The effect of increasing nucleotide-sugar concentrations on the incorporation of sugars into glycoconjugates in rat hepatocytes

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Treatment of rat hepatocytes with 0.5 mM concentrations of uridine and cytidine results in increased cellular concentrations of UTP, UDP-sugars and CTP, whereas that of CMP-*N*-acetylneuraminate remained unchanged [Pels Rijcken, Overdijk, Van den Eijnden and Ferwerda (1993) Biochem. J. **293**, 207–213]. The incorporation of radioactivity from ^{3}H-labelled sugars into the cell-associated and secreted glycoconjugate fraction was influenced by these altered cellular concentrations of the nucleotides. For [^{3}H]glucosamine, pretreatment with uridine resulted in a reduction of the glycosylation in both fractions. Increases in the secreted fractions were observed for fucose with both uridine and cytidine and for *N*-acetylglucosamine with uridine only. With [^{3}H]*N*-acetylglucosamine, similar specific radioactivities for UDP-*N*-acetylhexosamine and CMP-*N*-acetylneuraminate were found, regardless of the pretreatment conditions. With [^{3}H]*N*-acetylmannosamine, the specific radioactivity of CMP-*N*-acetylneuraminate showed an almost 2-fold increase on pretreatment. The latter increase did not result in an increased incorporation of radioactivity into the glycoconjugates. It was estimated that, in untreated cells, the ratio of radioactivity incorporated from [^{3}H]glucosamine into glycoconjugate-bound *N*-acetylhexosamine and *N*-acetylneuraminate amounted to 2:3. In pretreated cells this ratio changed to approx. 2:1. Overall, the data show that pretreatment resulted in an increased incorporation of *N*-acetylhexosamine into cell-associated and secreted glycoconjugates, accompanied by a reduction in sialylation. It was concluded that an increased availability of UDP-*N*-acetylhexosamine caused the increased incorporation of *N*-acetylhexosamine. The elevated cytosolic level of UDP-*N*-acetylhexosamine (and of compounds like CMP) is suggested to impair the transport of CMP-acetylneuraminate to the Golgi, resulting in reduced sialylation. This study demonstrates that protein glycosylation can be regulated at the level of the availability of the various nucleotide-sugars in the Golgi lumen.

INTRODUCTION

The biosynthesis of glycoproteins and their subsequent transport to the various locations in eukaryotic cells is regulated at several levels.

Firstly, during their synthesis, the proteins become covalently modified by the addition of oligosaccharide chains, which are processed during the subsequent transport to their final destination. This process has appeared to be an essential prerequisite for obtaining biologically competent molecules at their proper location (Lodish, 1988). The sequence of events in the N- and O-glycosylation of proteins has been extensively studied (Kornfeld and Kornfeld, 1985; Schachter, 1986). However, major questions remain on how cells are able to regulate the non-template synthesis of specific carbohydrate chains. The participation of glycosyltransferases and glycosidases in these processes has been well established. Their expression levels and subcellular localization are important factors in determining the structure of the mature oligosaccharide chains (Paulson and Colley, 1989).

Another level at which the oligosaccharide formation is regulated is the transit time during transport of the various glycoconjugates from one location to the other between the rough endoplasmic reticulum and the Golgi apparatus and within the various parts of the Golgi system. Vesicles bud off from early parts of the Golgi and fuse with later parts of it (Bourne, 1988; Rothman and Orci, 1990). This vesicular transport is regulated by a variety of multimeric GTP-binding proteins that are present in these vesicles (Goud et al., 1988).

A third level of regulation of the glycosylation and that which has received the least attention in literature, is the intraluminal concentration of the nucleotide-sugar substrates. Translocation of nucleotide sugars from the cytoplasm to the lumen of the endoplasmic reticulum and Golgi apparatus has appeared to be a required step prior to the incorporation of a sugar into an oligosaccharide chain (Verbert et al., 1987; Hirschberg and Snider, 1987).

As part of our studies [see also Pels Rijcken et al. (1990, 1993)] on the latter mode of regulation, we determined the effect of increases of the nucleotide-sugar pools in rat hepatocytes, as brought about by pretreatment of the cells with uridine and cytidine (Pels Rijcken et al., 1993) on the incorporation of radioactive sugars into glycoconjugates. For that purpose we calculated the specific radioactivities of the nucleotide-sugars on incorporation of radioactivity from various labelled sugars and related these values to changes in incorporation of label into glycoconjugates. The results of the present study indicate that increases in the UDP-sugar concentrations cause a decrease in the sialylation with a concomitant increase in the incorporation of *N*-acetylhexosamines. It is concluded that, in rat hepatocytes, protein glycosylation may be subject to regulation at the level of the availability of nucleotide-sugar donors in the Golgi lumen.

MATERIALS AND METHODS

Chemicals

L-[U-^{14}C]Leucine (344.0 Ci/mol), D-[6-^{3}H]glucosamine hydrochloride (30.0 Ci/mmol), L-[6-^{3}H]fucose (86.3 Ci/mmol), D-[2-^{3}H]mannose (30.0 Ci/mmol) and *N*-acetyl-D-[6-^{3}H(n)]mannosamine (30.0 Ci/mmol) were products of NEN Research Products, Boston, MA, U.S.A. Newborn-calf serum (heat-inactivated) was

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purchased from Gibco–BRL. 1,1,2-Trichlorotrifluoroethane, tri-*N*-octylamine, ethanol and perchloric acid (±70%) were obtained from Merck, Darmstadt, Germany. Soluene-350 was purchased from Packard, Groningen, The Netherlands. The nucleotides used as chromatographic standards were obtained from Sigma, St. Louis, MO, U.S.A., or Boehringer Mannheim, Germany. All other chemicals were of the highest quality commercially available.

Hepatocyte preparation and culture

Hepatocytes were isolated from adult male Wistar rats (specific-pathogen-free; 250–300 g body wt.; home-bred) as described previously (Pels Rijcken et al., 1993). The cells (2×10^6) were suspended in 3 ml of modified Waymouth culture medium supplemented with 10% (v/v) newborn-calf serum, plated on rat-tail-collagen-precoated culture dishes (6 cm diameter) in duplicate, and allowed to settle at 37 °C in a humid CO_2/air (1:19) atmosphere. After 4 h the non-attached cells were removed and each dish was washed twice. The cells were then preincubated with 3 ml of fresh medium, with or without 0.5 mM uridine or cytidine. After 16 h the medium was replaced by 3 ml of medium with the same additions as used for preincubation, but now containing also 1 μCi of [^{14}C]leucine together with one of the following [^{3}H]sugars: glucosamine (2 μCi), *N*-acetylmannosamine (5 μCi), fucose (5 μCi) or mannose (3 μCi). The incubations were then allowed to proceed for 24 h.

Extraction of soluble nucleotides and preparation of glycoconjugate fractions

At the end of the incubation the medium was drawn off. The attached cells were washed twice with 1 ml of ice-cold PBS (10 mM sodium phosphate/0.154 M NaCl, pH 7.4). The medium and washes of each incubation were collected. The (glyco)proteins in this solution, hereafter designated as 'secreted glycoprotein fraction', were precipitated by adding 50 μl of BSA (1%, w/v) to 500 μl of the glycoprotein solution. The mixture obtained was acidified with 100 μl of 2.0 M $HClO_4$ and left on ice for 15 min. After centrifugation and washing twice with 250 μl of 0.4 M $HClO_4$, the resulting pellet was solubilized, neutralized, mixed with scintillation fluid and counted for radioactivity.

The cells were harvested with a rubber policeman. The soluble nucleotides in the cells were extracted from them with 75% ethanol as described by Pels Rijcken et al. (1993). Lipids were removed from the soluble fraction with chloroform/methanol/water (10:5:3, by vol.) before injection on to an h.p.l.c. column. The pellets obtained after the ethanol extraction, containing the cell-associated glycoconjugates, were washed twice with 250 μl of 0.4 M $HClO_4$ and were then solubilized in 250 μl of Soluene-350 for 3 h at 65 °C. The solutions were neutralized with 250 μl of 2.0 M HCl, and mixed with 12 ml of scintillation cocktail, followed by counting of the radioactivity.

Non-specific binding of radioactivity to medium and cell-associated components was determined as a control. Negligible binding of radioactivity was found when mixtures of labelled leucine and sugar were added to the incubated hepatocytes immediately before harvesting.

H.p.l.c.

Separation of the nucleotides and radioactive metabolites in the cell extracts was done by h.p.l.c. on a Whatman Partisphere SAX anion-exchange cartridge (4.6 mm × 125 mm; particle size 5 μm) as described by Pels Rijcken et al. (1993). Fractions (0.5 ml each) were collected and counted for radioactivity. The concentration of the nucleotides was determined by comparing the u.v. absorbance with those of references of known concentrations. Since glucose and galactose and also *N*-acetylglucosamine and *N*-acetylgalactosamine were not completely separated, they were taken together and are denoted as 'hexose' and '*N*-acetylhexosamine' respectively.

RESULTS AND DISCUSSION

Incubation of rat hepatocytes for various times with the pyrimidine-nucleotide precursors uridine and cytidine at a 0.5 mM concentration resulted in a time-dependent increase of the cellular concentrations of UTP and CTP (Table 1). For UTP and the other uridine derivatives studied, this increase was much more pronounced than for the cytidine derivatives, irrespective of the use of either uridine or cytidine. This is in agreement with previous observations (Pels Rijcken et al., 1993). It shows that the synthesis of CTP is strictly regulated, probably by feedback inhibition. The increases in the concentrations of uridine and uridine derivatives on incubation with cytidine (Table 1) must result from a high rate of deamination of cytidine (which yields uridine). The increases in the levels of the UDP-sugars were less than those found for UTP. Interestingly, for CMP-*N*-acetylneuraminate, no increased concentration was observed, even though an almost threefold increase of the pool-size of CTP occurred (Table 1).

The elevated levels of UTP and CTP were accompanied by a decrease in the level of ATP. However, the total amount of soluble adenine nucleotides (ATP, ADP and AMP) was not altered (results not shown). The amount of GTP was not altered and the pool sizes of the GDP-sugars remained below the level of detection (~ 0.03 nmol/10^6 cells) under all conditions tested. Since the concentration of NAD^+ remained fairly constant, it could be used as an internal standard in the determination by h.p.l.c. of the soluble nucleotides.

After a 16 h period of pretreatment, various ^{3}H-labelled sugars were added to the hepatocytes. The incorporation of radioactivity

Table 1 Pool sizes of soluble nucleotides and their increase in rat hepatocytes after incubation for various times with pyrimidine-nucleotide precursors in the medium

The experiments were performed as described in the Materials and methods section. Pool sizes in the control cells are given in nmol/10^6 cells. The data for the control cells are presented as the means ± S.D. for *n* incubations. The increase factors of the pool sizes after incubation with pyrimidine precursors are calculated relative to these control values. In the columns where $n = 2$, the variation was always less then 15%. Abbreviations used: HexNAc, *N*-acetylhexosamine (= *N*-acetylglucosamine + *N*-acetylgalactosamine); Hex, hexose (= glucose + galactose); GlcA, glucuronic acid; NeuAc, *N*-acetylneuraminate.

Nucleotide derivative	Pool size in control cells		Increase factor relative to control on preincubation with: 0.5 mM Uridine		0.5 mM Cytidine	
	16 h	40 h	16 h	40 h	16 h	40 h
UDP-HexNAc	2.96 ± 0.21	2.57 ± 0.53	1.9	4.6	1.6	3.2
UDP-Hex	3.95 ± 0.54	3.49 ± 0.63	1.7	3.8	1.7	2.6
UDP-GlcA	2.52 ± 0.23	2.21 ± 0.32	1.5	2.5	1.3	2.6
UTP	1.68 ± 0.24	1.65 ± 0.40	3.6	6.7	2.7	3.7
CMP-NeuAc	0.24 ± 0.02	0.22 ± 0.03	1.0	1.0	1.1	1.1
CTP	0.36 ± 0.04	0.32 ± 0.03	1.4	2.5	1.7	3.0
NAD^+	5.25 ± 0.07	4.85 ± 0.11	1.0	1.0	1.0	1.0
ATP	9.83 ± 0.44	9.65 ± 0.61	0.8	0.7	0.8	0.6
n...	4	8	2	2	2	2

Table 2 Distribution of radioactive sugars and leucine in the secreted and cell-associated glycoconjugates of rat hepatocytes after incubation with pyrimidine-nucleotide precursors

The experiments were performed as described in the Materials and methods section. Incorporation of [^{14}C]leucine was measured as a control for protein synthesis (means ± S.D., $n = 8$). The values for the [^{3}H]sugars are the means for two incubations. The values for the $^{3}H/^{14}C$ (= sugar/leucine) ratio are given in parentheses. [^{3}H]Glucosamine was used as precursor of the *N*-acetylhexosamines and *N*-acetylneuraminate, and [^{3}H]*N*-acetylmannosamine as a specific precursor of *N*-acetylneuraminate. The last three columns present the ratios of radioactivity in the secreted versus cell-associated glycoconjugates. Abbreviations: U, uridine; C, cytidine; GlcN, glucosamine; ManNAc, *N*-acetylmannosamine; Fuc, fucose; Man, mannose; Leu, leucine.

	Secreted glycoprotein fraction			Cell-associated glycoconjugates					
	10^{-4} × Radioactivity (d.p.m./10^6cells) ($^{3}H/^{14}C$ ratio) on incubation with:			10^{-4} × Radioactivity (d.p.m./10^6 cells) ($^{3}H/^{14}C$ ratio) on incubation with:			Ratio of radioactivity in secreted and cell-associated glycoconjugates		
Precursor	None	0.5 mM U	0.5 mM C	None	0.5 mM U	0.5 mM C	None	0.5 mM U	0.5 mM C
[^{3}H]GlcN	30.5 ± 0.3 (3.81)	24.5 ± 0.7 (3.45)	27.9 ± 0.4 (3.88)	11.2 ± 0.2 (0.80)	8.8 ± 0.1 (0.65)	10.4 ± 0.0 (0.73)	2.7	2.8	2.7
[^{3}H]ManNAc	4.6 ± 0.4 (0.58)	4.8 ± 0.2 (0.68)	4.4 ± 0.1 (0.61)	1.3 ± 0.0 (0.09)	1.3 ± 0.1 (0.10)	1.3 ± 0.1 (0.09)	3.4	3.6	3.5
[^{3}H]Fuc	27.9 ± 0.5 (3.49)	28.1 ± 0.4 (3.96)	27.4 ± 1.1 (3.81)	8.7 ± 0.4 (0.62)	8.6 ± 0.6 (0.64)	8.1 ± 0.5 (0.57)	3.2	3.3	3.4
[^{3}H]Man	4.3 ± 0.3 (0.54)	3.6 ± 0.0 (0.51)	4.2 ± 0.3 (0.58)	2.1 ± 0.1 (0.15)	1.7 ± 0.0 (0.13)	1.9 ± 0.0 (0.13)	2.1	2.2	2.2
[^{14}C]Leu	8.0 ± 0.2	7.1 ± 0.3*	7.2 ± 0.6*	14.0 ± 0.4	13.5 ± 0.6	14.3 ± 1.0	0.6	0.5	0.5

* The differences between these values and the control are significant ($P < 0.05$; Dunnett *t*-test).

Table 3 Radioactivity and specific radioactivity (s.r.a.) in nucleotide-sugars in rat hepatocytes resulting from incubation with radioactive sugars after incubation with pyrimidine-nucleotide precursors

The experiments were performed as described in the Materials and methods section. The values are the means for two incubations. Values marked '–' could not be calculated (see the text). Abbreviations used: GlcN, glucosamine; ManAc, *N*-acetylmannosamine; Fuc, fucose; Man, mannose; HexNAc, *N*-acetylhexosamine (= *N*-acetylglucosamine + *N*-acetylgalactosamine); NeuAc, *N*-acetylneuraminate.

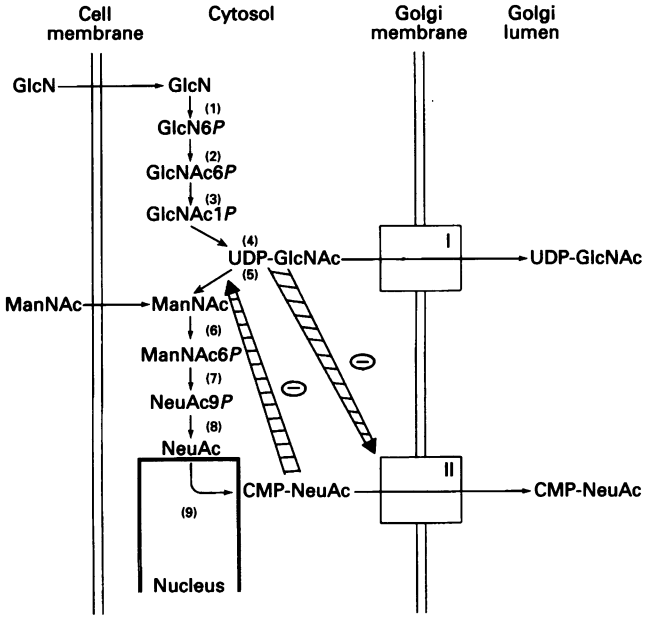
		Total radioactivity (T.R.; d.p.m./10^6 cells) and s.r.a. (d.p.m./nmol) on preincubation with:					
		None		0.5 mM Uridine		0.5 mM Cytidine	
Percursor used	Nucleotide sugar formed	T.R.	s.r.a.	T.R.	s.r.a.	T.R.	s.r.a.
[^{3}H]GlcN	UDP-HexNAc	$(35.5 \pm 1.3) \times 10^3$	13.8×10^3	$(114.0 \pm 5.9) \times 10^3$	9.6×10^3	$(87.1 \pm 5.1) \times 10^3$	10.6×10^3
	CMP-NeuAc	$(2.9 \pm 0.1) \times 10^3$	13.2×10^3	$(2.3 \pm 0.1) \times 10^3$	10.4×10^3	$(2.9 \pm 0.1) \times 10^3$	12.2×10^3
[^{3}H]ManNAc	CMP-NeuAc	692 ± 12	3145	1180 ± 82	5363	1229 ± 48	4861
	UDP-HexNAc	89 ± 10	35	267 ± 13	23	221 ± 17	27
[^{3}H]Fuc	GDP-Fuc	988 ± 62	–	1383 ± 104	–	1284 ± 58	–
[^{3}H]Man	GDP-Man	75 ± 1	–	90 ± 3	–	98 ± 2	–

into the glycoconjugates after 24 h was then measured (Table 2). A distinction was made between the secreted glycoproteins and the cell-associated glycoconjugates. [^{3}H]Glucosamine was used as precursor of the *N*-acetylhexosamines and *N*-acetylneuraminate, and [^{3}H]*N*-acetylmannosamine as a specific precursor of *N*-acetylneuraminate. In addition, the incorporation of [^{3}H]fucose and [^{3}H]mannose into glycoproteins was determined. [^{14}C]Leucine could be used as a measure for protein synthesis, since no ^{14}C label appeared to be present in the h.p.l.c. fractions that contained the nucleotide-sugars (results not shown).

As a result of the pretreatment with uridine or cytidine a slight decrease in the amount of ^{14}C label from leucine was observed for the secreted (glyco)proteins. These decreases, of approx. 10%, appeared to be statistically significant ($P < 0.05$; Dunnett *t* test). Since the amount of label incorporated in the cell-associated fraction showed only insignificant changes, we conclude that the pretreatment with uridine or cytidine results in a small reduction in the synthesis of secretory (glyco)proteins and that the synthesis of cell-associated (glyco)conjugates is unaffected (Table 2).

The incorporation of the ^{3}H label from the four sugars used showed changes upon preincubation with uridine and cytidine in some instances, indicating altered glycosylation. However, for the secreted fraction a significant effect of the pretreatment on the ^{14}C incorporation was observed. We therefore calculated the $^{3}H/^{14}C$ ratio in each instance in order to correct for the altered protein synthesis. Taking into account the fact that the variation between duplicates was very low in most instances, in a few cases a change in the degree of glycosylation became apparent. For [^{3}H]glucosamine, pretreatment with uridine resulted in an appreciable reduction of the glycosylation. Increased ratios in the secreted fractions (9–17%) were observed for fucose after pretreatment with both uridine and cytidine and for *N*-acetylneuraminate (from *N*-acetylmannosamine) with uridine only.

Manipulation of the pyrimidine-nucleotide pool sizes may induce changes in the specific-radioactivity values of nucleotide-



Scheme 1 Biosynthesis and transport of UDP-*N*-acetylglucosamine and CMP-*N*-acetylneuraminate in mammalian cells

The names of the enzymes of the various steps in the biosynthetic pathway of UDP-*N*-acetylglucosamine and CMP-*N*-acetylneuraminate are the following: (1) glucosamine kinase (EC 2.1.7.2); (2) glucosamine-6-phosphate *N*-acetyltransferase (EC 2.3.1.4); (3) *N*-acetylglucosamine 6-phosphate mutase (EC 2.7.5.2); (4) UDP-*N*-acetylglucosamine pyrophosphorylase (EC 2.7.7.23); (5) UDP-*N*-acetylglucosamine 2-epimerase (EC 5.1.3.14); (6) *N*-acetylmannosamine kinase (EC 2.7.1.60); (7) *N*-acetylneuraminate 9-phosphate synthase (EC 4.1.3.20); (8) *N*-acetylneuraminate 9-phosphatase (EC 3.1.3.29); (9) nuclear-localized CMP-*N*-acetylneuraminate synthase (EC 2.7.7.43). The nucleotide-sugar transport systems I and II have been described by Cacan et al. (1984) and Carey et al. (1980). System I denotes the transport system for UDP-*N*-acetylglucosamine and system II that for CMP-*N*-acetylneuraminate. The enzyme UDP-*N*-acetylglucosamine 2-epimerase (5) is inhibited by CMP-*N*-acetylneuraminate (Kornfeld et al., 1964; Sommar and Ellis, 1972; Kikuchi and Tsuiki, 1973). UDP-*N*-acetylglucosamine inhibits the CMP-*N*-acetylneuraminate transporter protein (Carey et al., 1980). Abbreviations: GlcN, glucosamine; 1*P* (etc.), 1-phosphate (etc.); GlcNAc, *N*-acetylglucosamine; ManNAc, *N*-acetylmannosamine; NeuAc, *N*-acetylneuraminate.

sugars that are synthesized on incubation with the radioactive sugars. Therefore the data given in Table 2 can only be discussed properly in relation with knowledge of the changes in the cellular concentrations of the nucleotide-sugars (Table 1) and in their specific radioactivities (Table 3).

Incubation for 24 h with [^{3}H]glucosamine resulted in similar specific-radioactivity values for UDP-*N*-acetylhexosamine and CMP-*N*-acetylneuraminate in control cells. Decreases in the specific-radioactivity values for UDP-*N*-acetylhexosamine and CMP-*N*-acetylneuraminate were found with uridine or cytidine, which were to be expected from the pool-size increase of UDP-*N*-acetylhexosamine on pretreatment of cells. The fact that the specific-radioactivity values for CMP-*N*-acetylneuraminate and UDP-*N*-acetylhexosamine were similar, and remained similar upon incubation, is not surprising, since UDP-*N*-acetylglucosamine is a precursor of CMP-*N*-acetylneuraminate, and the CMP-*N*-acetylneuraminate pool has a rapid turnover. The turnover can be estimated taking into account that the radioactivity, introduced by [^{3}H]*N*-acetylmannosamine, almost exclusively ends up in glycoconjugate-bound *N*-acetylneuraminate. The latter can be concluded from the fact that only a small amount of radioactivity ends up in UDP-*N*-acetylhexosamine, resulting in an extremely low specific radioactivity of UDP-*N*-acetylhexosamine compared with that of CMP-*N*-acetylneuraminate (Table 3). Therefore the amount of ^{14}C label in the glycoconjugates derived from radioactive *N*-acetylhexosamine can be neglected. The turnover in control cells for CMP-*N*-acetylneuraminate was 17 min, as was calculated by dividing the steady-state amount of radioactivity in CMP-*N*-acetylneuraminate (692 d.p.m.; see Table 3) by the sum of the radioactivity incorporated into the cellular and cell-associated glycoconjugate fractions (5.9×10^4 d.p.m./24 h; see Table 2). For cells preincubated with uridine or cytidine, these values were found to be 28 min and 31 min respectively.

From Tables 2 and 3 it can be calculated that the incorporation of radioactivity from [^{3}H]*N*-acetylmannosamine into nucleotide-sugars and glycoproteins after 24 h was approx. 6.8 times lower than that from [^{3}H]glucosamine. This value is the same regardless of whether the cells were pretreated with and without uridine or cytidine. The specific radioactivity of CMP-*N*-acetylneuraminate after incubation of the control cells with [^{3}H]*N*-acetylmannosamine was four times lower than with [^{3}H]glucosamine, in spite of the fact that 2.5 times more radioactivity was added to the medium. Thus the introduction of *N*-acetylmannosamine into the metabolic channel that goes from glucosamine via UDP-*N*-acetylglucosamine and *N*-acetylmannosamine to CMP-*N*-acetylneuraminate seems to be somehow hampered. Even though more steps are required starting from glucosamine, the introduction of this precursor into CMP-*N*-acetylneuraminate is more efficient (Scheme 1). This effect is due to the fact that transport across the hepatocyte membrane of *N*-acetylmannosamine is less efficient compared with that of glucosamine, which is probably caused by the acetyl group of *N*-acetylmannosamine. This difference in transport efficiency was established by comparing the total uptake of these radioactive sugars by the cells (results not shown). Pretreatment resulted in a 1.5–1.7-fold increase in the specific radioactivity in CMP-*N*-acetylneuraminate on incubation with [^{3}H]*N*-acetylmannosamine. With [^{3}H]glucosamine, however, a slight decrease in the specific radioactivity of CMP-*N*-acetylneuraminate was found. From this result it can be reasoned that there must exist more than one pool of CMP-*N*-acetylneuraminate in the cell: one presumably located within the *trans*-Golgi compartment, where sialylation occurs, the other residing within the cytosol and/or the nucleus (Coates et al., 1980; Ferwerda et al., 1986; Kean, 1970; Van den Eijnden, 1973). The activity of cytosolic UDP-*N*-acetylglucosamine 2-epimerase, which converts UDP-*N*-acetylglucosamine into *N*-acetylmannosamine, is sensitive to feedback inhibition by CMP-*N*-acetylneuraminate (Kornfeld et al., 1964; Sommar and Ellis, 1972; Kikuchi and Tsuiki, 1973). Hence, an increase in the cytosolic pool of CMP-*N*-acetylneuraminate would strongly inhibit the rate of synthesis of *N*-acetylmannosamine from UDP-*N*-acetylglucosamine. The latter inhibition would favour the introduction of exogenous [^{3}H]*N*-acetylmannosamine into this pathway (Scheme 1), which should be reflected by increased specific-radioactivity values for CMP-*N*-acetylneuraminate in pretreated cells. This indeed was found in our experiments (Table 3), thus suggesting an increased cytosolic pool of CMP-*N*-acetylneuraminate. This increased specific radioactivity is not reflected by an increase in the incorporation of radioactivity into the glycoconjugates (Table 2), indicating that under the conditions of pretreatment the sialylation of glycoconjugates is impaired. Diminished availability of CMP-*N*-acetylneuraminate at the site of its utilization in the Golgi is one of the possible causes. Cacan et al. (1984) and Carey et al. (1980) have shown that the transport of CMP-*N*-acetylneuraminate across the Golgi membrane can be inhibited by substrate analogues such as CMP and UDP-sugars. The latter

Table 4 Calculated incorporation of *N*-acetylhexosamine (HexNAc) and *N*-acetylneuraminate (NeuAc) into secreted and cell-associated glycoconjugates of rat hepatocytes

The incorporation of *N*-acetylhexosamine and *N*-acetylneuraminate {from [^{3}H]glucosamine ([^{3}H]GlcN)} and *N*-acetylneuraminate {from [^{3}H]*N*-acetylmannosamine ([^{3}H]ManNAc)} into glycoconjugates (first and second row respectively) were calculated by dividing the radioactivity present in the respective glycoconjugate fraction by the specific radioactivity of the nucleotide-sugars (the ultimate precursors). By subtracting the value of row 1 from that of row 2, the amount of incorporated *N*-acetylhexosamine alone (third row) was calculated.

	Incorporation (nmol/24 h per 10^6 cells)					
	Secreted glycoproteins			Cell-associated glycoconjugates		
Incorporated sugar(s)	Control	Uridine	Cytidine	Control	Uridine	Cytidine
HexNAc + NeuAc (from [^{3}H]GlcN)	22.8	24.7	25.0	8.3	8.9	9.4
NeuAc (from [^{3}H]ManNAc)	14.6	8.9	9.0	4.3	2.5	2.6
HexNAc* (row 1 minus row 2)	8.2	15.9	15.9	4.0	6.5	6.9

* The values given were calculated before rounding off of the data in rows 1 and 2.

compounds indeed have increased concentrations in hepatocytes after pretreatment. For CMP an increase by a factor of 3 was found (Pels Rijcken et al., 1993) (for the UDP-sugars, see Table 1). Inhibition of CMP-*N*-acetylneuraminate translocase would impair the transport of CMP-*N*-acetylneuraminate, resulting in a decreased pool of CMP-*N*-acetylneuraminate in the Golgi and subsequently in a reduced sialylation of the glycoconjugates. Since the total cellular amount of CMP-*N*-acetylneuraminate was unchanged after pretreatment (Table 1), an increase of the cytosolic pool seems to be balanced by a decrease in the Golgi pool.

From the data in Tables 2 and 3 for *N*-acetylmannosamine the amount of *N*-acetylneuraminate that had been incorporated in the respective glycoconjugate fractions was calculated by dividing the radioactivity incorporated as *N*-acetylneuraminate by the specific radioactivity of CMP-*N*-acetylneuraminate (Table 4). In the secreted and cell-associated fraction of control cells these amounts were 14.6 and 4.3 nmol/10^6 cells respectively. For pretreated cells these values were 8.9 and 2.5 (uridine) and 9.0 and 2.6 (cytidine) nmol/10^6 cells. It can be concluded that the observed reduction in sialylation is similar in both glycoconjugate fractions. A reduction in sialylation was also reported in Ehrlich-ascites-tumour cells after pretreatment with high concentrations of uridine (Wenzel and Schneider, 1989).

As mentioned above, the specific-radioactivity value for UDP-*N*-acetylhexosamine and CMP-*N*-acetylneuraminate were similar after labelling with [^{3}H]glucosamine. This allows one to quantify the effect of the pretreatment on the total amount of *N*-acetylhexosamine and *N*-acetylneuraminate, incorporated into glycoproteins (Table 4). Subtraction of the values for *N*-acetylneuraminate mentioned above from those for these total amounts gives the amounts of *N*-acetylhexosamine incorporated into the different glycoconjugate fractions (Table 4). From these calculated data it appears that, in control cells, 40% of the radioactivity has been incorporated in the form of *N*-acetylhexosamine and 60% as *N*-acetylneuraminate. For uridine- and cytidine-pretreated cells the percentage for *N*-acetylhexosamine was much higher, being approx. 65% in both instances and that for *N*-acetylneuraminate was decreased to approx. 35%. This result shows that increased incorporation of *N*-acetylhexosamine is accompanied by reduced sialylation. Although we could not discriminate between *N*-acetylglucosamine and *N*-acetylgalactosamine, this observation may have to be interpreted as an enhanced capacity for formation of branched N-linked glycans and of polylactosaminoglycan chains. Interestingly such an enhancement has been associated with malignant transformation of cells (Hubbard, 1987; De Korte et al., 1987; Van den Eijnden et al., 1988; Easton et al., 1991, 1992). The latter process in malignant cells, however, is generally accompanied by an increased sialylation, whereas here sialylation is depressed. The above-mentioned reduced availability of CMP-*N*-acetylneuraminate in the Golgi will form the basis for this phenomenon. Its effect might, however, be reinforced by the fact that the concomitant higher availability of UDP-*N*-acetylglucosamine may cause a higher activity of branching *N*-acetylglucosaminyltransferases IV and V (Schachter, 1986) and of β-3-*N*-acetylglucosaminyltransferase responsible for polylactosaminoglycan chain formation (Van den Eijnden et al., 1988). It has been reported that the action of *N*-acetylglucosaminyltransferase V yields branched structures that are favoured substrates for the β-3-*N*-acetylglucosaminyltransferase (Van den Eijnden et al., 1988), but are poor acceptors for α-6-sialyltransferase (Joziasse et al., 1987). Since this sialyltransferase is the predominant activity in rat liver (Van den Eijnden and Schiphorst, 1987), increased branching and polylactosaminoglycan formation will further diminish the capacity to terminate the chains by α-6-sialylation. A similar alteration in glycosylation is also seen in cases where the presence of antimetabolites induces changes in nucleotide-sugar concentrations (Peters et al., 1990).

With both [^{3}H]fucose as the precursor for GDP-fucose and [^{3}H]mannose as the precursor for GDP-mannose increases of radioactivity were obtained after pretreatment. As calculation of the specific-radioactivity values was not possible because the total amount of GDP-sugars remained below the detection limit, no estimation of the amounts of fucose and mannose incorporated was possible.

For GDP-[^{3}H]fucose, increases in radioactivity as a result of uridine and cytidine treatment were observed from 988 to 1383 and 1284 d.p.m./10^6 cells respectively (Table 3). We could not determine whether these increases are parallelled by an increase in the specific-radioactivity values of GDP-fucose. Since the concentration of GTP was unchanged and [^{3}H]fucose was only added in tracer amounts, an increase in the specific-radioactivity values of GDP-fucose was anticipated. If the latter were true, the observed increases in ^{3}H/^{14}C ratios in the secreted glycoprotein fraction (Table 2) would not necessarily indicate an altered fucosylation. However, the result for the cell-associated fraction, showing unchanged ^{3}H/^{14}C ratios, indicates that glycoconjugate fucosylation can somehow be affected.

The low values for GDP-[^{3}H]mannose in both control cells

and in cells that were pretreated with 0.5 mM uridine or cytidine appeared not to be due to losses or exchange of label during cell extraction (results not shown). An explanation for these low values would be metabolic conversions of mannose, leading to products other than GDP-mannose, such as GDP-fucose, as has been shown in both mammalian cells (Martin et al., 1989) and non-mammalian cells (Bulet et al., 1984). Such a possibility had been proposed earlier by Ginsburg (1961). It also might enter the glycolysis after being phosphorylated and converted into fructose 6-phosphate (Stoddart, 1984). The latter route leads to a loss of label.

In Table 2 (last three columns) we also present the ratios of incorporation of [^{3}H]sugars into the secreted versus cell-associated glycoconjugates. Pretreatment with either uridine or cytidine did not lead to changes of these ratios. For [^{3}H]*N*-acetylmannosamine the ratios of approx. 3.5 are higher than those for mannose (2.1–2.2). This is in agreement with the fact that secretion of glycoproteins is preceded by the processing of the carbohydrate chains, which results in *N*-acetyl-lactosaminic-type chains with terminal *N*-acetylneuraminate residues (Kornfeld and Kornfeld, 1985; Overdijk et al., 1986; Beem et al., 1987). The ratios for [^{3}H]fucose (3.2–3.4) are in agreement with the above.

In conclusion we showed that, in hepatocytes, increases in the UDP-sugar concentrations cause a decrease in the sialylation with a concomitant increase in the incorporation of *N*-acetylhexosamine. We suggest that this decreased sialylation is caused by impaired transport of CMP-*N*-acetylneuraminate across the Golgi membrane, due to the increased levels of UDP-*N*-acetylhexosamine. Furthermore we found evidence for feedback inhibition of UDP-*N*-acetylglucosamine 2-epimerase by CMP-*N*-acetylneuraminate in intact cells, that was earlier found in cell homogenates.

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