

# Ammonia Affects the Glycosylation Patterns of Recombinant Mouse Placental Lactogen-I by Chinese Hamster Ovary Cells in a pH-Dependent Manner

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Received June 30, 1993/Accepted November 11, 1993

The *N*-linked glycosylation of the recombinant protein mouse placental lactogen-I (mPL-I) expressed by Chinese hamster ovary (CHO) cells under nongrowth conditions was inhibited by increasing levels of ammonium chloride (3 and 9 mM) in a serum-free, protein expression medium. The effect of ammonia on glycosylation was dependent on the extracellular pH (pH<sub>e</sub>). In media containing 0 and 9 mM ammonium chloride, the percentage of the most heavily glycosylated forms of secreted mPL-I decreased from ca. 90% to ca. 25% at pH<sub>e</sub> 8.0, and from ca. 90% to ca. 65% at pH<sub>e</sub> 7.6, respectively. However, at pH<sub>e</sub> 7.2, the most heavily glycosylated forms of secreted mPL-I decreased from ca. 90% to ca. 80% in media containing 0 and 9 mM ammonium chloride, respectively. Inhibition of mPL-I glycosylation was found to correlate with the calculated concentration of the ammonia species (NH<sub>3</sub>). Control experiments showed that the ammonia effect on mPL-I glycosylation could not be attributed to increased chloride concentration or osmolarity, or to extracellular events after secretion of the recombinant protein into the supernatant. Ammonium chloride, 9 mM, inhibited the expression rate of mPL-I by CHO cells at low pH<sub>e</sub>. © 1994 John Wiley & Sons, Inc.

Key words: glycosylation • recombinant protein expression • CHO cells • ammonia • pH • placental lactogen

## INTRODUCTION

It is essential to identify cell culture conditions which may alter the posttranslational processing of polypeptides, because such changes may affect both the chemical and biological properties of protein therapeutics. One such posttranslational modification is glycosylation, in which an oligosaccharide chain is attached to the protein chain in the endoplasmic reticulum (ER) and is subsequently modified in both the ER and the Golgi (reviewed in Kobata<sup>31</sup> and Kornfeld and Kornfeld<sup>32</sup>). Structural changes in the oligosaccharide chain can potentially affect glycoprotein folding, solubility, biological activity, antigenicity, circulatory lifetime, as well as the susceptibility of glycoproteins

to denaturing agents and protease attack.<sup>16,22,58</sup> A number of factors that affect protein glycosylation have been identified (reviewed in Goochee and Monica<sup>23</sup>), including: cell growth rate,<sup>10,24</sup> low glucose concentration,<sup>7,11,26</sup> extracellular pH,<sup>3,48</sup> hypertonic medium,<sup>40</sup> serum concentration,<sup>37,44</sup> and ammonia.<sup>35,55</sup>

Ammonia is a toxic byproduct of amino acid metabolism which has been associated with decreased cell growth rates and cell viabilities in culture.<sup>6,15,20,21,33,36,39,47,54</sup> Because glutamine is a major energy source to most cultured mammalian cells,<sup>14,20,27,46</sup> glutamine metabolism as well as glutamine decomposition<sup>21,33,34</sup> are the primary sources of ammonia in culture media. The maximum theoretical yield of ammonia is 2 mol ammonia per mole of glutamine consumed, although a yield of less than 1 mol ammonia per mole glutamine consumed has been observed in mammalian cell culture.<sup>21,25,27,38,43</sup> Glutamine concentrations ranging from 1 to 7 mM are common in culture medium,<sup>18</sup> resulting in ammonia concentrations up to 5 mM.<sup>5,25,38</sup> However, there are reports of ammonia concentrations from 7 to 10 mM.<sup>4,19,21</sup> In addition, there are reports of media formulations with very high glutamine concentrations (8 to 40 mM), which would produce very high ammonia concentrations.<sup>17,35</sup>

In this study we have examined how ammonia, at concentrations encountered in culture media (initial ammonium chloride concentrations between 0 to 9 mM), can affect the *N*-linked glycosylation of recombinant mouse placental lactogen-I (mPL-I) expressed in Chinese hamster ovary (CHO) cells and the extent to which the effect of ammonia on mPL-I glycosylation was dependent on extracellular pH (pH<sub>e</sub>). Recombinant mPL-I is secreted as a glycoprotein of 194 amino acid residues with two potential sites for *N*-linked glycosylation.<sup>8,9</sup> Placental lactogens are closely related, both structurally and functionally, to prolactin and growth hormone (GH).<sup>29</sup> This family of hormones is involved in the introduction and maintenance of lactation, ovarian steroidogenesis, maternal metabolism, and fetal growth.<sup>42,50</sup>

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## MATERIALS AND METHODS

### Cells and Their Maintenance

The experiments were performed using a recombinant, anchorage-dependent Chinese hamster ovary (CHO) cell line expressing the glycosylated recombinant protein mouse placental lactogen-I (mPL-I). The mPL-I-expressing cells, which were coamplified with DHFR by methotrexate selection in CHO cells, have been described previously.<sup>8</sup> They were grown in a 1:1 mixture of MCDB 302 and Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO), supplemented with 40 mM sodium bicarbonate, 12  $\mu$ M proline, 2 mM glutamine, 20 U/mL penicillin, 0.02 mg/mL streptomycin, and 16  $\mu$ M methotrexate (Sigma) and 5% calf serum (Hyclone, Logan, UT). The calf serum was dialyzed against phosphate-buffered saline (0.2 g/L potassium phosphate monobasic, 0.2 g/L potassium chloride, 8.0 g/L sodium chloride, and 1.15 g/L anhydrous sodium phosphate dibasic) at 4°C using 3500 molecular-weight cutoff Spectra/Por dialysis tubing (Spectrum Medical Industries, Los Angeles, CA) to reduce the serum nucleoside concentration. The cells were cultured on 75-cm<sup>2</sup> T-flasks (Corning, Corning, NY) and grown in an incubator at 37°C in an atmosphere of 5% CO<sub>2</sub> and 95% relative humidity.

### Protein Expression Under Controlled pH

Protein expression phase began once the T-flasks became confluent to ensure that overgrowth of the cell monolayer had not occurred and that all experiments used similar cell numbers. For protein expression, calf serum and methotrexate were omitted from the medium, 0.01  $\mu$ M CdCl<sub>2</sub> was added to stimulate transcription of the mPL-I cDNA from the metallothionein promoter, and buffering was provided by 20 mM sodium bicarbonate and 20 mM HEPES (production medium). After the flasks were switched to the serum-free production medium, additional cell growth was minimal (ca. 10% increase in cell numbers over a 20-h period).<sup>3</sup> The production medium was supplemented with either 0, 3, or 9 mM ammonium chloride. Three confluent T-flasks were used for each data point. The growth medium was removed once the T-flasks were confluent and each flask was rinsed once with production medium. The cells were then incubated with ca. 15 mL per flask of the appropriate production medium to acclimate the cells to the desired pH<sub>e</sub> and ammonium chloride concentration. This medium was removed after 4 h, and 27 mL of fresh production medium was added to each flask.

To monitor pH, a 1.5-mL sample was withdrawn from one of the three T-flasks, and the pH was immediately measured to minimize any pH increase due to CO<sub>2</sub> loss. The same volume of 1 M HCl or 1 M NaOH was added to each T-flask to adjust the pH. If there was a large deviation in pH, a second sample was taken following acid/base addition to check for pH overshoot. The T-flask from which the pH samples were taken were rotated, so that each T-flask

would have been sampled once for each three pH samples. For the first 2 h, the pH was checked every 30 min, and the medium withdrawn for pH measurement was replaced with fresh medium. The flasks were subsequently monitored every 3 h without medium replacement. Using this strategy the pH was controlled within  $\pm 0.1$  pH units. The incubator CO<sub>2</sub> was adjusted to 5% for the high pH<sub>e</sub> values (8.4, 8.0, and 7.6) and to 1% to 3% for the low pH<sub>e</sub> values (6.7 and 7.2) to aid in pH control. Both the production medium (containing secreted mPL-I) and the cells were harvested after ca. 20 h.

### Assays

Cells were counted by trypsinizing the cells from the T-flask, centrifuging the cell suspension to obtain a cell pellet, aspirating off the supernatant, and resuspending the pellet in a 2% Triton X-100 (Sigma) in 0.1 M citrate to release the nuclei. Nuclei were counted using a Coulter Multisizer (Coulter Electronics, Hialeah, FL).

Biologically active mPL-I concentrations were determined by measuring [<sup>3</sup>H]-thymidine uptake of Nb2-node lymphoma cells, which were stimulated with dilutions of mPL-I containing, conditioned medium. This assay was performed as described.<sup>3</sup> Nb2 cells are dependent on lactogenic hormones for growth<sup>52</sup> and their growth response was found to be approximately linear for prolactin concentrations between 2 to 200 pg/mL (data not shown). The mPL-I concentration was estimated by comparing the uptake of [<sup>3</sup>H]-thymidine by Nb2 cells that were stimulated to grow with known dilutions of mPL-I containing, conditioned medium to a standard curve generated by measuring the uptake of Nb2 cells stimulated with known amounts of ovine prolactin.

### SDS-PAGE Analysis and Immunoblotting

Supernatant samples containing approximately 1  $\mu$ g of concentrated recombinant protein (as determined by the Nb2 mitogenic assay), concentrated using 10,000 NMWL Ultrafree-CL ultrafiltration membranes (Millipore, Bedford, MA), were diluted 1:1 in sample buffer (0.125 M Tris, 25% glycerol, 2.5% sodium dodecylsulfate [SDS], 0.0025% [w/v] bromophenol blue, 15% mercaptoethanol, and 20 mM dithiothreitol) or cell lysates from ca. 1  $\times$  10<sup>6</sup> cells in 30 mL sample buffer were separated by SDS-PAGE (12% polyacrylamide gels). The cells were lysed by sonication of cell pellets in sample buffer. The protein bands were transferred to 0.2  $\mu$ m nitrocellulose paper (Bio-Rad, Richmond, CA) in Towbin buffer (25 mM Tris, 192 mM glycine, and 20% methanol, pH 8.3). All remaining steps were carried out in TBS (0.02 M Tris, and 0.5 M NaCl, pH 7.5). After blocking the unoccupied protein binding sites on the nitrocellulose sheet with 1% gelatin in TBS, the blot was incubated with rabbit anti-mPL-I antiserum,<sup>41</sup> and subsequently with either a goat antirabbit IgG alkaline phosphatase-conjugated antibody (Sigma),

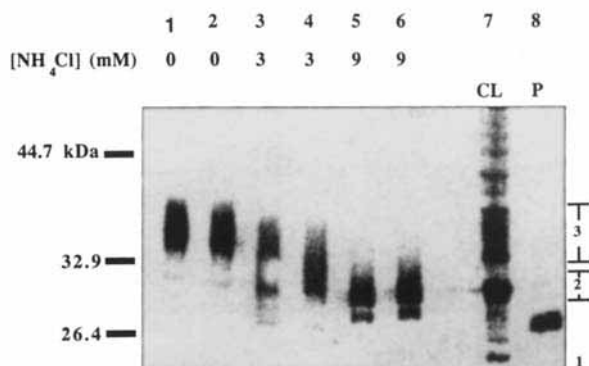
or a goat antirabbit IgG  $^{125}\text{I}$ -conjugated antibody (NEN, Wilmington, DE). The relative intensity of the bands probed with the  $^{125}\text{I}$ -conjugated antibody were quantified using a PhosphorImager with ImageQuant Version 3.0 software (Molecular Dynamics, Sunnyvale, CA).

Peptide-*N*-glycosidase F (PNGase F) was used to hydrolyze the glycosamine linkage of high mannose, complex, and hybrid asparagine-linked oligosaccharide chains, producing a carbohydrate-free protein chain.<sup>53</sup> mPL-I containing supernatant samples were enzymatically deglycosylated by treatment with PNGase F as described.<sup>3</sup> The migration of the treated samples on SDS-PAGE was then determined by immunoblotting.

## RESULTS

### Effect of Ammonium Chloride on mPL-I Glycosylation

mPL-I containing culture supernatants and cell samples were collected from cultures maintained at  $\text{pH}_e$  6.7, 7.2, 7.6, 8.0, and 8.4 ( $\pm 0.1$  pH units) in media supplemented with 0, 3, or 9 mM ammonium chloride for 20 h following a 4-h acclimation period. The higher  $\text{pH}_e$  (above 7.6) values were examined because we had earlier found that mPL-I expression and glycosylation were optimal at the  $\text{pH}_e$  range of 7.6 to 8.0.<sup>3</sup> mPL-I concentration in the supernatants was determined using the Nb2 mitogenic assay. The intracellular and secreted glycoforms of mPL-I were assayed by SDS-PAGE and immunoblotting, and quantified using densitometry. The immunoblot of cell lysates (Fig. 1a) reveals that mPL-I was present intracellularly in three groups of glycoforms, classified here as group 1 (ca. 21.9 kilodaltons [kD]), 2 (ca. 28.3 to 30.4 kD), and 3 (ca. 32.9 to 39.0 kD). Group 3 mPL-I constitutes the majority of the mPL-I secreted under nonstressed conditions (0 mM  $\text{NH}_4\text{Cl}$ ), and apparently was the most heavily glycosylated group of mPL-I. Group 2 glycoforms were typically not detected

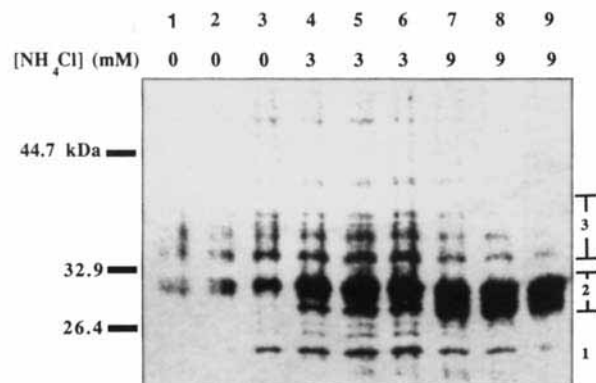


**Figure 1.** (a) Immunoblot specific for mPL-I from cell lysates of cultures maintained at  $\text{pH}_e$  8.4.  $\text{NH}_4\text{Cl}$  concentrations of the culture media were: 0 mM (lanes 1, 2, and 3), 3 mM (lanes 4, 5, and 6), and 9 mM (lanes 7, 8, and 9). The glycoform bands classified as groups 1, 2, and 3 are indicated on the right. Each lane was loaded with lysate from ca.  $1 \times 10^6$  cells.

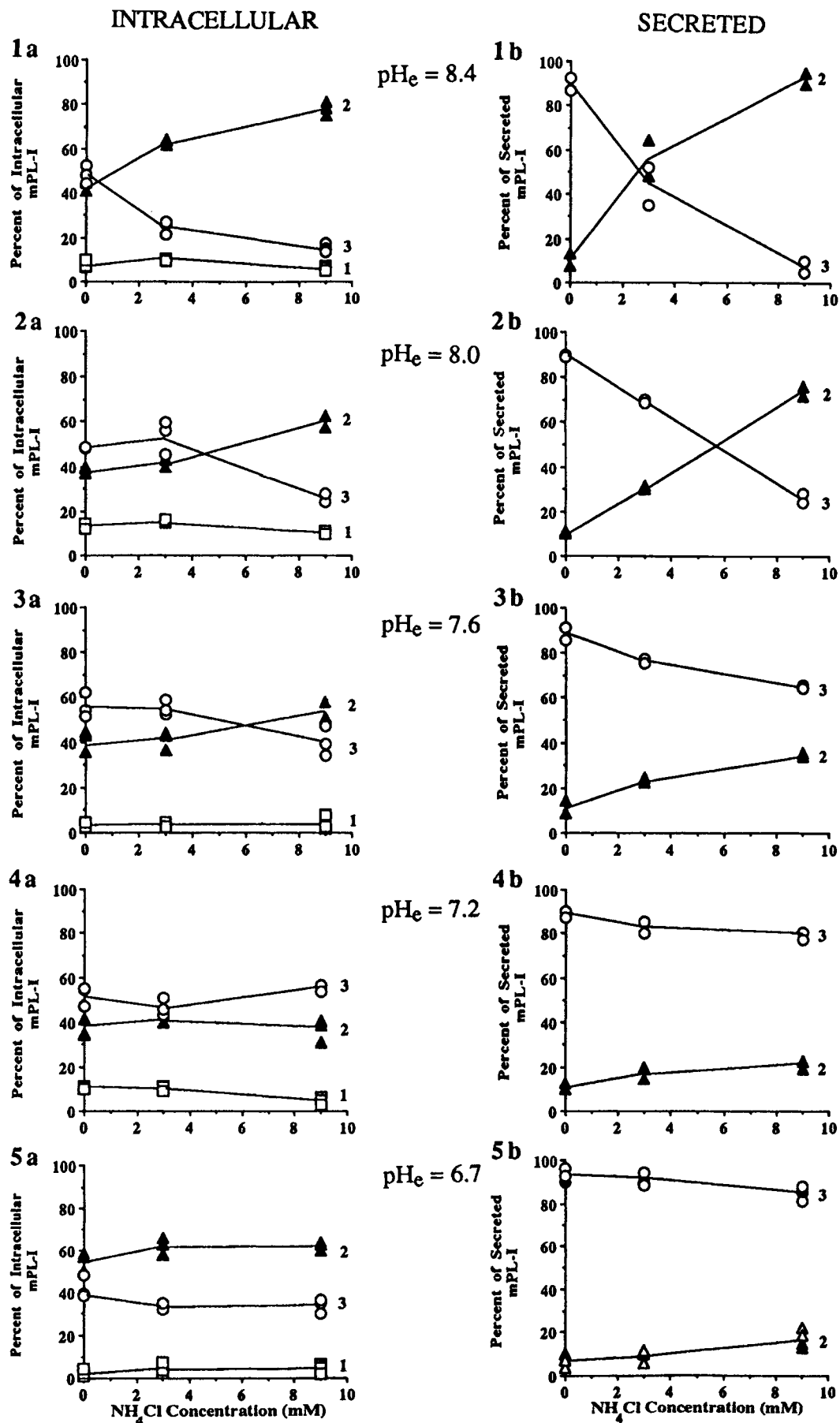
in the culture medium under nonstressed conditions. The apparent molecular size of group 1 was below that of enzymatically deglycosylated mPL-I by treatment with PNGase F (ca. 24.0 to 26.0 kD) (Fig. 1b, lanes 7 and 8), and probably represents intracellular degradation products or incompletely synthesized mPL-I. Immunoblots of cell lysates from wild-type CHO cells did not contain any proteins recognized by mPL-I antiserum.

The immunoblots of intracellular mPL-I (Fig. 1a) (in triplicate) and of secreted mPL-I (Fig. 1b) (in duplicate) from cultures maintained at  $\text{pH}_e$  8.4, show that, as the  $\text{NH}_4\text{Cl}$  concentration was increased from 0 mM to 3 mM and 9 mM, there was a large decrease in the molecular size range of mPL-I. Intracellularly, the most dramatic changes were the decreasing amounts of mPL-I in group 3, as well as the increasing amounts in group 2 mPL-I (Fig. 1a). Lower-molecular-weight bands in group 2 mPL-I appeared at 3 and 9 mM  $\text{NH}_4\text{Cl}$ . Such changes were also apparent for secreted mPL-I (Fig. 1b). At 9 mM  $\text{NH}_4\text{Cl}$ , most of the secreted mPL-I was in group 2, with negligible amounts of mPL-I in group 3. In addition to the decrease in group 3 mPL-I, there was also a decrease in the molecular size range of group 3 mPL-I with increasing  $\text{NH}_4\text{Cl}$  concentration. At both 3 and 9 mM  $\text{NH}_4\text{Cl}$  ( $\text{pH}_e$  8.4), there was a mPL-I species (ca. 27 kD) which migrated slightly slower than enzymatically deglycosylated mPL-I (ca. 26 kD) (Fig. 1b). This 27-kD species represented ca. 20% of the total secreted mPL-I in medium containing 9 mM  $\text{NH}_4\text{Cl}$ .

Changes in the percentage of both intracellular and secreted mPL-I in groups 1, 2, and 3 were quantified by densitometry (Fig. 2). At  $\text{pH}_e$  8.4, the percentage of intracellular mPL-I in group 3 decreased from 50%



**Figure 1.** (b) Immunoblot specific for mPL-I containing supernatants from cultures maintained at  $\text{pH}_e$  8.4. The  $\text{NH}_4\text{Cl}$  concentrations of the culture media were: 0 mM (lanes 1 and 2), 3 mM (lanes 3 and 4), and 9 mM (lanes 5 and 6). Approximately 1  $\mu\text{g}$  of mPL-I, as determined by the Nb2 mitogenic assay in supernatant samples, was loaded per lane. For reference, cell lysates ( $\text{pH}_e$  8.4, 0 mM  $\text{NH}_4\text{Cl}$ , ca.  $1 \times 10^6$  cells per lane) (lane 7 [CL]) and a sample of enzymatically deglycosylated mPL-I (by treatment with PNGase F) (ca. 0.5  $\mu\text{g}$  mPL-I per lane) (lane 8 [P]) were also included. The glycoform bands classified as groups 1, 2, and 3 are indicated on the right. The approximate molecular sizes in kilodaltons are indicated on the left. The immunoblot was developed using  $^{125}\text{I}$ -linked secondary antibody.

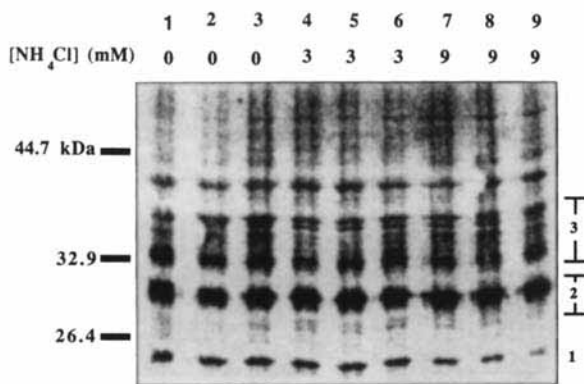


**Figure 2.** The percentage of mPL-I in groups 1, 2, and 3 as a function of  $NH_4Cl$  concentration for (a) cell lysates and (b) culture supernatants from cultures maintained at (1)  $pH_e$  8.4, (2)  $pH_e$  8.0, (3)  $pH_e$  7.6, (4)  $pH_e$  7.2, and (5)  $pH_e$  6.7. The immunoblots were developed using an  $^{125}I$ -linked secondary antibody and the signals were quantified by densitometry using a PhosphorImager.

at 0 mM NH<sub>4</sub>Cl to 15% at 9 mM NH<sub>4</sub>Cl (Fig. 2.1a). The percentage of secreted mPL-I in group 3 decreased from >90% at 0 mM NH<sub>4</sub>Cl to <10% at 9 mM NH<sub>4</sub>Cl (Fig. 2.1b). In contrast, the percentage of mPL-I in group 2 increased from 50% to 80% for intracellular mPL-I and from approximately 10% to 90% for secreted mPL-I when NH<sub>4</sub>Cl was increased from 0 mM to 9 mM. Intracellular mPL-I in group 1 did not change significantly with increasing concentrations of NH<sub>4</sub>Cl, and group 1 mPL-I was not detected in the supernatant.

The effects of increasing ammonium chloride concentrations on the percentage of intracellular or secreted mPL-I in groups 1, 2, or 3, from cultures maintained at pHe 8.0, 7.6, 7.2, or 6.7 as quantified by densitometry, are shown in Figures 2.2, 2.3, 2.4, and 2.5, respectively. The effects of increasing NH<sub>4</sub>Cl concentration on the molecular sizes of the expressed mPL-I at pHe 8.0, although still substantial, were less severe than the effects noted at pHe 8.4. At pHe 8.0, the percentage of intracellular mPL-I in group 3 (Fig. 2.2a) decreased from approximately 50% to 25%, and the percentage of secreted mPL-I in group 3 (Fig. 2.2b) decreased from approximately 90% to 25% for cultures at 0 mM and 9 mM NH<sub>4</sub>Cl, respectively. The ca. 27-kD band in the supernatant that was observed in Fig. 1b (pHe 8.4, 9 mM NH<sub>4</sub>Cl), was barely detectable at 9 mM NH<sub>4</sub>Cl at pHe 8.0 (represented ca. 5% of the total secreted mPL-I in medium with 9 mM NH<sub>4</sub>Cl).

At lower pHe values, there was a decreased effect of NH<sub>4</sub>Cl on the molecular size of group 3 mPL-I and on the percent of intracellular and secreted mPL-I in groups 1, 2, and 3. This was obvious at pHe 7.6 where the percentage of secreted groups 3 mPL-I decreased from approximately 90% (0 mM NH<sub>4</sub>Cl) to 60% with 9 mM NH<sub>4</sub>Cl (Fig. 2.3b). There was no apparent change in the size distribution of intracellular (Fig. 3a) or secreted mPL-I (Fig. 3b) with increasing NH<sub>4</sub>Cl (up to 9 mM) from cultures maintained at pHe 7.2. However, the densitometry analysis did show that the percentage of secreted group 3 mPL-I decreased



**Figure 3.** (a) Immunoblot specific for mPL-I from cell lysates of cultures maintained at pHe 7.2. NH<sub>4</sub>Cl concentrations of the culture media were: 0 mM (lanes 1, 2, and 3), 3 mM (lanes 4, 5, and 6), and 9 mM (lanes 7, 8, and 9). The glycoform bands classified as groups 1, 2, and 3 are indicated on the right. Each lane was loaded with cell lysates from ca.  $1 \times 10^6$  cells.

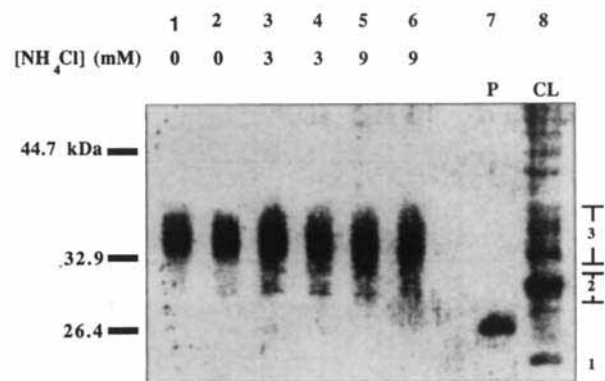
from approximately 90% to 80% as NH<sub>4</sub>Cl increased from 0 to 9 mM (Fig. 2.4b).

The percentage of intracellular group 3 mPL-I was always greater than group 2 mPL-I for pHe 7.2 to 8.4 from cultures with 0 mM NH<sub>4</sub>Cl. The results from duplicate experiments at pHe 6.7 showed the percentage of intracellular group 2 mPL-I to be greater than group 3 mPL-I (Fig. 2.5a). This was most likely a result of low pHe. Nevertheless, the densitometry data showed the percentages of groups 1, 2, and 3 mPL-I to be much more resistant to NH<sub>4</sub>Cl concentrations at pHe 6.7 (Fig. 2.5a and b) than was noted at high pHe (7.6, 8.0, and 8.4). The immunoblot of secreted mPL-I at pHe 6.7 (Fig. 4) showed a slight increase in the molecular size of secreted mPL-I at 3 mM NH<sub>4</sub>Cl compared with mPL-I secreted into medium containing 0 mM NH<sub>4</sub>Cl. This result was also found to be consistent for the duplicate experiments at pHe 6.7.

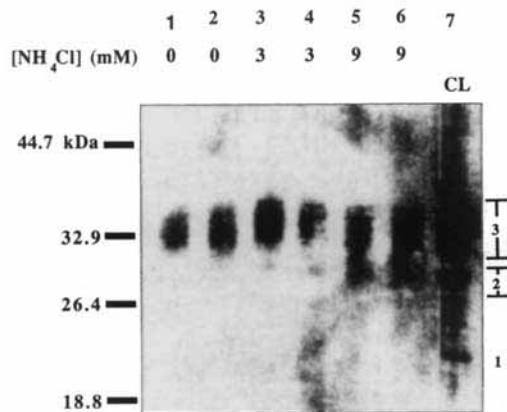
### Control Experiments

To determine whether different protein loadings on the immunoblot would affect the percentages of the different mPL-I groups, the gel loadings for both the cell lysates (in duplicate) and supernatant samples were varied by  $\pm 50\%$  of the amount used in the study (i.e.,  $1.0 \times 10^6$  cells/lane and  $1.0 \mu\text{g}$ /lane for the cell lysates and supernatant samples, respectively). The samples for this immunoblot were from the culture at pHe 8.0 and 3 mM NH<sub>4</sub>Cl. The results presented in Table I (A, B, and C) show that the percentages of intracellular mPL-I in groups 1, 2, and 3, and for secreted mPL-I in groups 2 and 3 were similar for all three loadings. In addition, the percentages reported in Table IB and C correspond to the values in Figures 2.2a and 2.2b (at 3 mM NH<sub>4</sub>Cl).

Samples of secreted mPL-I were enzymatically deglycosylated by PNGase F treatment to demonstrate that



**Figure 3.** (b) Immunoblot specific for mPL-I containing supernatants of cultures maintained at pHe 7.2. NH<sub>4</sub>Cl concentrations of the culture media were: 0 mM (lanes 1, 2, and 3), 3 mM (lanes 3 and 4), and 9 mM (lanes 5 and 6). Each lane was loaded with  $1 \mu\text{g}$  of mPL-I as determined by the Nb2 mitogenic assay. The immunoblot was developed using <sup>125</sup>I-linked secondary antibody. For reference, a cell lysate sample (pHe 7.2, 0 mM NH<sub>4</sub>Cl [from ca.  $1 \times 10^6$  cells]) (lane 8 [CL]) and a sample (0.5  $\mu\text{g}$ ) of enzymatically deglycosylated mPL-I (lane 7 [P]) were also included. The glycoform bands classified as groups 1, 2, and 3 are indicated on the right.



**Figure 4.** Immunoblots from the second set of mPL-I containing supernatants of cultures maintained at  $pH_e$  6.7. Supernatant samples from cultures maintained with  $NH_4Cl$  concentrations of 0 mM (lanes 1 and 2), 3 mM (lanes 3 and 4), and 9 mM (lanes 5 and 6). The glycoform bands classified as groups 1, 2, and 3 are indicated on the right. For reference, a cell lysate sample ( $pH_e$  7.2, 0 mM  $NH_4Cl$  [from ca.  $1 \times 10^6$  cells]) (lane 7) was also included. Lanes containing supernatant samples were loaded with 1  $\mu g$  of mPL-I as determined by the Nb2 mitogenic assay. The immunoblot was developed using  $^{125}I$ -linked secondary antibody.

the changes in the migration of secreted mPL-I on SDS-PAGE were in fact due to changes in *N*-linked glycosylation. The immunoblot of the PNGase F-treated samples (Fig. 5) at  $pH_e$  7.2 with 0 mM  $NH_4Cl$  and at  $pH_e$  7.2, 7.6, 8.0, and 8.4 all with 9 mM  $NH_4Cl$  revealed two similar bands at ca. 25 to 26 kD (similar to what has been previously reported<sup>3,9</sup>), indicating that  $NH_4Cl$  did not affect the *N*-linked oligosaccharide-free mPL-I molecule as determined by SDS-PAGE. Thus, the shifts in migration patterns on SDS-PAGE of secreted mPL-I at different  $pH_e$  and ammonia concentrations were attributed to differences in the *N*-linked oligosaccharide side chains.

It was necessary to determine whether the changes observed in glycosylation were caused by  $NH_4Cl$  and not the result of changes in either medium osmolarity or chloride concentration. This was examined by incubating CHO cells expressing mPL-I in medium containing 9 mM NaCl at a  $pH_e$  similar to those where changes were observed in media containing 9 mM  $NH_4Cl$ . There were no apparent changes in the molecular sizes of mPL-I (as determined by SDS-PAGE) secreted from cells incubated at  $pH_e$  8.0 in medium containing 9 mM NaCl compared with a control culture (0 mM NaCl) (data not shown).

The  $pH_e$  dependence of the inhibition of mPL-I glycosylation by ammonia was observed for both secreted

**Table I.** Different loadings per lane of cell lysates from CHO cells expressing mPL-I and mPL-I containing supernatant samples ( $pH_e$  8.0, 3 mM  $NH_4Cl$ ) were analyzed by immunoblotting with  $^{125}I$ -conjugated secondary antibody and scanned using the PhosphorImager.

(A)

mPL-I Subgroup	$0.5 \times 10^6$ cells/Lane		$1.0 \times 10^6$ cells/lane		$1.5 \times 10^6$ cells/lane	
	No. 1	No. 2	No. 1	No. 2	No. 1	No. 2
Group 1	202351	226843	584330	434725	1109395	750861
Group 2	4801726	6899999	8771857	8936077	13881296	10190287
Group 3	4347865	6215388	7880286	8709763	12164119	9727459

(B)

mPL-I Subgroup	$0.5 \times 10^6$ cells/lane		$1.0 \times 10^6$ cells/lane		$1.5 \times 10^6$ cells/lane	
	No. 1	No. 2	No. 1	No. 2	No. 1	No. 2
Group 1	2%	2%	3%	2%	4%	4%
Group 2	51%	52%	51%	49%	51%	49%
Group 3	47%	47%	46%	48%	45%	47%

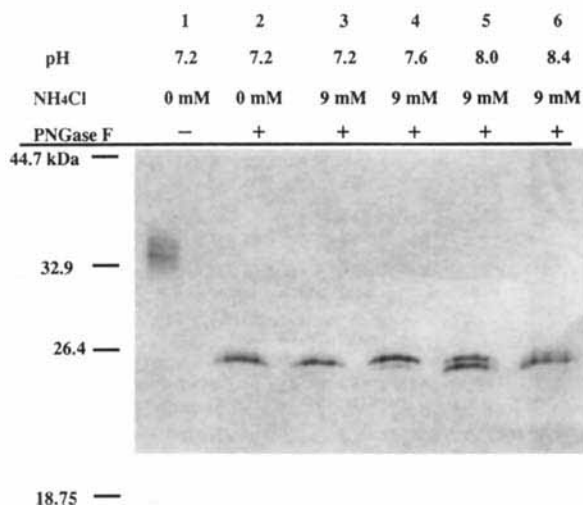
(C)

mPL-I Subgroup	0.5 $\mu g$ mPL-I/lane		1.0 $\mu g$ mPL-I/lane		1.5 $\mu g$ mPL-I/lane	
	Signal	Percentage	Signal	Percentage	Signal	Percentage
Group 2	3229554	21%	4470674	23%	7747808	27%
Group 3	11892445	79%	15329760	77%	21345297	73%

(A) The immunoblot signals (values reported in pixels) of each of the three intracellular mPL-I groups with different cell lysate loadings.

(B) The corresponding percentage for each of the three intracellular mPL-I groups with different cell lysate loadings.

(C) The immunoblot signals of mPL-I supernatant samples with different mPL-I loadings from the PhosphorImager and the corresponding percentage for each mPL-I group.



**Figure 5.** Immunoblot of enzymatically deglycosylated mPL-I by treatment with PNGase F. Each lane was loaded with 0.5  $\mu$ g of mPL-I as determined (before treatment) by the Nb2 mitogenic assay. An untreated supernatant sample from a culture maintained at pH<sub>e</sub> 7.2 was loaded in lane 1. The NH<sub>4</sub>Cl concentration and pH<sub>e</sub> are indicated at the top of the figure.

mPL-I and intracellular mPL-I, thus suggesting that the changes occurred intracellularly. Nevertheless, a control experiment was performed to determine whether ammonia in the medium may have altered mPL-I glycosylation after secretion, either by affecting the activity of glycosidases which may have been secreted into the supernatant or by differentially causing the cells to secrete glycosidases. Ammonium chloride was added to a final concentration of 9 mM to two aliquots of mPL-I containing supernatant from cultures maintained at pH<sub>e</sub> 8.0.

One aliquot was incubated at 37°C for 20 h (without cells present) to determine whether glycosidases, which may be present in the culture supernatant, were affected by the presence of 9 mM NH<sub>4</sub>Cl. The second aliquot was added to a CHO culture that expressed a related recombinant protein, mouse placental lactogen II (mPL-II),<sup>3</sup> and incubated for 20 h at pH<sub>e</sub> 8.0. The migration on SDS-PAGE (as detected by immunoblotting) of the samples was compared with an untreated control of medium containing mPL-I, and demonstrated that incubation with or without cells did not affect the migration patterns of mPL-I on SDS-PAGE (data not shown). These experiments demonstrated that the effect of ammonia on mPL-I glycosylation was not the result of an extracellular event in culture supernatants.

### Effect of Ammonium Chloride on Specific mPL-I Expression Rates

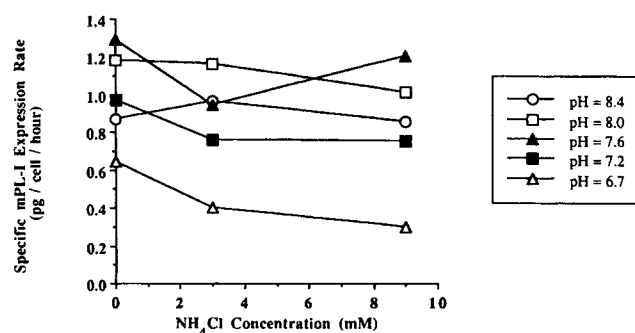
The effect of NH<sub>4</sub>Cl concentration on the specific rates of mPL-I expression from CHO cells was determined between pH<sub>e</sub> 6.7 to 8.4 (Fig. 6). mPL-I expression rates were calculated from the mPL-I concentration (as determined by the Nb2 bioassay) and the average volume in the T-flasks (decrease due to samples taken for pH adjustment). The

mPL-I expression rate was underestimated by ca. 20% due to the use of the average medium volume. Because the same pH control strategy was used for each data point, the reported trends would still be valid. Overall, the specific rate of mPL-I expression decreased with increasing NH<sub>4</sub>Cl. However, it appears that the decrease in specific mPL-I expression rates with increasing NH<sub>4</sub>Cl concentrations was greater at the lower pH<sub>e</sub> examined. For example, increasing the NH<sub>4</sub>Cl concentration from 0 to 9 mM resulted in a 50%, 20%, 5%, 15%, and 1% decrease in the specific mPL-I expression rates for pH<sub>e</sub> 6.7, 7.2, 7.6, 8.0, and 8.4, respectively. The maximum specific expression rates occurred at pH<sub>e</sub> 7.6 or 8.0 for cultures with ammonium chloride up to 9 mM, and the specific expression rates decreased with decreasing pH<sub>e</sub>. The effect of pH<sub>e</sub> on mPL-I specific expression rates was consistent with earlier results,<sup>3</sup> and the optimal pH<sub>e</sub> for the maximum mPL-I expression rate was not affected by the presence of ammonium chloride up to a concentration of 9 mM.

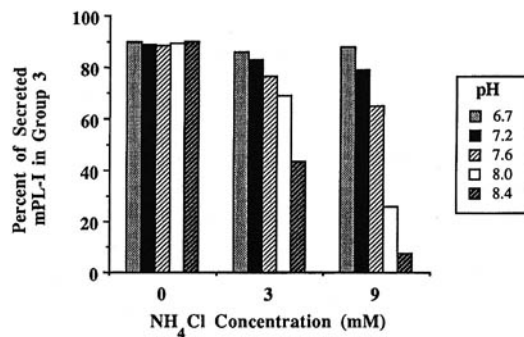
## DISCUSSION

Ammonia has typically been identified as a harmful component in cell culture media because of its inhibitory effect on cell growth.<sup>6,20,21,33,36,39,47,54</sup> Ammonia has also been reported to affect many cellular processes including the pH of intracellular organelles (such as the Golgi compartments), enzyme activity (independent of pH), vesicle fusion and transport, and lysosomal proteolysis,<sup>12,28,49</sup> all of which may have contributed to the pH<sub>e</sub>-dependent effect of ammonia on mPL-I glycosylation reported here. Clearly, classification of several mPL-I bands into three groups, for the purpose of quantitation (Fig. 1a), was a simplistic treatment of a very complex event. Nevertheless, both the magnitude of and the trends for the effect of ammonia on mPL-I glycosylation could be effectively quantified by the changes in the different band groups of both secreted and intracellular mPL-I.

The pH<sub>e</sub> dependence of the inhibitory effect of ammonia on mPL-I glycosylation is summarized in Figure 7, in which the percentage of secreted mPL-I in group 3 (most



**Figure 6.** Effect of NH<sub>4</sub>Cl concentration on the specific expression rate of mPL-I by CHO cells at different pH<sub>e</sub>. Conditioned media were collected after 20 h for each pH<sub>e</sub> value.



**Figure 7.** Summary of the effect of  $\text{NH}_4\text{Cl}$  on mPL-I glycosylation at different  $\text{pH}_e$  values. The percentage of secreted mPL-I in group 3 for different  $\text{pH}_e$  is plotted as a function of  $\text{NH}_4\text{Cl}$  concentration.

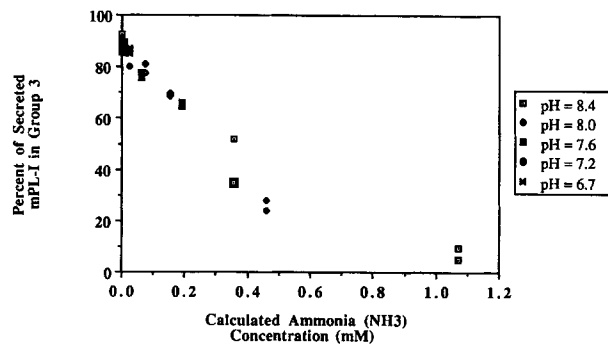
glycosylated) was plotted versus ammonium chloride concentration for each  $\text{pH}_e$  studied. This figure demonstrates the decreasing effect of  $\text{NH}_4\text{Cl}$  concentration on mPL-I glycosylation with decreasing  $\text{pH}_e$ . This is consistent with results described in a recent patent on the  $\text{pH}_e$ -dependent ammonia effect on the sialylation of monoclonal antibodies expressed by hybridomas.<sup>35</sup>

Several authors<sup>13,15,20,45</sup> have hypothesized that the unprotonated ammonia species ( $\text{NH}_3$ ) as well as the unprotonated forms of other weak bases (chloroquine, methylamine, tributylamine) are responsible for their inhibitory effects on animal cells and that the protonated forms (ammonium ion,  $\text{NH}_4^+$ ) have little effect. This is because the ammonium ion must be transported across cell membranes through ion-transport systems.<sup>13,30</sup> However, the uncharged base can freely diffuse across plasma and lysosomal membranes.<sup>2</sup> The  $\text{pH}_e$  dependence of  $\text{NH}_4\text{Cl}$  inhibiting mPL-I glycosylation is consistent with the hypothesis that the unprotonated ammonia ( $\text{NH}_3$ ) species is responsible for the inhibitory effects of ammonia/ammonium ion. The unprotonated ammonia ( $\text{NH}_3$ ) concentration in the supernatant was calculated for each  $\text{pH}_e$  by the Henderson-Hasselbach equation [Eq. (1)] using a value of 9.27 for the  $\text{p}K_a$  of ammonium at 37°C.<sup>57</sup>

$$\text{pH} = \text{p}K_a + \log(\text{NH}_3/\text{NH}_4^+) \quad (1)$$

The percentage of secreted mPL-I in group 3 was found to decrease in a linear fashion with  $\text{NH}_3$  concentration (Fig. 8). This supports evidence that the  $\text{NH}_3$  concentration was responsible for the  $\text{pH}_e$ -dependent inhibition of mPL-I glycosylation by  $\text{NH}_4\text{Cl}$ . This was also consistent with the results of Doyle and Butler,<sup>15</sup> who found the inhibition of hybridoma cell growth to correlate with increasing  $\text{NH}_3$  concentration.

There was a reproducible increase in mPL-I glycosylation noted in medium containing 3 mM  $\text{NH}_4\text{Cl}$  at  $\text{pH}_e$  6.7 (Fig. 4). It has been reported that the trans-Golgi is an acidic compartment<sup>1,51</sup> in which ammonia can accumulate and increase the pH.<sup>55</sup> We have previously reported<sup>3</sup> that mPL-I glycosylation was found to decrease at  $\text{pH}_e$  6.7



**Figure 8.** The percentage of mPL-I in group 3 plotted against the calculated [using Eq. (1)] ammonia ( $\text{NH}_3$ ) concentration. The equilibrium ammonia concentration at each  $\text{pH}_e$  was calculated based on the initial concentration of  $\text{NH}_4\text{Cl}$ .

compared with cultures maintained at  $\text{pH}_e$  6.9 (without  $\text{NH}_4\text{Cl}$  addition). The small increase in mPL-I glycosylation at  $\text{pH}_e$  6.7 with 3 mM  $\text{NH}_4\text{Cl}$  compared with  $\text{pH}_e$  6.7 with 0 mM  $\text{NH}_4\text{Cl}$  may have resulted from ammonia increasing the Golgi pH to a more physiologically optimal value. The subsequent decrease in mPL-I glycosylation at  $\text{pH}_e$  6.7 with 9 mM  $\text{NH}_4\text{Cl}$  may be the result of either inhibition due to the ammonia species itself or due to further increases in Golgi pH.

Previous studies have reported that ammonia can disrupt the terminal sialylation of glycoproteins.<sup>35,55</sup> Although the specific sugar units affected by ammonia were not identified in this present study, it is clear that the potential for ammonia to disrupt glycosylation is greater than disrupting only terminal sialylation. The severity with which  $\text{NH}_4\text{Cl}$  inhibited mPL-I glycosylation was demonstrated by comparing mPL-I expressed under extreme conditions ( $\text{pH}_e$  8.4, 9 mM  $\text{NH}_4\text{Cl}$ ) to enzymatically deglycosylated mPL-I. When CHO cells expressing mPL-I were incubated at  $\text{pH}_e$  8.4 in medium containing 9 mM  $\text{NH}_4\text{Cl}$ , the majority of the secreted mPL-I was in group 2. However, there was also a significant mPL-I band that migrated slightly above (ca. 26 to 27 kD on SDS-PAGE) the enzymatically deglycosylated mPL-I (24 to 26 kD). These results indicate that ammonia has the potential for affecting the entire glycosylation process.

Previous studies have suggested switching to a lower pH toward the end of the culture period to minimize the effects of extracellular ammonia.<sup>15,19,35</sup> However, mPL-I was found to be optimally expressed at  $\text{pH}_e$  7.6 (in this and a previous study<sup>3</sup>), and at this  $\text{pH}_e$  there was significant inhibition of mPL-I glycosylation even at low ammonia concentrations (3 mM). Whereas at  $\text{pH}_e$  7.2 the effects of ammonia on mPL-I glycosylation were less severe, the expression rate of mPL-I decreased by ca. 40%. Thus, where protein glycosylation is important, it would be necessary to optimize culture pH, glutamine levels, and medium exchange schedules to minimize the effects of ammonia levels on protein glycosylation while maintaining high protein expression rates.

This work was supported by a grant from the National Science Foundation to E. T. P. (EET-8896100) and by a grant from the National Institutes of Health to D. L. (HD24518), who is also the recipient of an American Cancer Society faculty research award. The authors also thank the National Hormone and Pituitary Program for the ovine prolactin, and to Frank Talamantes for the antiserum against mPL-I.

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