Biochemical and Biophysical Research Communications **258,** 132–137 (1999)
Article ID bbrc.1999.0550, available online at http://www.idealibrary.com on IDEAL®

Aberrant Metabolic Sialylation of Recombinant Proteins Expressed in Chinese Hamster Ovary Cells in High Productivity Cultures

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Received March 5, 1999

The incorporation of sialic acid into therapeutic recombinant glycoprotein expressed in Chinese hamster ovary (CHO) cells during growth in large bioreactors (10 l) has been monitored under high productivity conditions induced by the presence of sodium butyrate. Samples of the bioreactor culture (~4 × 10^6 cells) were labeled with ^{3}H-*N*-acetylmannosamine, a metabolic precursor of sialic acid. After 24 h, the recombinant glycoprotein, an immunoadhesion chimeric molecule, was purified and the amount of sialic acid incorporated was determined as radioactive counts. The labeling profile of the protein over the course of the culture was compared with the sialic acid content of the molecule as determined by direct chemical analysis. Early in the culture, the two methods of analysis gave a similar sialylation profile. However, after sodium butyrate was included in the culture, the metabolically incorporated sialic acid rapidly and dramatically decreased to near undetectable levels. In contrast, sialic acid content of the protein, as determined by chemical analysis, decreased only moderately and gradually over the culture period, from a maximum of 6.1 to about 5.0 mol sialic acid/mole of protein after 10 days in culture. These results suggest that butyrate may enhance reutilization of existing glycoproteins in the culture, generating sialic acid for biosynthesis through lysosomal degradation and thereby bypassing *de novo* biosynthesis.

The elegantly complex mechanism of assembly of the glycan structures on mammalian glycoproteins has been well defined over the last twenty years. Currently, the carbohydrate moieties on therapeutic glycoproteins are of great interest because they can have a significant impact on the efficacy of the molecule (1,2). N-linked complex type oligosaccharides lacking sialic acid terminate with galactose, N-acetylglucosamine, or mannose residues. Glycoproteins with these exposed carbohydrates are rapidly removed from the plasma by receptor mediated endocytosis by various tissues (3). With many therapeutic proteins maximizing the residence time in the circulation is essential for achieving complete therapeutic effectiveness. Therefore, it is highly desirable to identify mammalian expression hosts or culture conditions that produce recombinant proteins with completely extended oligosaccharide branches capped with sialic acid. Chinese hamster ovary, CHO, cells have been employed for the production of numerous therapeutic products. However, several investigators have shown that a wide variety of cell culture parameters and media components can influence glycosylation in this host (4-6). Defining cell culture conditions that consistently give maximal sialylation is requisite for establishing a viable production process for therapeutic glycoproteins.

We have observed that sialylation of a number of recombinant glycoproteins produced in CHO cells is often incomplete, giving rise to partially truncated oligosaccharides side chains on the therapeutic molecule terminating in galactose residues. This is particularly apparent in proteins made under high productivity conditions in which sodium butyrate has been included in the culture inorder to amplify protein synthesis.

In many of these cases, 'undersialylation' cannot be accounted for by degradation of the protein by the actions of a soluble sialidase because the viability of the culture is maintained at high levels (7). The sialidase is predominantly a cytosolic enzyme and is released into the cell culture fluid only upon cell lysis.

In an effort to gain insight into the sialylation processes under these conditions, we have investigated here, sialylation of a recombinant protein produced in CHO cells in a batch culture setting under high productivity conditions. Two methods for monitoring the sialic acid content of a recombinant immunoadhesion protein, GP1-IgG (8), over the course of a CHO cell

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0006-291X/99 $30.00

culture, were employed. Sialic acid that was incorporated into GP1-IgG was determined by metabolic labeling growing cultures using radioactively labeled N-acetylmannosamine (ManNAc) as a metabolic precurssor of sialic acid. This method is advantageous because it is sensitive, requiring only a small number of cells for analysis, and provides insight into sialylation events at 24 h intervals rather than the conventional method of compositional analysis which gives sialic acid levels on total recombinant protein that has accumulated in the culture up to the time of sampling. The pattern of sialylation was also determined by quantifying the sialic acid on the protein by chemical derivatization followed by chromatographic analysis.

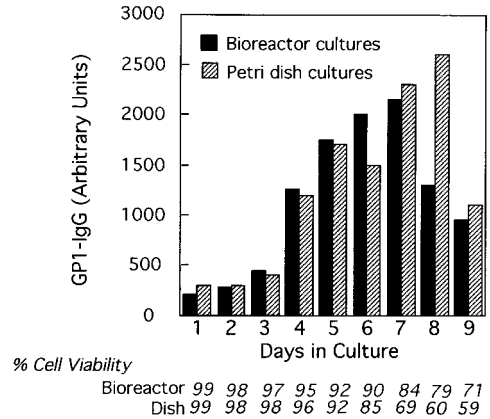

FIG. 1. Comparison of protein productivity and cell viability of GP1-IgG secreting cells maintained in Petri dishes and in a 2 l bioreactor. Samples of cells were periodically withdrawn, every 24 h, from the bioreactor and placed in 60 mm Petri dishes and maintained undisturbed at the temperature of the bioreactor. After 24 h the cell viabilities of the cells in the dishes and those in the bioreactor were compared using the LDH method. Similarly, the amount of recombinant protein produced under both culture conditions was quantified with HPLC as described under Experimental Procedures in duplicate.

EXPERIMENTAL PROCEDURES

Expression host and recombinant GP1-IgG. Stable expression of the recombinant protein was accomplished by transfecting dhfr minus CHO cells, derived by clonal selection from CHO-DUKX (9). The cDNA encoding the protein was incorporated into an SV-40 based expression vector also containing the DHFR gene. GP1-IgG is an immunoadhesion chimeric glycoprotein containing three N-linked complex type oligosaccharide chains in the variable region. This molecule contains a theoretical maximum of 7.5 moles of sialic acid per mole of protein (8).

Cell culture. CHO cells were cultured in serum free modified DMEM medium supplemented with 2 g/l glucose to obtain an inoculum in the mid-logrithmic phase. Cells were isolated by centrifugation or tangential flow diafiltration with fresh media prior to inoculation (seeded at 10^6 cells/ml) of 2 or 10 l bioreactors, respectively. In all experiments, GP1-IgG cell cultures were held at 37°C for two days at 60% dO_2, and shifted to 31°C for 8 additional days. At day 3, or 24 h after the temperature shift and feed, sodium butyrate was added to a final concentration of 12 mM. Cell viability was determined using the lactate dehydrogenase assay (10).

Development and validation of metabolic labeling protocol for monitoring sialic acid incorporation in GP1-IgG. The primary objective of these studies was to monitor, on a periodic basis, sialylation events of a recombinant protein under large scale production conditions in batch cultures using metabolic labeling and direct chemical analysis of the GP1-IgG-bound sialic acid. Since it was not practical to carryout metabolic labeling in a large bioreactor, we examined several small scale model systems that closely paralleled the bioreactor culture over a 24 h interval. Samples of the culture were periodically removed from the bioreactor and transferred to one of the following three vessels, 25 ml culture flasks (closed), 60 mm Petri dishes, both agitated with gentle shaking (data not shown), and 60 mm Petri dishes that were not shaken. Cell viability, and recombinant protein productivity were compared in the three model systems with cultures in 2 or 10 l bioreactors. Cultures in the undisturbed 60 mm Petri dishes gave the best results with nearly identical expression levels of GP1-IgG as the cells maintained in the bioreactor format. Cell viability in the dishes and the bioreactor were very similar up to seven days in the culture, Figure 1. The results were repeated in at least three separate experiments (data not shown).

Efficiency of labeling sialic acid in GP1-IgG. ^{3}H-ManNAc was used as a metabolic precursor to specifically label sialic acid of GP1-IgG. The efficiency of labeling the recombinant protein and the distribution of the label incorporated in various cellular compartments was carried out by transferring samples (3.5 ml, $\sim 4-6 \times 10^6$ cells/ml) of a mid-logrithmic phase culture of CHO cells, grown in batch culture in 2 l bioreactors, to 60 mm Petri dishes containing 10 mCi N-acetyl [mannosamine-6-^{3}H] (15 Ci/mmol). The dishes were incubated at 37°C for 24 h without agitation. After incubation, the culture fluid containing the recombinant protein was separated from the cells with centrifugation. The resulting supernatant was subjected to G-25 size exclusion chromatography (NAP 25 column, Pharmacia) and concentrated to ~300 μl using a microconcentrator (Pall-Filtron Corp). The labeled recombinant protein was specifically isolated by immunoprecipitation with a monoclonal antibody to GP1-IgG, Mab-GP1-IgG. In other samples, all of the secreted proteins in the concentrated culture fluid fraction, including the recombinant protein, were isolated by precipitation with 40% trichloroacetic acid (TCA). The remaining cell pellet was washed with phosphate buffered saline (PBS) and the cells ruptured with mild sonication. The cytosol was separated from the membrane fraction with high speed centrifugation (100,000 × g, 4°C). The sialic acid in the secreted protein fraction, the cytosol, the cell membrane and the recombinant GP1-IgG protein was determined as radioactive counts.

Sialidase treatment of metabolically labeled GP1-IgG. Verification that the radioactive counts in the recombinant protein were sialic acid exclusively, and not other metabolites of ManNAc, was made by treating the labeled GP1-IgG (isolated by immunoprecipitation) with 0.5 U/ml of neuraminidase (Cl. perfingens, Sigma) for 8 h at 37°C. After digestion, the GP1-IgG protein was precipitated with TCA (40%) and the counts determined in the resulting supernatant and pellet fractions.

Determining uptake rates of N-acetylmannosamine of GP1-IgG cells grown in bioreactor cultures. The uptake rate of ManNAc by GP1-IgG producing CHO cells was examined, in 24 h intervals, over the initial 7 days of a 2 l bioreactor culture. In addition, uptake rates by the cells used as inoculum were measured immediately prior to medium exchange and transfer. Cells ($\sim 4 \times 10^6$ cells) were taken from a mid-logrithmic phase culture grown in a 2 l bioreactor and transferred to 60 cm Petri dishes containing 35 mCi of ^{3}H-ManNAc. The cells in the dish were maintained at 37 or 31°C and incubated for 2, 4, 8 and 24 h. The amounts of free, intracellular ^{3}H-ManNAc and its soluble metabolites were determined by disrupting the cells with five freeze/thaw cycles, precipitating the proteins and lipids in the lysate with TCA, centrifuging, and counting the supernatant (cytosolic fraction). Since the amount of free sialic acid in the cells is very small, the majority of the unincorporated counts are mostly ManNAc that has been internalized.

The remaining cells of the inoculum culture were collected with centrifugation (1000 × g, 4°C), suspended in fresh medium, and transferred to a second 2 l bioreactor. Immediately after transfer, the cells were tested for ^{3}H-ManNAc uptake as described above. In this way, the effects of the medium exchange process on the kinetics of ^{3}H-ManNAc uptake could be examined. Uptake rates were determined on samples removed from the culture every 24 h.

Sialylation of GP1-IgG in CHO cells grown in batch culture in a 10 l bioreactor. Culture samples (~10^6 cells) were taken from a 10 l bioreactor culture and transferred to 60 mm Petri dishes containing 35 mCi of ^{3}H-ManNAc. Labeling was carried out for 24 h (at the temperature of the bioreactor) and the sample concentrated using the protocol described earlier for determining the efficiency of labeling.

In these experiments, the GP1-IgG was isolated from the concentrated sample by immunoaffinity chromatography in an HPLC format, using an HPLC column containing Protein A (Poros A/M, PerSeptive Biosystems). After loading, the column was washed extensively with phosphate buffered saline and the GP1-IgG protein was eluted from the column with 1N acetic acid. The protein was quantified based on the adsorbance at 280 nmeters, using highly purified GP1-IgG as a calibration standard. Fractions containing the GP1-IgG protein were also collected and the amount of sialic acid was determined as radioactive counts.

The results of the earlier uptake experiments suggested that the cultures required at least a 24 h adaptation period to recover from centrifugation and the medium exchange process before uptake rates of the tracer stabilized. In the experiments with 10 l bioreactors a much larger volume of the inoculum culture was required. In this case, the media exchange process for the inoculum was facilitated by tangential flow filtration rather than by centrifugation which had been used earlier for small scale cultures. Although it was not tested, we anticipated that the tangential flow filtration process may be more severe and have longer lasting disruptive effects on the cell membrane permeability than mild centrifugation. For this reason, the metabolic labeling experiments at the 10 l bioreactor scale were initiated about 48 h after inoculation of the culture in order to insure that the cells had sufficient time to equilibrate and adapt to the bioreactor environment. The initial sample for metabolic labeling was taken immediately prior to the temperature shift and glucose addition. Thus, the value for the sialic acid incorporated represents only material biosynthesized over the 24 h period from day 2 to day 3. Correspondingly, the molar sialic acid content of GP1-IgG as determined by chemical analysis of the sample taken on day 3 of the culture represents an average content of sialic acid on the protein that has accumulated in the culture from day 0, after the culture was seeded.

Determination of sialic acid on GP1-IgG by chemical analysis. Since chemical detection of sialic acid on the recombinant protein was significantly less sensitive than metabolic labeling, much larger samples of the 10 l bioreactor were required to obtain sufficient amounts of recombinant product for analysis. The amount of culture fluid varied dramatically as the titer of recombinant product increased as the culture progressed. The amounts of culture fluid taken ranged from about 1 l for samples removed 48 h after innoculation to about 0.1 l for samples removed at day 7 of the culture. Samples for chemical analysis were withdrawn in parallel with the samples used for metabolic labeling. Sialic acid on the isolated GP1-IgG fraction was determined chemically using o-phenylenedine (OPD) derivatization to yield a stable fluorescent derivative that was quantitated by HPLC (11).

RESULTS

Efficiency of labeling and distribution of metabolically labeled sialic acid. Experiments were carried out to determine the level of ^{3}H-ManNAc converted into radioactively labeled sialic acid under the conditions used for labeling. These experiments provided insight into the sensitivity of the method as well as the overall distribution of metabolically labeled sialic acid in various cell fractions and the recombinant protein as shown in Figure 2. In a 24 h labeling period, only about 0.1% of the added ManNAc was incorporated into cellular components. Nearly 96% of the incorporated material was found in the secreted protein fraction and of this, only ~3% was specifically in GP1-IgG. Even though the amount of labeled sialic acid incorporated into GP1-IgG was a small percentage of the total counts incorporated, it was still 10-20 times background levels (~2-3,000 cpm per 10^6 cells). Thus, the method provides adequate sensitivity for monitoring sialylation over the course of the culture. A nearly identical distribution of sialic acid was found in wild-type CHO K1 cells except that no recombinant protein was produced in these cells and the fraction isolated by immunoprecipitation that would normally contain the GP1-IgG protein had near background levels of radioactivity.

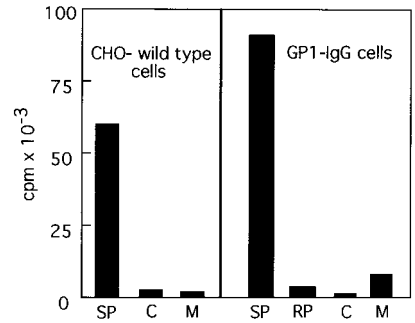

FIG. 2. Distribution of labeled sialic acid in CHO cell fractions and GP1-IgG (SP, secreted proteins; C, cytosol; M, membrane; RP, recombinant protein). Growing cells were incubated with ^{3}H-ManNAc and the cell fractions were obtained as described under Experimental Procedures. GP1-IgG was isolated by immunoprecipitation. Total counts in each fraction from ~10^6 cells are shown.

In order to verify that the radioactivity present in the GP1-IgG was associated with sialic acid exclusively, and not other ManNAc metabolites, the protein was digested with neuraminidase. Near quantitative release of the radioactivity from the protein was observed with enzyme treatment (data not shown). In the control sample, without neuraminidase digestion, a small amount of sialic acid (~5% of the total) was apparently released from the protein. This is most likely the result of acid catalyzed hydrolysis of the sensitive sialic acid residues by the strongly acidic conditions (40% TCA) used to precipitate the protein.

CHO cell uptake of ^{3}H-ManNAc. The uptake of ^{3}H-ManNAc by the GP1-IgG producing cells was determined in 24 h intervals over 7 days of culture, including samples taken from the inoculum culture before and after medium exchange, Figure 3. Cells taken immediately after seeding the bioreactor had a substantially higher uptake rate than that observed prior to

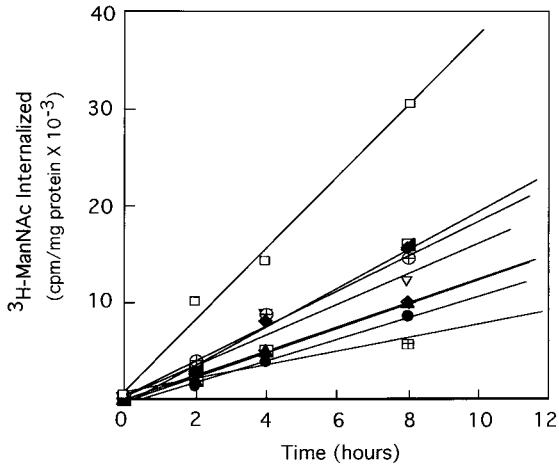

FIG. 3. Effect of culture age on the uptake rate of ^{3}H-ManNAc by GP1-IgG secreting CHO cells in a 2 l bioreactor culture over 7 days of the culture. Prior to inoculation of the bioreactor, samples of cells were taken from the inoculum culture and incubated with ^{3}H-ManNAc for the times shown (◆). The ^{3}H-ManNAc taken up by the cells was determined as radioactive counts as described under Experimental Procedures. The remaining cells of the inoculum were resuspended in fresh media and transfered to the bioreactor. An additional sample was then immediately removed and uptake rates of the tracer were determined (□, day 0). Subsequently, samples of the bioreactor culture were periodically withdrawn every 24 h and the rate of uptake of the tracer was determined. Uptake rates on each of the days are shown. (●, d 1), (▲, d 2), (⊞, d 3), (⊕, d 4), (⊕, d 5), (▽, d 6), (◪, d 7).

the medium exchange and with all other samples taken later in the culture. This enhanced rate of uptake may be due to the compromised integrity of the cell membrane that results from the stress associated with centrifugation and the media exchange process. About 24 h after transfer, the cells apparently recover, adapting to the bioreactor environment. The uptake rates were very similar in the samples studied thereafter. Although uptake of the tracer was not identical in each of the labeling experiments it was consistent and there was no apparent trend in the rate of accumulation as the time in culture progressed. After the temperature shift and feed at day three, the uptake rate slowed slightly. However, after about 8 h the internalization of labeled material returned to a level similar to that observed prior to these perturbations. Thus, the amount of tracer within the cells available for conversion into sialic acid was relatively constant throughout the labeling experiments. Therefore, any differences in the metabolic sialylation profile of the recombinant protein over the course of the culture cannot be attributed to changes in the levels of ^{3}H-ManNAc within the cells because the uptake rate is apparently unaffected by the age of the culture.

Sialylation profile of GP1-IgG produced in a large scale batch culture by metabolic labeling with ^{3}H-ManNAc and by chemical analysis. The profile of sialic acid derived from ^{3}H-ManNAc and incorporated into GP1-IgG over the course of the culture was compared with the profile of sialic acid determined by direct chemical analysis of the protein, Figure 4. Early in the culture, the metabolically incorporated sialic acid profile closely matched the sialylation profile determined by chemical assay. However, 48 h after the addition of butyrate, the amount of metabolically incorporated labeled sialic acid decreased to nearly undetectable levels. This profound diminution of labeling did not correlate with decreases in cell viability or protein productivity. Rather, cell viability remained relatively high and protein productivity increased about 4 fold in the first 24 h after butyrate addition. In marked contrast to the metabolically incorporated sialic acid, the molar sialic acid content of the protein reached a high of about 6.1 mol sialic acid/mol of protein and only gradually decreased to about 5.0 mol sialic acid/mol protein at day 10.

Experiments were carried out to evaluate the sialylation profile of the culture without the addition of butyrate. However, the level of protein productivity was extremely limited under these conditions, and it was not possible to obtain sufficient amounts of GP1-IgG for sialic acid analysis (data not shown).

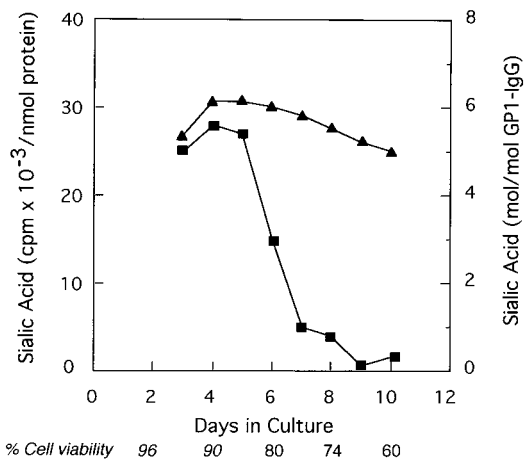

FIG. 4. Sialylation profile of GP1-IgG produced in a 10 l bioreactor using metabolic labeling with ^{3}H-ManNAc as a sialic acid precusor (■, sialic acid cpm/nmol GP1-IgG) and by chemical analysis of sialic acid (▲, sialic acid mol/mol GP1-IgG). Metabolic labeling of culture samples taken from the bioreactor was carried out as described under Experimental Procedures. The amount of sialic acid metabolically incorporated into the protein was determined as radioactive counts. Sialic acid on the protein was also determined from samples taken from the bioreactor every 24 h in parallel with the metabolic labeling experiments using the OPD method for quantification of the molar amounts of sialic acid on the protein as described elsewhere (11). Sampling of the culture was initiated on day 2, immediately prior to the temperature shift from 37 to 31°C and the addition of glucose. On day 3, sodium butyrate was added to a final concentration of 12 mM. The cell viability of samples from the bioreactor is also shown.

DISCUSSION

Metabolic labeling of glycoproteins and glycolipids using N-acetylmannosamine has been employed for a variety of analysis including, identifying molecular forms of sialic acid in various cell types (12), sequencing oligosaccharides on glycoproteins (13), and investigating sialylation in normal and transformed cells (14). The results obtained in the studies here have raised intriguing questions about the sialylation pathway in CHO cells grown under high productivity conditions. In some CHO cell systems, which express recombinant protein, butyrate has been shown to stimulate sialyltransferase activity (15). Enhancement of the transferase levels should lead to an increase in sialylation of the recombinant protein not the progressive decrease that is observed. Thus, we initially speculated that some step, other than the transferase, possibly one involved in the formation of sialic acid, could become rate limiting under these conditions. This led us to consider, in detail, the overall sialylation pathway in CHO cells. It is apparent that the precise mechanism for sialic acid biosynthesis in CHO cells is not known; however, it may be similar to the pathways of other tissues and cell types.

UDP-GlcNAc-2-epimerase has been shown to play a key role in the biosynthesis of sialic acid from ManNAc in liver (16). Interestingly, the enzyme activity has not been detected in other tissues (17). More recently, Northern blot analysis of mRNA levels for the enzyme has failed to detect mRNA in many tissues, supporting the observations of earlier enzymatic studies (18). Other potential routes to sialic acid from ManNAc have been identified. These include the direct epimerization of GlcNAc to ManNAc (19), followed by phosphorylation at C-6 by ManNAc kinase (17). Both enzyme activities have been verified in many tissues, including those lacking the UDP-GlcNAc-2-epimerase. A third avenue for sialic acid synthesis involves the neuraminylaldolase (20). Although the enzyme is generally considered to be a catabolic protein, degrading sialic acid into ManNAc and pyruvate, under some conditions the reaction can be driven to the formation of sialic acid from these metabolites.

All of these enzyme systems may be present in CHO cells and their levels of expression may be cell line or cell culture dependent. However, it is not readily obvious how inhibiting any specific step in these pathways could account for both the rapid and near complete decrease in metabolic incorporation of ManNAc while at the same time maintaining high levels of sialic acid on the protein, which then progressively decreases as the culture proceeds.

One possible explanation that could account for the observed results, and one that does not envoke perturbation of sialic acid biosynthesis directly, is that butyrate may stimulate cellular endocytosis and reutilization of sialic acid derived from pre-exisiting sialylated glycoproteins secreted into the culture fluid. As we have shown here, the amount of sialic acid on GP1-IgG is only a very minor component of the total sialic acid of the entire secreted protein fraction. Thus, the secreted proteins may serve as a source of sialic acid through a lysosomal-mediated recycling pathway. Material derived in this manner would dilute the pools of *de novo* synthesized sialic acid. The amount of radioactively labeled carbohydrate on the protein would decrease substantially because the tracer would be diluted by the pools of lysosomal-derived unlabeled sialic acid.

Furthermore, a recycling pathway may not have the capacity to supply the amounts of sialic acid required during robust protein biosynthesis and thus, the levels of sialic acid added to the protein would decrease as the culture progressed. It has been suggested that reutilization of secreted proteins may be a major source of sialic acid for biosynthesis in other tissues and cell types (14). Studies aimed at evaluating butyrate effects on sialic acid reutilization are a logical extension of the observations made in this study.

Finally, understanding the mechanism of sialylation of recombinant proteins in high productivity cultures is paramount in order to devise genetic or other means to develop expression hosts that give therapeutic molecules with optimal glycan stuctures.

ACKNOWLEDGMENTS

We thank Dr. Marty Vanderlaan for his help with the development of the HPLC chromatography system and Dr. Rob Arathoon for suggesting the use of Petri dishes as models for bioreactor cultures.

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