride and vortexed until the material was fully dissolved. The glycans were dissolved in 5 μL of the labeling reagent (Bigge et al., 1995). The solution was then incubated at 65 °C for 2 h. After incubation, the labeled glycans were recovered by a GlycoClean S cartridge using the procedure described above. The 2-AB labeled samples were then dried and reconstituted in 120 μL of distilled water.

6.3. Analysis of Sialylated N-Glycans by Ion-Exchange HPLC. Anion-exchange chromatography was carried out using a GlycoSep C column (4.6 mm × 100 mm; Oxford Glycosystems Ltd.). Buffer A was 20% acetonitrile/80% water and buffer B was 250 mM ammonium acetate in 20% acetonitrile, pH 4.5. The solvent gradient was 0−40% B in 18 min followed by 40−100% B over the next 16 min. The 2-AB labeled glycans from EPO (100μg) were dissolved in water, and 100 μL of each sample was injected by an autoinjector (Shimadzu SIL-9). Oligosaccharide elution was monitored with a fluorescent detector (Pharmacia LKB). The excitation wavelength was 330 nm, and the emission filter had a cut off of 408 nm. Neutral oligosaccharides eluted in the void volume. Negative-charged glycans were separated into mono-, di-, tri-, or tetrasialylated peaks by the gradient.

6.4. Oligosaccharide Derivatization by 4-Aminobenzonitrile (4-AB). The reagent solution was prepared freshly prior to derivatization by dissolving 10 mg of sodium cyanoborohydride in 1 mL of a methanolic solution containing 6% 4-AB, 5% acetic acid, and 50% methanol. This reagent (5 μL) was added to each dried glycan sample. Samples were incubated at 65 °C for 2 h and then dried by a Speed Vac.

6.5. N-Linked Asialo-Oligosaccharide Analysis by Normal-Phase HPLC. An amide adsorption normal-phase column (4.6 mm × 250 mm; Phenomenex) was used for the separation of neutral oligosaccharides. The retention of asialo-glycans on this column was based on the hydrophilicity of each molecule, which is related to the molecular size. After labeling with 4-AB, the dried oligosaccharides were dissolved in 25 μL of water. Each sample was further diluted with 95 μL of the buffer B. Each sample (100 μL) was then injected by an autoinjector. Buffer A was 250 mM ammonium formate pH 4.4. Buffer B was 80% acetonitrile/20% buffer A. The gradient was 0−41% buffer A in 182 min. Oligosaccharide elution was monitored with an UV detector (Linear Instruments model 2000 detector) at 285 nm. Oligosaccharide peaks were identified by comparing retention times with a standard mixture under the same condition.

Results

1. Cell Growth, EPO Production, and Ammonium Concentration in CHO Cell Culture. The effects of ammonia on CHO cell growth in batch culture were reported previously (Yang and Butler, 2000). The cell yield was reduced by 25% and 56% at 20 and 40 mM NH_4Cl, respectively. The inhibitory concentration for a 50% decrease in growth (IC-50) was estimated at 33 mM NH_4Cl in this culture system. The cell viability determined by trypan blue exclusion decreased significantly in relation to the concentration of added NH_4Cl. The viability was 68.5% in 40 mM NH_4Cl compared to 80.1% in a control at day 4 of culture.

The EPO production in a control culture and one containing 30 mM NH_4Cl were compared by a sandwich ELISA. The EPO samples were assayed following deglycosylation using PNGase F and *O*-glycosidase. This step was found to be necessary because of the variable response of fully or partially glycosylated EPO in the ELISA. The EPO concentrations determined from a control culture was significantly higher than a culture supplemented with 30 mM NH_4Cl (Figure 1). However, the specific EPO production did not show a significant difference between control and ammonia-supplemented cultures.

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

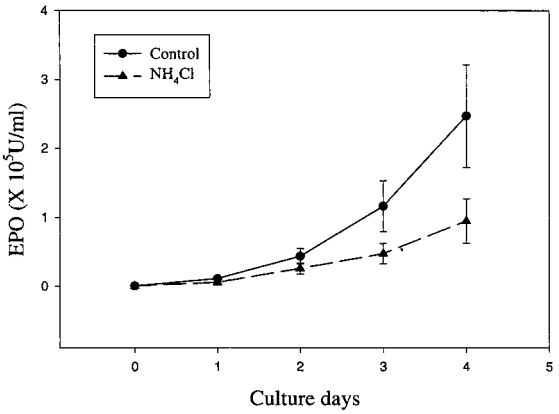

Figure 1. EPO production in CHO cell cultures. CHO cells were inoculated at 1×10^5 cells mL^{-1} into CHO-SFM2.1 containing 0 and 30 mM NH_4Cl. Culture supernatants were collected from day 1 to day 4, and EPO concentrations were determined by ELISA after deglycosylation.

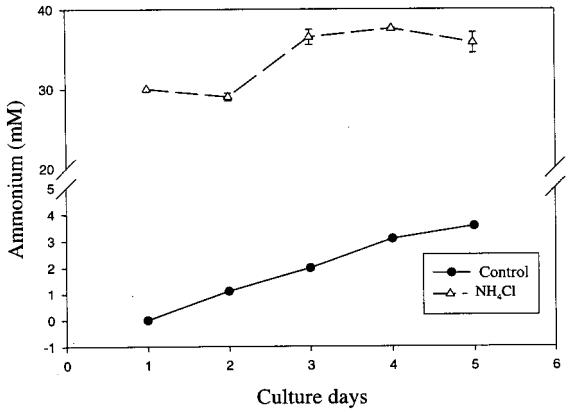

Figure 2. Ammonium concentration determination. Ammonium level in the culture supernatants of a control and the culture containing 30 NH_4Cl were measured as described in Materials and Methods. Values are mean ± SE from two independent cultures.

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 (Figure 2). Our previous data showed 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 (Yang and Butler, 2000).

2. 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 L) from each culture was collected on day 4 and purified by an immunoaffinity column. The N-linked oligosaccharides were released enzymatically and labeled with ANTS. The ANTS-labeled glycans were separated by 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 (Figure 3). The sialylated glycan sample has one prominent band at a position corresponding to eight 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 1−3 lactosamine repeats (Morimoto et al., 1999). Our results of 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 compared with the control. Each lane was loaded with 20% of the glycan sample obtained from 1 L 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 (Figure 4). 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 tetraantennary structures was reduced by about 60%, while the tri- and biantennary structures were increased proportionally in the presence of ammonia (Table 2). The result indicated that the ammonia reduced the proportion of the tetrasialylated and the tetraantennary glycan structures.

3. N-Glycan Analysis by HPLC. 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 (Figure 5). The oligosaccharides were separated into groups according to charge. The glycans were identified by comparison with retention times of oligosaccharide standards containing variable 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 is that the relative proportion of EPO with four sialic acids was reduced from 46% to 29% in the culture. The relative proportion of mono- and disialylated glycans were increased (Table 3). 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 tetraantennary standard (Figure 6). The result showed that the major oligosaccharide structures were of the tetraantennary type, although these were significantly reduced from 60% to 26% in the presence of ammonia (Table 2). This correlated with the analysis by FACE which showed that the tetraantennary structure comprised 64% of the total glycans. The relative proportions of the bi- and triantennary structure were increased in

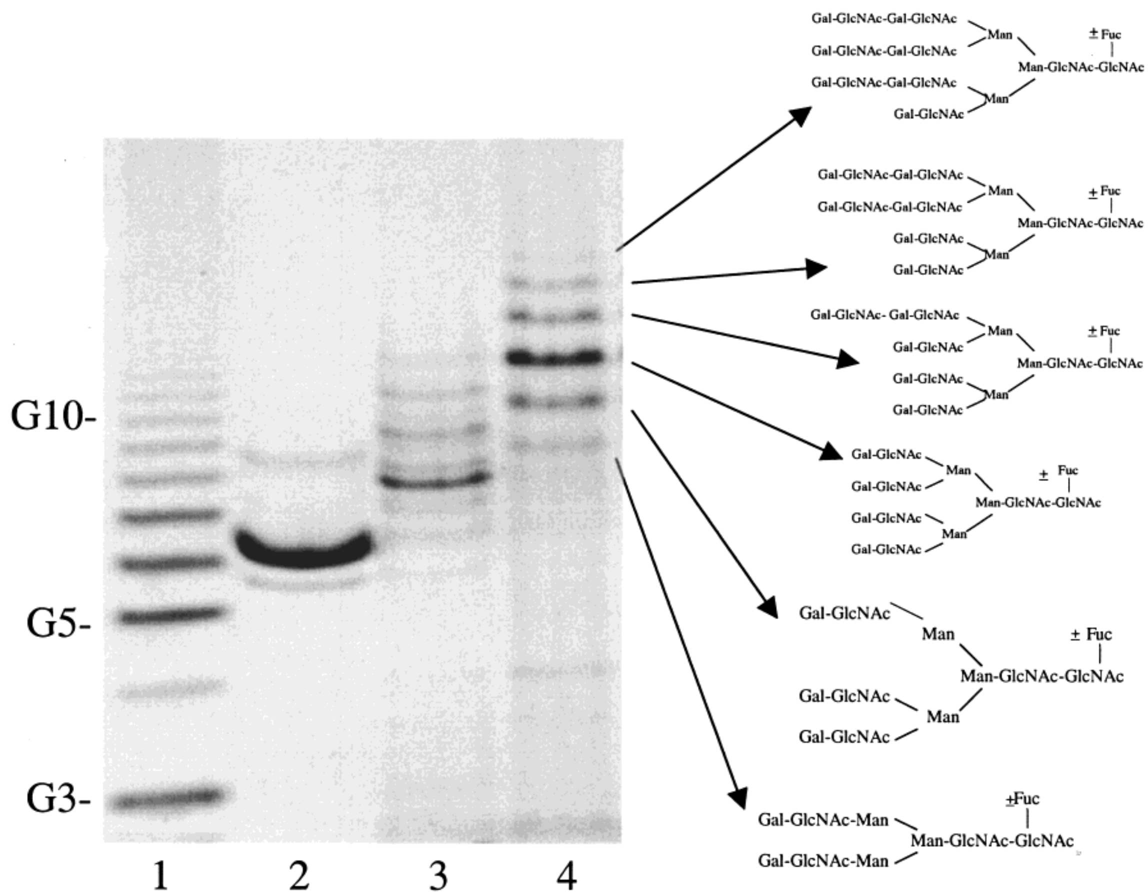

Figure 3. EPO N-linked oligosaccharide profile. EPO N-linked oligosaccharides were analyzed by FACE. Images were acquired using the Glyco Doc imager. Lane 1, glucose ladder; lane 2, biantennary oligosaccharide standards with or without galactose; lane 3, EPO N-linked oligosaccharide profiles; and lane 4, asialo-oligosaccharides.

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.

4**. Determination of the N-Linked Oligosaccharide Sequence by FACE.** 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 trisaccharide core structure (Figure 7). 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.

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 1, 2−4). The results indicated that EPO contained a typical N-linked complex oligosaccharide structure. In this study, we successfully digested down to the N-linked oligosaccharide core structure. Glycans from the ammonia-supplemented cultures showed the same sequence pattern which indicated that ammonia did not affect the N-linked oligosaccharide sequence.

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 erythropoietin it is well established that full sialylation of the 3-N 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.

Our previous work showed that CHO cell growth was inhibited above a culture concentration of 5 mM NH_4Cl (Yang and Butler, 2000). 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.

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, provid-

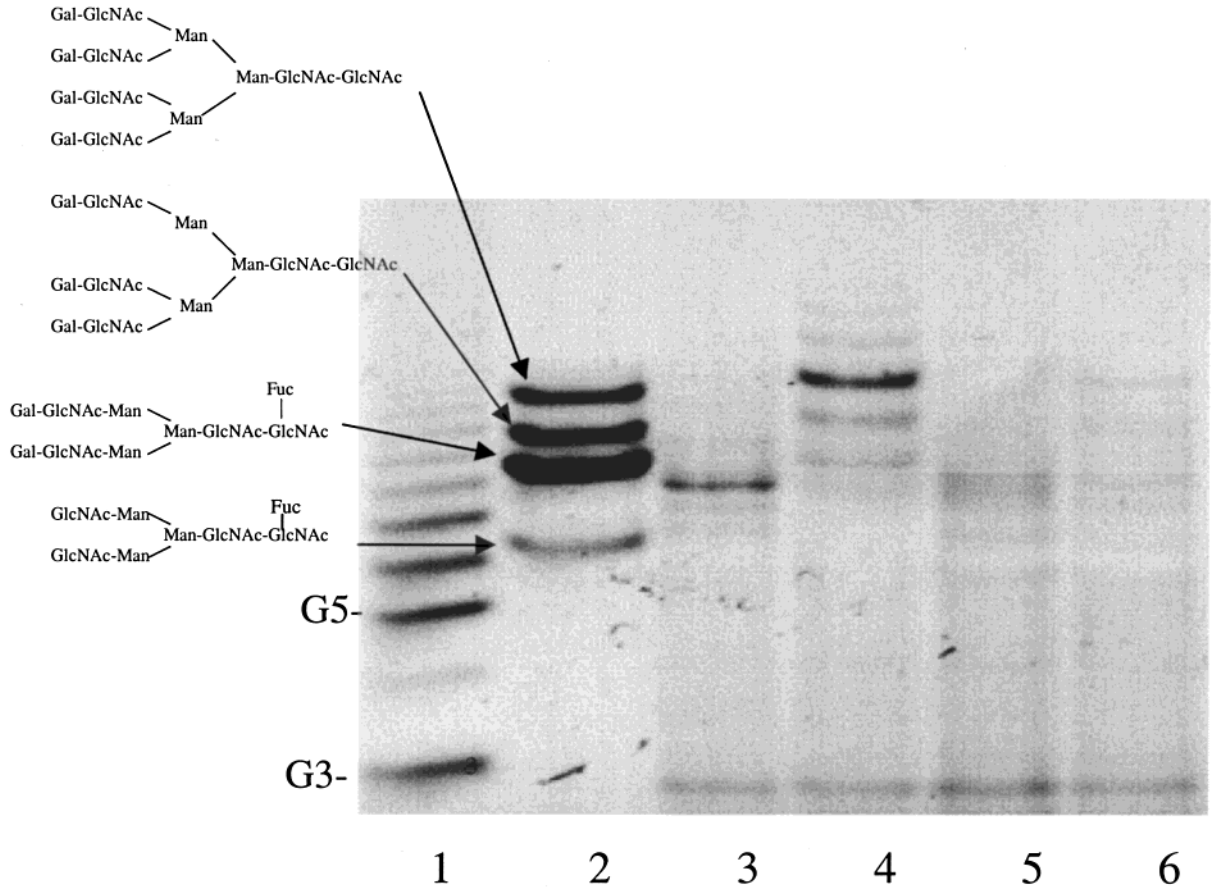

Figure 4. Effects of ammonia on EPO N-linked oligosaccharides. Culture supernatants from cultures with or without added ammonia chloride (30 mM) were collected on day 4. The carbohydrates released from EPO were analyzed by FACE. Lane 1, glucose ladder; lane 2, carbohydrate standards; lane 3, N-glycans 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.

Table 2. Relative Proportion of Oligosaccharide Antennarity Analyzed by FACE and HPLC[a]

analysis technique	culture	relative proportion of different antennarity (%) 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

[a] The proportion of each antennary structure was determined by Gel Doc analytical software for FACE analysis.

ing 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 tetraantennary 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 that 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 tetrasialylated 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 tetrasialylated glycans were reduced in the presence of ammonia-supplemented culture compared to the control culture. The corresponding proportions of mono- and disialylated oligosaccharides were elevated. This result combined with the observed change of pI by 2-D electrophoresis suggested that ammonium 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 ammonium 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 tetraantennary structures in the presence of ammonia, while the relative proportions of the bi- and triantennary 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

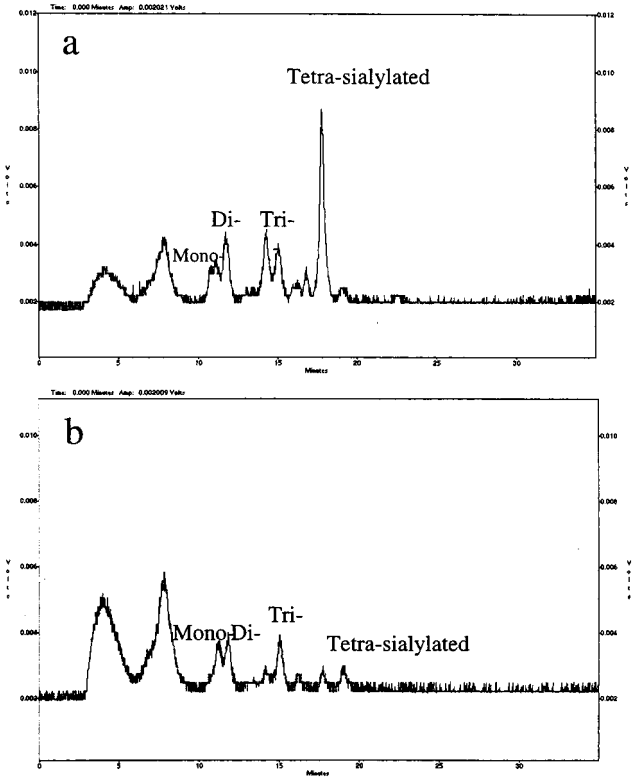

Figure 5. EPO glycans analyzed by anion exchange chromatography. Glycans released from purified EPO were labeled with 2-AB followed by GlycoClean S cartridge treatment to remove free dye. Samples were analyzed by HPLC using a GlycoSep C column. (a) glycans released from EPO and (b) glycans from culture containing 30 mM NH_4Cl.

Table 3. Relative Peak Area of EPO Carbohydrates with Variable Sialic Acids As Analyzed by Anion Exchange Chromatography

	relative proportion of sialylated glycans (%)			
culture	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

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 ammonium with an increase in the complexity of mutant IL-2 N-glycosylation. In contrast, no effect of ammonium 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 tetraantennary glycan structures and sialylation in EPO. First, the activities of the glycosylation enzymes may be reduced. Second, the balance of intracellular nucleotide sugar pools may be perturbed. Third, the availability of oligosaccharide

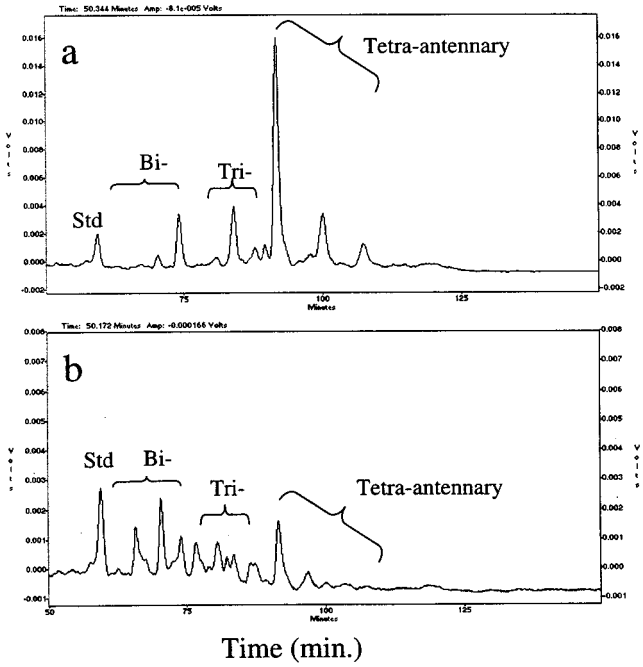

Figure 6. EPO oligosaccharide analysis by normal-phase chromatography. Glycans released from EPO in a control culture (a) and a culture containing 30 mM NH_4Cl (b) were labeled with 4-AB and analyzed by HPLC using a GlycoSep N column. The peak "Std" is the asialo-agalacto-binatennary oligosaccharide as the internal standard. The peaks were identified by comparison of retention times with oligosaccharide standards.

precursors may be reduced, and fourth, 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 *N*-acetylglucosaminytransferase IV and V add *N*-acetylglucosamine 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 microenvironment within the ER or Golgi compartments, leading to reduced enzymic activity. An intracellular pH change could affect the activity of glycosyltransferases or glycosidases, for example, the branching enzymes GlcNAc-transferases III and IV (Schachter, 1985) and sialyltransferase. Andersen and Goochee (1995) found a 2-fold 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 7.0−7.2, a likely trans-Golgi pH range in the presence of 10−15 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 tetraantennary 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 ammonium effects in the synthesis of UDP-GNAc using $^{15}NH_4Cl$ in

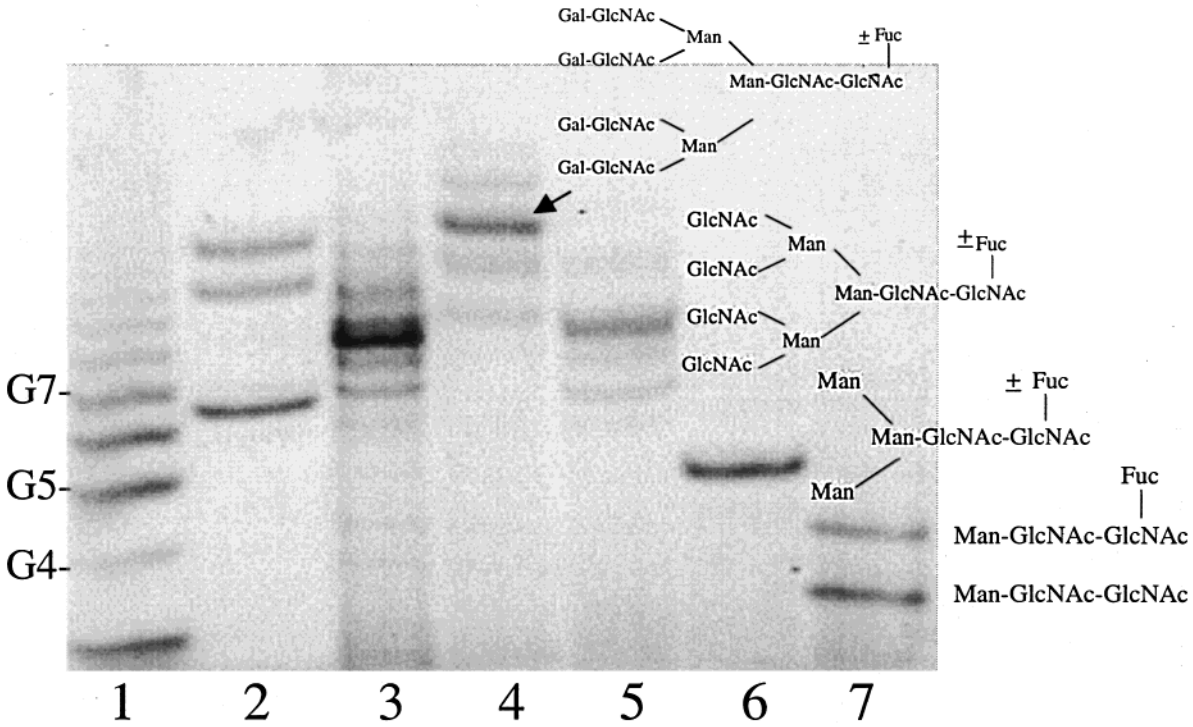

Figure 7. Sequence determination of oligosaccharides by FACE. Oligosaccharides were digested by exoglycosidases using an oligosaccharide sequencing kit. Lane 1, glucose ladder; lane 2, agalacto-bi-, tri-, and tetra-asialo-antennary standards; lane 3, N-linked glycan profile of EPO; lane 4, + neuraminidase; lane 5, + β-galactosidase; lane 6, + β-*N*-acetylhexosaminidase; and lane 7, + α-mannosidase.

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-*N*-acetylhexosamine (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 tetraantennary 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, 2000).

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 2000).

In conclusion, 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- and tetrasialylated and tetraantennary 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.

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