Glycoconj J (2007) 24:531–541
DOI 10.1007/s10719-007-9048-x

Liver-specific increase of UTP and UDP-sugar concentrations in rats induced by dietary vitamin B6-deficiency and its relation to complex N-glycan structures of liver membrane-proteins

Agnes B. Renner · Kathrin Rieger · Detlef Grunow · Martin Zimmermann-Kordmann · Martin Gohlke · Werner Reutter

Received: 2 January 2006 / Revised: 14 May 2007 / Accepted: 24 May 2007 / Published online: 19 June 2007

Abstract This is the first known report on the influence of vitamin B6-deficiency on the concentration of UDP-sugars and other uracil nucleotides in rats. Animals aged 3 weeks or 2 months were fed a vitamin B6-free diet for periods varying from 3 days to 7 weeks. Nucleotides were quantified by enzymatic-photometry and by SAX-high precision liquid chromatography. In 3 week-old rats, vitamin B6-deficiency resulted in an up to 6.3-fold increase in the concentrations of UTP, UDP, UMP and UDP-sugars and less of CTP in rat liver, while no changes were observed in older rats. In young rats, the concentration of uracil nucleotides started to increase after 1 week diet, with a maximum after 2 weeks. After 5 weeks, the concentrations returned to normal values. In heart, lungs, kidney and brain, concentrations were measured after 2 weeks diet in young rats. In contrast to liver, the heart muscle uracil nucleotide concentrations were decreased by 40%. In kidney, the sum of UTP, UDP and UMP showed a decrease of 40%, whereas UDP-sugars were increased 1.4-fold. In the lungs, nucleotide concentrations were mostly unaffected by vitamin B6-deficiency, despite a 70% increase of UDP-GA. In brain, UDP-Glc, UDP-Gal and the sum of CTP and CDP showed an increase of 30–50%. We became surprised that the increased UDP-sugar concentrations did not influence the structure of liver plasma membrane-N-glycans. Despite the 4 to 6-fold increase of UTP and UDP-sugars, no changes in the complexity or sialylation of these N-glycans could be detected. This study demonstrates that, especially in liver, pyridoxal phosphate is closely involved in the control of uracil nucleotides during a defined period of development. In contrast to *in vitro* experiments, *in vivo* N-glycan biosynthesis in liver is regulated by a more complex or higher mechanism than substrate concentrations.

Keywords Vitamin B6-deficiency · UTP · UDP-sugars · N-glycosylation of plasma membrane glycans · Rat liver

Agnes B. Renner and Kathrin Rieger contributed equally.

A. B. Renner · K. Rieger · D. Grunow · M. Zimmermann-Kordmann · M. Gohlke · W. Reutter
Institut für Biochemie und Molekularbiologie,
Charité - Universitätsmedizin Berlin,
Campus Benjamin Franklin, Arnimallee 22,
D-14195 Berlin, Germany

A. B. Renner (✉)
Augenklinik, Charité - Universitätsmedizin Berlin,
Campus Benjamin Franklin, Hindenburgdamm 30,
D-12200 Berlin, Germany
e-mail: a.renner@berlin.de

K. Rieger
Medizinische Klinik III, Hämatologie,
Onkologie und Transfusionsmedizin,
Charité - Universitätsmedizin Berlin,
Campus Benjamin Franklin, Hindenburgdamm 30,
D-12200 Berlin, Germany

Introduction

Pyridoxal-5′-phosphate (PALP), the physiologically active form of vitamin B6, functions as a coenzyme for enzymes in a variety of metabolic reactions, particularly in amino acid metabolism, including the biosynthesis of neurotransmitters, such as dopamine, serotonin and GABA. It functions in δ-aminolevulinic acid synthesis and conse-

quently heme biosynthesis. It is a coenzyme of serine palmityltransferase (sphingosine biosynthesis), and of serine hydroxymethyltransferase, as well as functioning in the formation of glucose-1-phosphate by serving as a coenzyme of glycogen-phosphorylase (for detailed references see Friedrich [6] and Snell [29]). In addition to these well-documented functions, a number of studies have demonstrated a new role for PALP as a modulator of gene expression. The first indication of this came with the observation that PALP interacts with steroid hormone receptors. It was subsequently shown that PALP modulates the expression of glucocorticoid receptors as well as other members of the steroid hormone receptor superfamiliy [1–3]. In particular, steroid hormone-dependent gene expression is enhanced during vitamin B6-deficiency and reduced under conditions of vitamin B6 elevation [17]. Oka *et al.* [17] demonstrated that PALP influences not only steroid hormone receptor-dependent gene expression but also hormone-independent gene expression by modulating the activity of RNA polymerases I and II and the level of mRNA, both of which are increased in nuclei isolated from the liver of vitamin B6-deficient rats. Tissue-specific effects of vitamin B6-deficiency were first described by Oka *et al.* [18]. The level of glycogen-phosphorylase mRNA in the muscle of vitamin B6-deficient rats was reduced to 40% compared with control rats, while a 5-fold increase was detected in the livers of deficient animals. In contrast, the expression of the ß-actin gene was unaffected by vitamin B6-deficiency in the muscle, but was enhanced in the liver of the deficient animals [18]. However, Trakatellis *et al.* [31] treated rat lymphocytes with the pyridoxine antimetabolite, deoxypyridoxine, and found a decrease of mRNA and DNA-synthesis leading to a suppression of cell proliferation.

During studies on hexosamine metabolism in rats, hepatitis-like liver injury was observed after intraperitoneal administration of D-galactosamine (GalN) [11, 25]. GalN metabolism in hepatic tissue led to a rapid and dramatic decrease of the concentrations of UTP, UDP, UMP, UDP-glucose, UDP-galactose and UDP-glucuronic acid [5, 13, 24]. All the signs of hepatitis and the appearance of Councilman bodies could be observed by light microscopy. The strong decrease of uracil nucleotides and UDP-sugars resulted in a decrease of uracil nucleotide-dependent biosynthesis of macromolecules (nucleic acids, glycoproteins, glycolipids, and especially glycogen), leading to the induction of liver cell necrosis [5, 26]. During these experiments, it was observed accidentally that vitamin B6-deficient rats receiving GalN did not suffer from hepatitis. In these rats, the concentrations of the uracil nucleotides were also decreased after GalN injection but much less decreased as in normal rats (Reutter, unpublished data). Since the decrease of uracil nucleotides plays a crucial role in the development of galactosamine hepatitis, we decided to examine the influence of vitamin B6-deficiency on the concentration of nucleotides and UDP-sugars in rat liver and other organs and on the biosynthesis of N-glycans of rat liver plasmamembranes.

Materials and methods

Chemicals

The following enzymes were bought from Boehringer Mannheim (Mannheim/Germany): NMPK (EC 2.7.4.4) from bovine liver, 1.0 U/mg; UDPG-DH (EC 1.1.1.22) from bovine liver, 0.6 U/mg; UDPGP (EC 2.7.7.9) from bovine liver, 100 U/mg, Trypsin, peptide-*N*4-(*N*-Acetyl-ß-glucosaminyl)asparagine amidase F (PNGase F) and neuraminidase. The following enzymes were obtained from Sigma (Deisenhofen/Germany): NDPK (EC 2.7.4.6) from yeast, 1,000–2,000 U/mg; UDP-Gal-4-epimerase (EC 5.1.3.2) from galactose-adapted yeast, 10–20 U/mg. Guanosine-5′-diphospho-L-[1-^{14}C]-fucose was purchased from Amersham Pharmacia Biotech (Freiburg i.Br./Germany). Ultima Gold XR from Canberra-Packard (Frankfurt a.M./Germany) was used as the scintillation liquid. The nucleotides and nucleotide sugars used as chromatographic standards were from Sigma (Deisenhofen/Germany). SignalTM-2-AB-Labelling Kit was obtained from Oxford Glyco Sciences (Oxford, U.K.). All other chemicals were obtained from Boehringer Mannheim (Mannheim/Germany), Merck (Darmstadt/Germany), Roth (Karlsruhe/Germany) and Sigma (Deisenhofen/Germany) and were of analytical grade. Only filtered water in Millipore quality was used for high precision liquid chromatography (h.p.l.c.).

Animals

Male and female Wistar rats were used. They were kept on a natural light/dark schedule with constant room temperature and atmospheric humidity. Test rats were fed a nearly vitamin B6-free diet (≤0.6 mg pyridoxine/kg) from Hoffmann-La Roche (Dr. H. Bachmann, Basel/Switzerland), while control rats were fed an unrestricted commercial diet, Haltungsdiaet-altromin 1321, from Altromin GmbH (Lage/Germany). The young rats were nursed for 21–24 days, then separated from the mother and fed the vitamin B6-free diet ad libitum for 3, 5, 7, 14 days, 3, 5, or 7 weeks. Control rats were fed the unrestricted diet. The rats intended for study at an older age were also nursed for 21–24 days, then separated from the mother animal and fed with Haltungsdiaet-altromin ad libitum until the age of 2 months. The vitamin B6-free diet was then administered for 3 weeks. An additional group was nursed for 21 days, underwent adrenalectomy on day 20–21, and then was fed with vitamin B6-free diet ad libitum or unrestricted diet for the following 2 weeks. Adrenalecto-

mized rats received physiological sodium solution instead of pure water ad libitum. Another group was separated after 21 days from the mother and then fed the vitamin B6-free diet ad libitum or unrestricted diet for the following 13 days. These animals were fasted for 2 days before removal of their livers. For the analysis of plasma membrane N-glycans, rats were nursed for 21–24 days, then separated from the mother and fed the vitamin B6-free diet or Haltungsdiaet-altromin for controls for 3 weeks. The daily food intake of each rat was measured and the control rats were given one and a half this amount of Haltungsdiaet-altromin. Each animal was weighed every day. All animals had free access to water or salt solution, as appropriate.

Analysis of nucleotides and UDP-sugars in rat tissue

Homogenization

For the determination of metabolites in rat tissue the rats were anesthetized with an intraperitoneal injection of Nembutal (30 mg Nembutal / 1 ml PBS) and liver, heart, lungs, kidney and brain were obtained by freeze-stop technique and stored in liquid N_2 until use. The homogenates were prepared as described by Keppler *et al*. [10]. The samples were stored at −20°C until use for enzymatic-photometric measurements.

Delipidation

Delipidation of tissue homogenate was necessary before the application of h.p.l.c.. The delipidation was performed on ice, using ice-cold solutions. Five hundred microliter tissue homogenate, 20 µl ^{14}C-GDP-L-fucose (to quantify the loss of metabolites during delipidation) and 680 µl double distilled water were mixed. First, 2 ml methanol were added and mixed, followed by 4 ml chloroform (H_2O:methanol: chloroform=3:5:10 [v/v] as described by Pels Rijcken *et al*. [20]). After centrifugation, 1.5 ml from the upper phase were placed in a Centricon® concentrator 10 from Millipore (Eschborn/Germany) and centrifuged for 2 h at 5,000 U/min. Double distilled water (500 µl) was added, followed by centrifugation for 1 h at 5,000 U/min. The extract was then evaporated to 100 µl and redissolved in double distilled water to a final volume of 300 µl. The samples were stored at −20°C until use for h.p.l.c. measurements.

Enzymatic-photometric measurements

The concentrations of UTP, UDP, UMP, UDP-Glc and UDP-Gal were measured in rat tissue homogenates according to the method of Keppler *et al*. [10, 12], after removal of protein with perchloric acid. The irreversible oxidation of UDP-Glc to UDP-GA, in which 2 moles of NAD^+ are reduced to NADH per mole of UDP-Glc by UDPG-DH, was used as the indicator reaction. The NADH formation was measured sequentially at 340 nm in a UV/Visible Spectrophotometer Ultrospec 3000 from Pharmacia Biotech (Cambridge/England). Concentrations were calculated according to the Lambert-Beer law.

High precision liquid chromatography

Cytosine and guanosine nucleotides and other UDP-monosaccharides and hexosamines were determined by h.p.l.c., using two h.p.l.c. pumps from Bischoff (Leonberg/Germany) operated with Instrument Interface from Bio-Rad (München/Germany). The absorbance was measured with a spectrophotometer UVIKON 720 LC from Kontron (München/Germany) operating at 254 nm combined with Software ValueChrom 4.0 from Bio-Rad (München/Germany). The nucleotides and nucleotide sugars in the delipidated tissue homogenate were separated on a PartiSphere 5 SAX anion-exchange cartridge (125×4.6 mm; particle size 5 µm) from Whatman (New Jersey/USA) as described by Pels Rijcken *et al*. [20, 22], with modification of gradient and flow rate. Additionally, the cartridge was kept at a constant temperature of 35°C. Separation was achieved with a phosphate buffer concentration gradient generated by mixing eluent A (5 mM KH_2PO_4, pH 4.0) and eluent B (500 mM KH_2PO_4, pH 4.4) as follows: 0–5 min, 98% eluent A, 2% eluent B; 5–75 min, 98–93% eluent A, 2–7% eluent B; 75–135 min, 93–0% eluent A, 7–100% eluent B; 135–150 min, 0% eluent A, 100% eluent B; 150–151 min, 0–98% eluent A, 100–2% eluent B; 151–160 min, 98% eluent A, 2% eluent B, with a constant flow rate of 1 ml/min. Each sample was manually injected. Before starting a new run, the column was equilibrated for 20 min with the starting solution. For measurement of the radioactivity, 1 ml fractions were collected from minute 64 to 80. The radioactivity of the fractions was determined in a TRI-CARB® liquid scintillation counter 1900 CA from Canberra-Packard (Frankfurt a.M./Germany). The concentration of the various nucleotides was calculated by comparison of their u.v. absorbances (integrated areas) with those of the external standard.

UDP-Glc and UDP-Gal eluted together. It was not possible to obtain a complete separation from UMP. A reliable concentration of UDP could not be determined by h.l.p.c., because it coeluted with an unknown metabolite. UDP-Xyl eluted immediately after UDP-Glc, while UDP-Gal and CDP eluted immediately after UDP-GA. In samples from vitamin B6-deficient rats, the peaks of UDP-Xyl and CDP were overlapped by the huge peaks of UDP-hexoses and UDP-GA. Detector sensitivity was optimized for detection of uracil-, cytidine- and guanine nucleotides. Quantification of the much higher concentrations of adenine nucleotides was therefore not possible.

Analysis of the N-glycans of rat liver-plasmamembranes

Purification of plasmamembrane-proteins from rat liver

Plasma membanes from rat liver were isolated according to a method of Pfleger *et al*. [23] with some minor modifications [4].

Delipidation of plasmamembrane-proteins

Delipidation of the isolated plasma membranes was done according to the method of Svennerholm and Fredmann [30].

PNGase F digestion

Tryptic glycopeptides obtained from plasma membrane-homogenate was digested with 7 m-units of PNGase F from *Flavobacterium meningosepticum* per 10 mg peptides in 400 µl of a buffer containing 50 mM *N*-methyl-2,2-iminodiethanol, pH 8.0, for 18 h at 37°C. This digestion was repeated once. The completeness of PNGase F digestion was checked by carbohydrate composition analysis of two aliquots (about 5% each) of the PNGase F digest. One aliquot was passed over a cation-exchange resin (AG-50W-X12; Bio-Rad, München, Germany), which retards peptides, whereas the other aliquot was not subjected to chromatography. Since both samples resulted in comparable amounts of monosaccharide, the PNGase F digestion was concluded to be complete. Subsequently, the oligosaccharides of the main aliquot (95% of the digest) were purified by cation-exchange chromatography (AG-50W-X12).

Fluorescent labelling of the glycans and desialylation

Released N-glycans were fluorescently labelled by reductive amination with 2-aminobenzamide (2-AB) using a Signal™ Labelling Kit according to the protocol of the manufacturer. Excess 2-AB was removed by paper chromatography [14].

2-AB-labelled N-glycans were treated with 100 m-units of neuraminidase from *Arthrobacter ureafaciens* in 100 µl of 25 mM sodium phosphate buffer, pH 5.0, for 48 h at 37°C.

Neutral-pH anion-exchange chromatography

Negatively charged sugars were separated by a Mono Q anion-exchange column (HR 5/5, 10×0,5 cm; Pharmacia). Oligosaccharides were eluted at a flow rate of 1 ml/min by a linear gradient, consisting of solvent A (water) and solvent B (0.6 M ammonium acetate, pH 7.0), starting 5 min after injection, from 0 to 35% solvent B in 65 min.

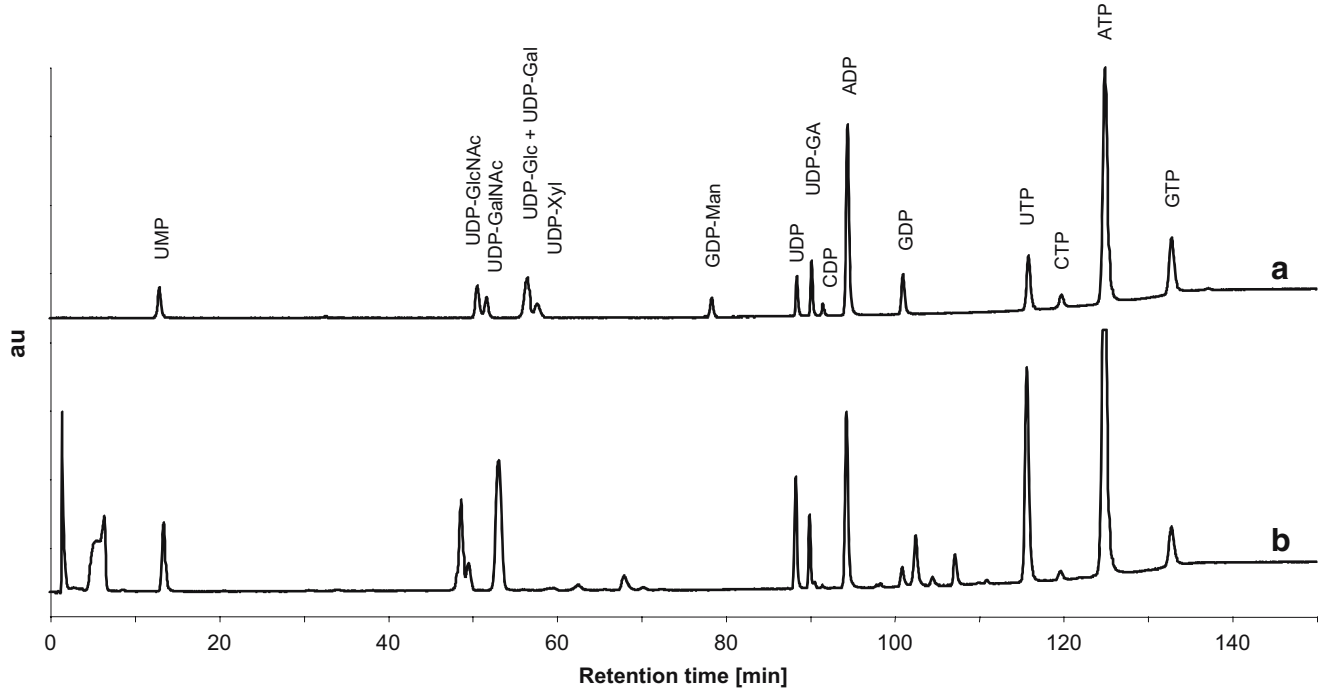

Fig. 1 SAX-h.p.l.c. separation of nucleotides and nucleotide sugars in rat liver. **a** standard solution. **b** homogenized and delipidized rat liver sample from a young rat after 2 weeks of vitamin B6-deficiency. The separation was performed as described in "Materials and methods." Spectrophotometer sensitivity: A, B: RecScale 0.1; ZeroSupp −0.01. C: RecScale 0.2; ZeroSupp −0.02

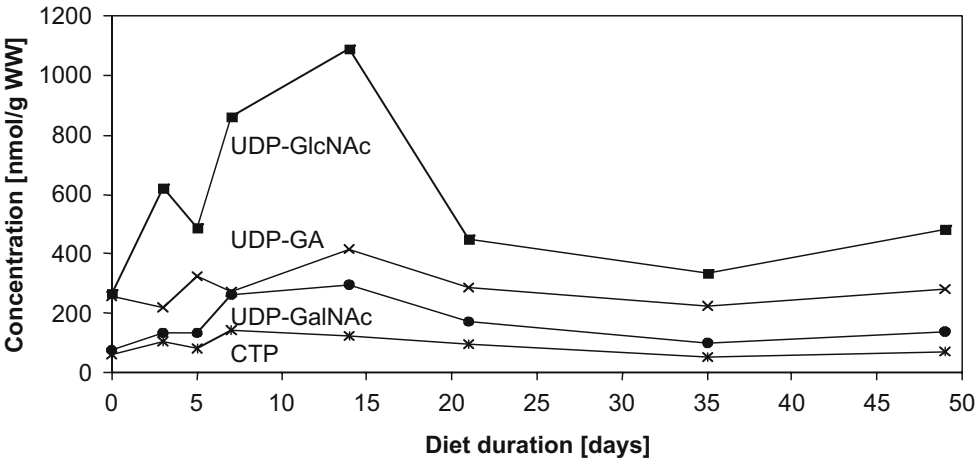

Fig. 2 Concentrations of nucleotides and UDP-sugars in liver tissue from young vitamin B6-deficient rats as a function of diet duration. Mean values without standard deviation. Rats were placed on the vitamin B6-free diet at the age of 21–25 days

Separation of neutral N-glycans

Neutral 2-AB-labelled N-glycans were separated by amino-phase h.p.l.c. using an APS 2-Hypersil column (4×250 mm, 3 μm; Bischoff Berlin, Germany) at room temperature with a flow rate of 1.5 ml/min. Glycans were eluted by a two-step gradient, consisting of solvent A (acetonitrile) and solvent B (15 mM sodium acetate, pH 5.2), of 0–20% solvent A in 10 min, and of 20–50% solvent B within 100 min.

Carbohydrate composition analysis with high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

Monosaccharide analysis was performed by hydrolysis of the oligosaccharides with 2 M trifluoroacetic acid for 4 h at 100°C followed by high-performance anion-exchange chromatography with pulsed amperometric detection as described previously [8]. The detection limit for the monosaccharides is about 5–10 pmol (response factors vary slightly for different monosaccharides).

Mass spectrometry

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was carried out on a Bruker Biflex instrument (Bruker, Bremen, Germany). Ionization was accomplished with a 337 nm beam from a nitrogen laser. Mass spectra were recorded in the positive ion mode using the reflector. 2,5-dihydroxybenzoic acid (10 mg/ml) in 60% ethanol was used as an aqueous solution of the glycan (2–20 pmol/μl) was 1/1 the matrix and 0.5–1 μl was placed on the target.

Results

Analysis of nucleotides and UDP–sugars in rat tissue

Nucleotides and UDP-sugars were quantified by enzymatic-photometric methods and by SAX-h.p.l.c. The enzymatic-photometric method according was used to determine sequentially the concentrations of UTP, UDP, UMP, UDP-Glc and UDP-Gal. Cytosine and guanosine nucleotides,

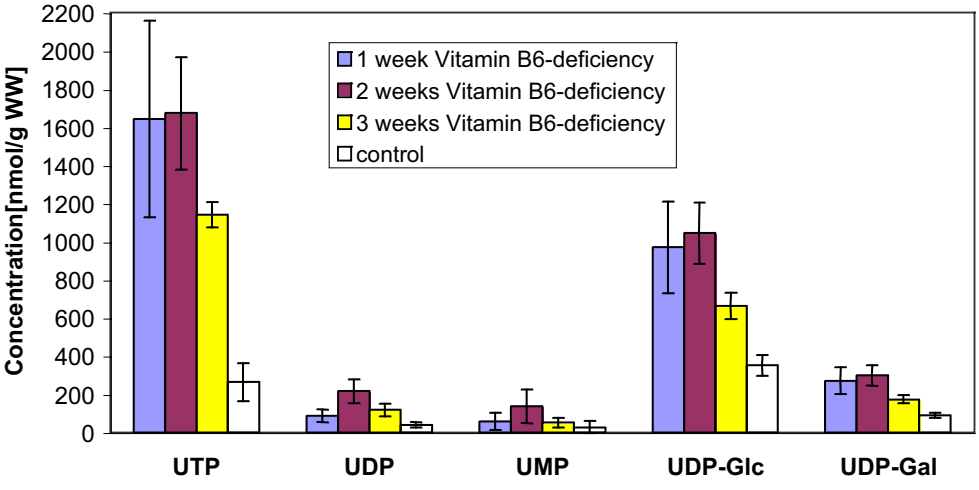

Fig. 3 Concentrations of uracil-nucleotides in liver tissue from young vitamin B6-deficient rats after 1, 2 and 3 weeks on a vitamin B6-free diet. Rats were placed on the vitamin B6-free diet at the age of 21–25 days. Test rats (n=4 for each period of B6 deficiency), control rats (n=10). All controls were pooled because there were no differences between the controls after 1, 2 and 3 weeks. Error bars denote ± 1 SD

Table 1 Factors of concentration changes of nucleotides and UDP-sugars in different tissues of vitamin B6-deficient rats

Nucleotides	Liver	Heart	Kidney	Lungs	Brain
UTP, UDP, UMP	5.4 (a)	0.6 (c)	0.6 (c)	0.8	0.9
UDP-GlcNAc	4.1 (a)	0.6 (c)	1.0	1.0	1.1
UDP-GalNAc	3.9 (a)	0.6 (c)	1.4 (c)	0.9	1.1
UDP-Glc	3.0 (a)	0.6 (a)	1.4 (c)	1.0	1.3 (a)
UDP-Gal	3.3 (a)	0.5	1.3 (a)	1.0	1.5 (c)
CTP	2.4 (a)	0.5	0.5 (c)	1.3	1.1
CDP	2.3 (a)	1.2	1.0	0.9	1.6 (a)

Results after 2 weeks on a vitamin B6-deficient diet. UTP, UDP, UMP, UDP-Glc, and UDP-Gal were measured by an enzymatic-photometric method. UDP-GlcNAc, UDP-GalNAc, CTP und CDP were measured by anion-exchange-chromatography.
Significance measured by Mann-Whitney-Test
(a) $p \leq 0.046$
(b) $p \leq 0.039$
(c) $p \leq 0.024$

UDP-GA, UDP-Xyl, UDP-GlcNAc and UDP-GalNAc were measured by h.p.l.c. During delipidation 17.3% of material was lost on average, as measured with ^{14}C-GDP-L-fucose as internal standard. The chromatograms of the h.p.l.c. separation of the soluble nucleotides und nucleotide sugars in the used standard and in homogenized and delipidized rat liver from control and vitamin B6-deficient rats are shown in Fig. 1.

Vitamin B6-deficiency induced a dramatic increase of uracil nucleotide concentrations, of UDP-sugars and to a smaller extend of CTP in the livers of young rats. The extent of the concentration increase depended on the duration of the diet. This increase was first detectable after 1 week on the vitamin B6-free diet, the maximal concentration being reached after 2 weeks, except for CTP, which peaked at 1 week (Fig. 2). Although the vitamin B6-deficient diet was continued, uracil nucleotide concentrations decreased slowly. After 5 weeks on the vitamin B6-free diet, the uracil nucleotide concentrations returned to normal.

This behavior was typical for all the measured nucleotides and UDP-sugars in liver tissue of young vitamin B6-deficient rats. Concentrations of CDP, GTP and GDP in the liver tissue of young vitamin B6-deficient rats remained unaffected.

Figure 3 shows the concentrations of uracil nucleotides after 1, 2 and 3 weeks of vitamin B6-deficiency in liver tissues from young rats (n=4), in comparison with control rats (n=10). All controls were pooled because there were no differences between the controls after 1, 2 and 3 weeks. Nucleotides and UDP-sugars showing increased concentrations in vitamin B6-deficient rats reached maximal concentrations after 2 weeks on the vitamin B6-deficient diet. The UTP concentration showed the highest increase (6.3-fold). UDP and UMP concentrations increased 5.2 and 4.7-fold, CTP 2.4-fold and the UDP-sugars 1.6- to 4.1-fold.

In contrast to the liver, the sum of UTP, UDP and UMP, UDP-GlcNAc, UDP-GalNAc and UDP-Glc in heart muscle showed a decrease of 40%. The sum of CTP and CDP, as well as GTP and GDP showed no significant changes.

In kidney, changes were more heterogeneous. Thus, while the sum of UTP, UDP and UMP showed a decrease of 40%, the concentrations of the activated UDP compounds (UDP-GalNAc, UDP-Glc, UDP-Gal and UDP-GA) were increased 1.3- to 1.4-fold. In the lungs, there was an isolated increase of UDP-GA. In the brain the concentrations of UDP-Glc, UDP-Gal and the sum of the CTP and CDP concentrations showed an increase of 30–50%, while the concentrations of other nucleotides were unaffected (Table 1).

Figure 4 shows the changes in the sum of the concentrations of UTP, UDP and UMP in different organs, tested after 2 weeks of vitamin B6-deficiency.

While vitamin B6-deficiency led to increased concentrations of nucleotides and UDP-sugars in young rats,

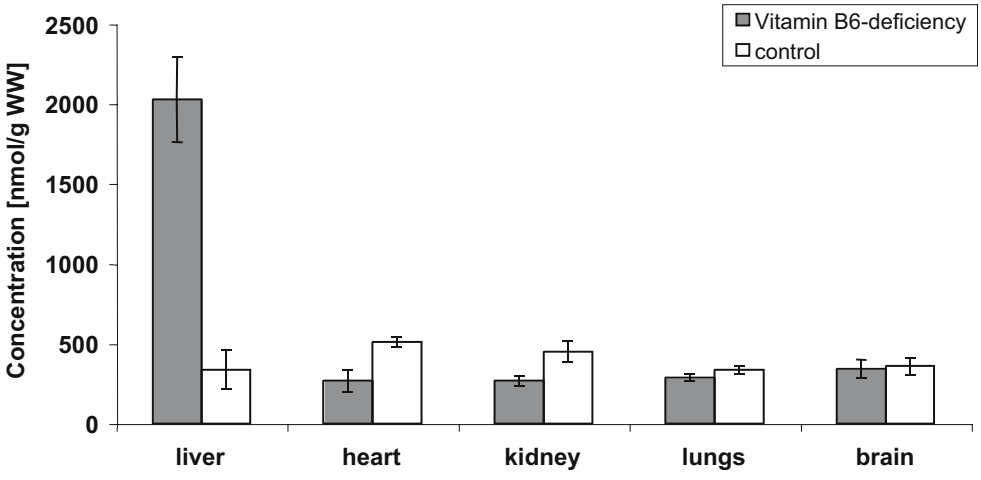

Fig. 4 Sums of the concentrations of UTP, UDP and UMP in different tissues of young vitamin B6-deficient rats. The nucleotides were determined by an enzymatic-photometric method. UTP, UDP and UMP are shown as their sum, because of the rapid degradation in ischemia during removal of the organs. Error bars denote ± 1 SD

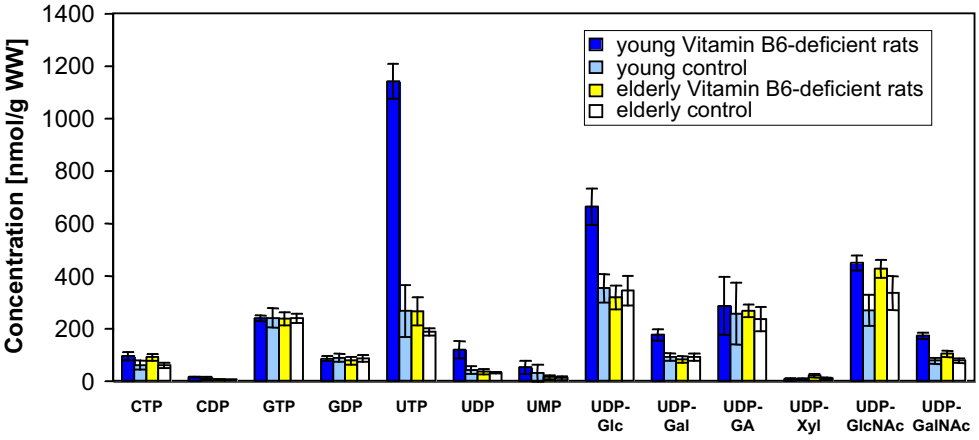

Fig. 5 Comparison between young and older rats after maintenance for 3 weeks on a vitamin B6-deficient diet. Shown are the concentrations of all measured metabolites in liver tissue from young and older vitamin B6-deficient rats after 3 weeks on a vitamin B6-free diet. Young rats were 21–25 days and older rats 2 months of age when treatment was started. Test rats (young n=4; older n=6), control rats (young n=10; older n=4). Error bars denote ± 1 SD

nucleotides and UDP-sugars remained unchanged in liver tissue from older rats, which had been restricted to a vitamin B6-free diet from the age of 2 months on (Fig. 5). In summary, both the duration of the vitamin B6-free diet and the age of the rats affected the changes of the concentrations of nucleotides and UDP-sugars.

To examine a possible influence of glucocorticoids on the changes in uracil nucleotide concentrations in rat liver, adrenalectomized rats were compared with animals that had fasted for 2 days and in addition with a control group. In vitamin B6-deficient rats, which had undergone adrenalectomy before starting the diet, an increase of uracil nucleotides and UDP-sugars was found, comparable to the concentrations measured in the vitamin B6-deficient group without adrenalectomy. Cytosine and guanosine nucleotides remained unchanged. Vitamin B6-deficient animals that had fasted showed no increase of uracil nucleotide concentrations. The control groups of normal, adrenalectomized and fasting rats showed no differences in their nucleotide concentrations. Results are summarized in Table 2 and illustrated in Fig. 6.

Analysis of the N-glycans of rat liver-plasmamembranes

Plasmamembrane-glycoproteins were digested with PNGase F following a tryptic digestion. The oligosaccharides were separated from peptides by passing over a cation-exchange resin. Typical constituents of complex-type sugars were detected by carbohydrate composition analysis. The amount of L-fucose, glucosamine, galactose and mannose were found in molar proportions of 0.7 : 3.2 : 2.6 : 3 in vitamin B6-deficiency and 0.7 : 3.6 : 2.9 : 3 in control. Galactosamine, which indicates a potential content of O-linked glycans, was detected only in a molar proportion of 0.08 in vitamin B6-deficiency and 0.1 in control compared to three mannoses.

Analysis of 2-AB-labelled glycans

N-Linked glycans were enzymatically released from tryptic glycopeptides by PNGase F digestion and labelled with the fluorescent 2-AB by reductive amination. 2-AB-labelling allowed a very sensitive detection of subnanomolar amounts of glycans. The purified fluorescent-labelled N-glycans were devided in two aliquots: The native, in parts negatively charged sugars were analysed for sialylation patterns, the second aliquot was enzymatically desialylated to characterize the neutral glycans.

Characterisation of the neutral oligosaccharides after desialylation

Mass determination by MALDI-TOF-MS

Mass spectrometric data were obtained from the desialylated glycan mixture prior to separation by aminophase h.p.l.c., as well as from isolated oligosaccharide fractions.

Table 2 Factors of concentration changes of nucleotides and UDP-sugars in the livers of vitamin B6-deficient rats after adrenalectomy, after fasting and under control conditions, compared to their controls

	UDP-Glc	UDP-Gal	Σ UTP + UDP + UMP	UDP-GlcNAc	UDP-GalNAc
Vitamin B6-deficiency	2.6	2.8	5.8	2.2	3.2
Vitamin B6-deficiency and adrenalectomy	2.0	2.1	4.3	2.7	3.7
Vitamin B6-deficiency and 2 days of fasting	0.8	0.8	1.0	0.9	1.0

Results after 2 weeks on a vitamin B6-deficient diet. UTP, UDP, UMP, UDP-Glc, and UDP-Gal were measured by an enzymatic-photometric method. UDP-GlcNAc and UDP-GalNAc were measured by SAX-h.p.l.c.

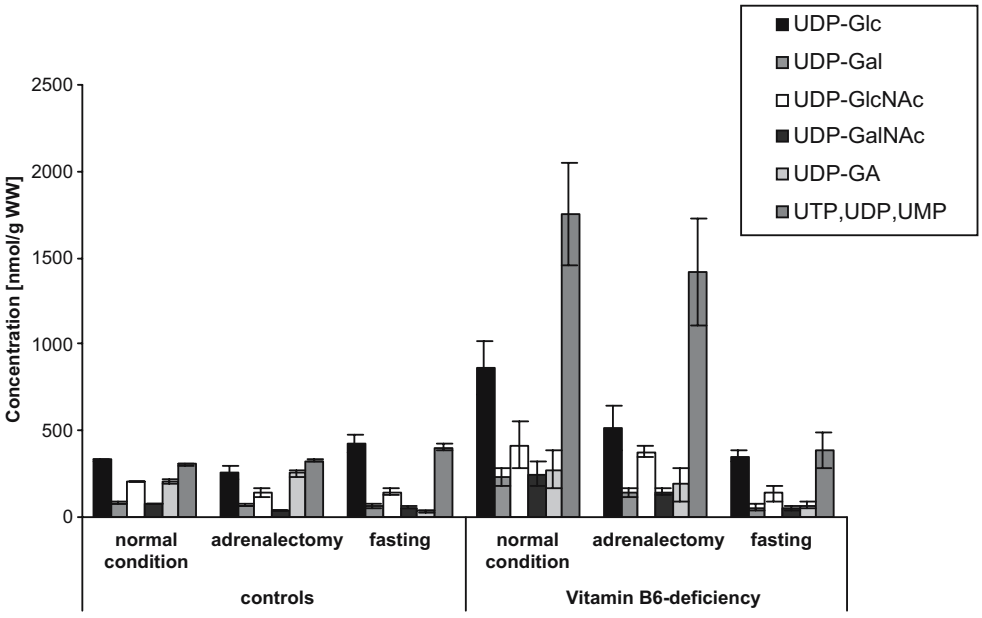

Fig. 6 Concentrations of nucleotides and UDP-sugars in the livers of vitamin B6-deficient rats after adrenalectomy, after fasting and under normal conditions. UTP, UDP, UMP, UDP-Glc, UDP-Gal were measured by an enzymatic-photometric method. UDP-GlcNAc, UDP-GalNAc and UDP-GA were measured by SAX-h.p.l.c. UTP, UDP and UMP are shown as their sum, because of the rapid degradation in ischemia

The oligosaccharide mixture consisted of a wide variety of bi-, tri- and tetra-antennary structures with and without fucose, as well as high-mannose structures.

The mass-spectra of glycans from vitamin B6-deficient rats did not show differences in comparison to controls (Fig. 7).

Separation of neutral oligosaccharides by aminophase h.p.l.c.

Desialylated glycans were separated and fractionated using aminophase h.p.l.c. The elution pattern displayed a variety of signals, representing the heterogeneity of plasmamembrane-

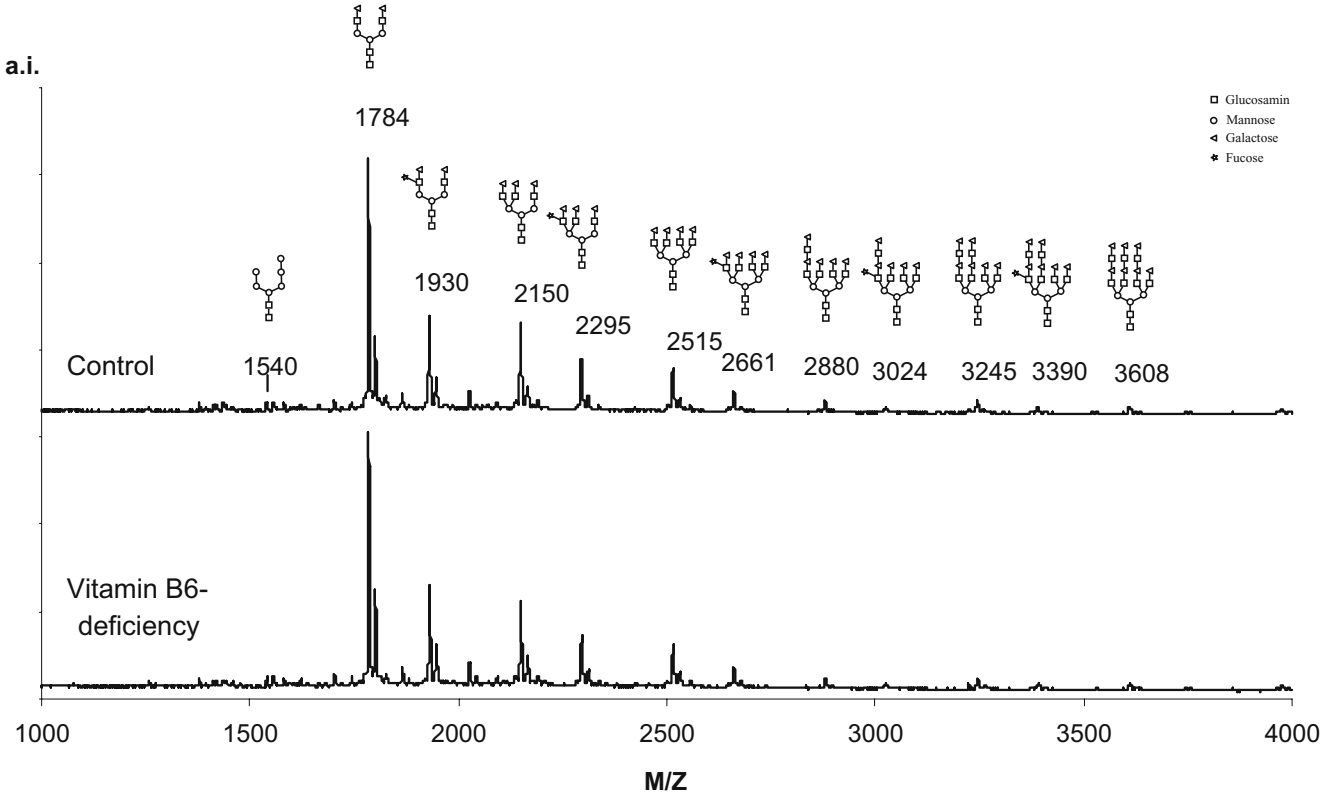

Fig. 7 Mass determination of desialylated N-glycans released from liver-plasma membrane glycoproteins. Mass spectra of liver-plasma membrane glycoproteins of control rats (*above*) and vitamin B6-deficient rats (*below*) were measured by MALDI-TOF-MS prior to separation by aminophase h.p.l.c. N-linked glycans were enzymatically released from tryptic glycopeptides by PNGase F digestion, were labelled with the fluorescent 2-AB by reductive amination and underwent desialylation. Cristallization matrix 2,5-Dihydroxybenzoic acid

Table 3 Composition of complex N-glycans derived from plasma-membrane glycoproteins of rat livers in vitamin B6-deficiency and in controls in relation to the total of oligosaccharides

Composition	Vitamin B6-deficiency (%)	Control (%)
biantennary +/− fucose	54.5	55.8
triantennary +/− fucose	21.2	20.1
tetraantennary +/− fucose	10.4	9.2
tertraantennary + repeats +/− fucose	3.5	3.4
total of complex oligosaccharides	89.6	88.5

oligosaccharides. 15 main fractions (*about* 1% of total glycan compound) were obtained and the retention times were compared to acid-α-1-GP, which was used as external standard (data not shown). The structural characterisation received by retention time comparison were verified in the following by MALDI-TOF-MS. There were no qualitative or quantitative changes in oligosaccharide compound between neutral glycans of vitamin B6-deficiency and controls (Table 3).

Characterization of the charged oligosaccharides

Sialylated oligosaccharides were separated according to their number of negative charges by neutral-pH anion-exchange chromatography. Six fractions were obtained representing neutral, mono-, di-, tri-, tetra-, penta- and hexasialylated N-glycans (data not shown).

The glycans of each fraction were enzymatically desialylated and rechromatographed using aminophase h.p.l.c. Since the elution profile of the neutral oligosaccharides on aminophase h.p.l.c. was defined during the analysis, the chromatographic patterns of each desialylated glycan subfraction (A0, A1, A2, A3, A4, A5 and A6) could be assigned to glycan structures and was also verified by MALDI-TOF-MS. In summary, 94–96% of the whole glycan mixture were sialylated glycans. The main fractions were bi-antennary di-sialylated oligosaccharides and bi-antennary tri-sialylated glycans. Forty percent of sialylated glycans were oversialylated for example there were three sialic acid residues linked to bi-antennary glycans or six sialic acids linked to tetra-antennary glycans. Also regarding sialylation profiles of N-glycans derived from plasma membranes of liver, there were no qualitative or quantitative changes in vitamin B6-deficient and control rats (Table 4).

Discussion

The data presented here show that pyridoxal phosphate deficiency leads to an increase of the concentrations of UDP-sugars and other uracil nucleotides and consecutively of CTP, specifically in liver tissue in contrast to some other organs.

The duration of the vitamin B6-free diet affected the magnitude of the concentration increase in young rats placed on a vitamin B6-free diet from the age of 21–25 days. The concentration increase appeared from the first until the third week of feeding, attaining a maximum after the second week for uracil nucleotides and UDP-sugars and after the first week for CTP. The maximal concentration increase was 4.7- to 6.3-fold for uracil nucleotides, 1.6- to 4.1-fold for UDP-sugars and 2.4-fold for CTP.

The data suggest that pyridoxal phosphate plays an important role in the regulation of uracil nucleotide concentration. The highest concentration increase in young vitamin B6-deficient rats was found for UTP. Since UTP is the starting molecule for the synthesis of UDP-sugars and CTP, the increase in the concentrations of UDP-sugar and CTP shows a time lapse. The high concentrations of uracil nucleotides in vitamin B6-deficiency may be the result of enhanced de novo-biosynthesis or reutilisation caused by increased activity of enzymes of uracil nucleotide synthesis or reutilisation. Enhanced biosynthesis of these enzymes

Table 4 Composition of charged complex N-glycans derived from plasmamembrane glycoproteins of rat livers in vitamin B6-deficiency and in controls regarding to the total of oligosaccharides

	Biantennary +/− fuc (%)		Triantennary +/− fuc (%)		Tetraantennary +/− fuc (%)		Tetraantennary+ repeats +/− fuc (%)	
	Control	Vitamin B6-deficiency	Control	Vitamin B6-deficiency	Control	Vitamin B6-deficiency	Control	Vitamin B6-deficiency
A0	0.8	0.6	–	–	–	–	–	–
A1	7	8	1	1	–	–	–	–
A2	25	23	8	7	–	–	–	–
A3	14	16	6	6	–	–	–	–
A4	6	6	7	7	2	2	2	2
A5	–	–	4	4	3	3	2	2
A6	–	–	1	1	2	3	0.5	0.7

may be due to an increased gene expression of related enzymes. This assumption is supported by earlier findings showing that pyridoxal phosphate enhances steroid hormone-dependent gene expression and glucocorticoid receptor mRNA. Moreover, pyridoxal phosphate has been shown to enhance hormone-independent gene expression by modulating the activity of RNA polymerase I and II and the level of mRNA, e.g. β-actin mRNA [17]. It enhanced the DNA-binding activity of glucocorticoid receptors [15, 32], as well as increasing the level of glycogen phosphorylase mRNA without changing the enzyme activity [18]. Furthermore, it increased the levels of albumin mRNA and aspartate aminotransferase mRNA [16] as well as the cystathionase mRNA level and cystathionase synthesis [28]. Based on the present study, we strongly suggest that PALP also modulates the gene expression of the enzymes of uracil nucleotide metabolism, affecting either in their biosynthesis or breakdown or both. The increased concentration of CTP may rather be the result of the increased concentration of its precursor UTP.

The susceptibility of the very young rats may be related to the age-dependency of the gene expression of PALP-dependent enzymes, which is not evident from the experiments mentioned above. The decreased concentration of uracil nucleotides in heart and kidney is in accord with the results of Oka *et al*. [18], showing tissue specificities during vitamin B6-deficiency.

The increased concentrations of uracil nucleotides and UDP-sugars in young vitamin B6-deficient rats has metabolic consequences, at least for glycogen metabolism, which is affected twice. Firstly, UDP-Glc, the substrate for glycogen synthase is increased. Secondly, PALP is the coenzyme of glycogen phosphorylase. There is only a limited number of studies in the literature dealing with glycogen metabolism in liver tissue of vitamin B6-deficient rats. The results obtained from these studies are inconsistent with one another. We support the results of Robinson *et al*. [27], who reported an increased glycogen concentration accompanied by a decreased glycogen phosphorylase activity in vitamin B6-deficient mice. However, Okada *et al*. [19] found neither an increased glycogen concentration nor changed glycogen phosphorylase activity in liver tissue from vitamin B6-deficient Wistar rats. Oka *et al*. [18], investigating liver tissue from vitamin B6-deficient Wistar rats, also reported an unchanged activity of glycogen phosphorylase, although they found an increased level of glycogen phosphorylase mRNA. The antiport-system for UDP-sugars in the Golgi complex could be affected by the increased concentration of the UDP-sugars (UDP-Gal/UMP, UDP-GalNAc/UMP and UDP-GlcNAc/UMP) in vitamin B6-deficient rats. As UDP-sugars are essential for the formation of glycoconjugates, we checked, if increased concentrations of these UDP-sugars due to vitamin B6-deficiency would influence the glycosylation pattern of plasmamembrane-proteins of liver.

Despite up to 6-fold increase of uracil nucleotides and -sugars, the N-glycosylation pattern of the most prominent glycans of plasma membranes of rat liver did not disclose any difference to the glycans of untreated rats. This surprising *in vivo* findings is in strong contrast to *in vitro* studies which show that in cell culture systems an increased concentration of UTP, UDP, and UDP-sugars induced by uridine and cytidine supplementation, lead to an increase of complexity of N-glycans of the rat-hepatocytes, while sialylation decreased [21].

Grammatikos *et al*. increased the intracellular UDP-N-acetylhexosamine pool by treating recombinant BHK cells with glucosamine and uridine and found an increase in complexity of the N-glycosylation of IL-2 [9]. Also, the treatment with ammonium leads to more complex glycans in cell cultures, due to an increase of UDP-GlcNAc and UDPGalNAc [7].

This finding strongly suggests, that in contrast to *in vitro* experiments, N-glycan biosynthesis in the liver is regulated as part of the whole animal by additional factors besides the concentrations of the activated sugars or other factors in the cell culture medium.

Acknowledgements This work is dedicated to Dr. Martin Zimmermann-Kordmann, who suddenly died. We are deeply saddened by the loss of an excellent scientist and warm-hearted friend and colleague.
We are grateful to F. Hoffmann-La Roche Ltd, Switzerland, for providing us with vitamin B6-deficient feed and thank Dr. T. Scott, Leeds, for critically reading the manuscript. This work was supported by the Sonnenfeld-Stiftung.

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