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N-Acetylneuraminic Acid Biosynthesis in Rat Liver and Kidney

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Rat liver and kidney tissue slices incubated with *N*-acetyl [^{3}H]mannosamine incorporated radioactivity into free and bound *N*-acetylneuraminic acid and CMP-*N*-acetylneuraminic acid (CMP-NeuAc). Liver and kidney also incorporated radioactivity from intravenously injected [^{3}H]ManNAc into *N*-acetylneuraminic acid and CMP-NeuAc. From the decrease in the specific radioactivity of CMP-NeuAc after a single injection of *N*-acetyl[^{3}H]mannosamine the half-life of CMP-NeuAc was determined. From this half-life and the pool size of CMP-NeuAc a synthesis rate of CMP-NeuAc was calculated, being 1.2 nmol/min/g wet weight of kidney. In previous experiments a value of 1.0 nmol/min/g wet weight was determined for liver [Ferwerda *et al.* (1983) Biochem J 216: 87-92]. The synthesis rate of CMP-NeuAc *in vivo* was in the same range as the synthesis rate calculated from the turnover of bound *N*-acetylneuraminic acid, which was 2.7 and 0.4 nmol/min/g wet weight for liver and kidney respectively.

The assay conditions for UDP-*N*-acetylglucosamine 2-epimerase and *N*-acetylmannosamine kinase were adapted to measure low activities *in vitro*. It appeared that the kinase activity detected in kidney can synthesize *N*-acetylmannosamine6-phosphate at a rate sufficient for the observed production of *N*-acetylneuraminic acid *in vivo*. Also a low, but measurable activity of UDP-*N*-acetylglucosamine 2-epimerase was detected in kidney *in vitro*, suggesting that the biosynthetic pathway of *N*-acetylneuraminic acid in kidney is the same as in liver. The synthesis rate of *N*-acetylneuraminic acid in liver determined *in vivo* is approximately 12 times slower than the maximal potential rate calculated from the activities of the *N*-acetylneuraminic acid (precursor-) forming enzymes as detected *in vitro*. This indicates that in liver *in vivo* the enzymes are working far below their maximal capacity.

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N-Acetylneuraminic acid (NeuAc) is the important negatively charged, terminal sugar of many glycoproteins and glycolipids. The generally accepted pathway for the biosynthesis of NeuAc from UDP-*N*-acetylglucosamine (UDP-GlcNAc) involves the intermediates *N*-acetylmannosamine (ManNAc), ManNAc-6-phosphate and NeuAc-9-phosphate (for review, see ref. 1). All four enzymes participating in this pathway are detectable in sialoglycoprotein-secreting tissues, e.g. liver, salivary gland and intestinal mucosa [2-6]. In non-sialoglycoprotein-secreting tissues the activities of the enzymes UDP-GlcNAc 2-epimerase (UDP-2-acetamido-2-deoxy-D-glucose 2-epimerase, EC 5.1.3.14) and ManNAc kinase (ATP:2-acylamino-2-deoxy-D-mannose-6-phosphotransferase, EC 2.7.1.60) are below the detectable level when measured *in vitro* with radioactive substrates [5]. This might be interpreted assuming that in non-sialoglycoprotein-secreting tissues ManNAc-6-phosphate is formed by an alternative pathway in which the precursors UDP-GlcNAc and ManNAc are not involved, or that the synthesis rate of ManNAc-6-phosphate is too low for detection *in vitro*.

A pathway for ManNAc-6-phosphate synthesis, in which UDP-GlcNAc 2-epimerase and ManNAc kinase are not involved, is the direct epimerization of GlcNAc-6-phosphate. The enzyme catalysing this reaction is GlcNAc-6-phosphate 2-epimerase (2-acylamido-2-deoxy-D-glucose-6-phosphate 2-epimerase, EC 5.1.3.9), which has been reported to occur only in bacteria [7]. We tried to detect this enzyme in kidney extracts, with *N*-acetyl-[^{3}H]glucosamine-6-phosphate as substrate. However, all our attempts were unsuccessful. We only observed a conversion of GlcNAc-6-phosphate into GlcNAc-1-phosphate and a rapid de-*N*-acetylation (unpublished results), as was also reported by Kikuchi and Tsuiki [4]. During our experiments *in vivo* we observed that in kidney, [^{14}C]NeuAc and CMP-[^{14}C]NeuAc were formed after injection of [^{14}C]ManNAc in rats. In addition, we could calculate that under normal conditions the synthesis rate of NeuAc in liver *in vivo* is in the range of a few nmoles/min/g wet weight. If the synthesis rate of NeuAc in other tissues is the same or lower and the enzymes involved in the pathway exhibit *in vitro* an activity of the same order, they would have been undetectable in the assays previously used. Therefore, we reinvestigated the NeuAc biosynthesis in a non-sialoglycoprotein secreting tissue, i.e. kidney, in comparison to the NeuAc biosynthesis in liver. The present study demonstrates that in both tissues the same pathway for NeuAc biosynthesis is involved. Part of this work has been presented in a preliminary form [8].

Materials and Methods

Materials

UDP-*N*-acetylglucosamine, *N*-acetylglucosamine, *N*-acetylmannosamine, ATP and acid phosphatase from potatoes were obtained from Sigma Chemical Company (St. Louis, MO, USA). UDP-*N*-acetyl-D-[U-^{14}C]glucosamine (sp. act. 213 Ci/mol), *N*-acetyl-D-[6(N)-^{3}H]mannosamine (sp. act. 19 000 Ci/mol), *N*-acetyl[4-^{14}C]neuraminic acid (sp. act. 57 Ci/mol) and CMP-*N*-acetyl[4-^{14}C]neuraminic acid (sp. act. 1-2 Ci/mol) were products from New England Nuclear (Boston, MA, USA). The [^{14}C]ManNAc batches used contained 1% or less [^{14}C]GlcNAc, as determined by high-voltage electrophoresis. Dowex

AG®1-X8 (Cl^- form; 100-200 mesh) was purchased from Bio-Rad Laboratories (Richmond, CA, USA), and was converted into the bicarbonate form as recommended by the manufacturer.Heparin was obtained from Kabi AB (Stockholm, Sweden). All other chemicals were obtained from commercial sources and were of analytical grade. Adult male rats (Wistar strain), weighing approximately 200 g and fed *ad libitum* were used.

Incubation of Tissue Slices

Rats were killed by decapitation; the livers and kidneys were rapidly removed and sliced (approximately 2 mm × 6 mm) at 0°C. The slices (1 g wet weight) were incubated with 6 μmol of [^{14}C]ManNAc (15 Ci/mol) in 3 ml phosphate-buffered Krebs-Ringer [9] solution, pH 7.4, supplemented with 0.16 M pyruvate, 0.1 M fumarate, 0.16 M glutamate and minimal essential medium amino acids [10], in an atmosphere of 5% CO_2/95% O_2 at 37°C for 2 h under constant shaking. At the end of the incubation the slices were removed from the incubation medium and washed twice with cold phosphate-buffered Krebs-Ringer solution, pH 7.4; all subsequent procedures were conducted at 0-4°C. The incubation medium, combined with the washes was extensively dialysed against distilled water to eliminate residual [^{14}C]ManNAc. Proteins present in the retentate were precipitated by adding an equal vol of 1 M HCl, containing 2% (w/v) trifluoroacetic acid and separated by centrifugation. The slices were homogenized in 5 vol of cold 75% (by vol) ethanol, sonicated for 1 min and centrifuged at 105 000 × *g* for 1 h. The various metabolites were isolated from the supernatant and bound NeuAc was isolated and purified from the pellet obtained from the slices and from the incubation medium, as described below.

Turnover Studies

To determined the half-life of CMP-NeuAc in kidney, rats were injected intravenously with 0.5 ml phosphate-buffered saline (10 mM sodium phosphate, 0.154 M NaCl), pH 7.4, containing 50 μCi of [^{14}C]ManNAc per 100 g of body weight. They were killed by immersing the whole animal in liquid nitrogen and stored at -40°C. The kidneys were removed in a frozen state and stored at -40°C until use.

To determine the half-life of bound NeuAc in liver, kidney and plasma, rats were injected intraperitoneally with 0.5 ml phosphate-buffered saline, pH 7.4, containing 50 μCi of [^{14}C]ManNAc per 100 g of body weight. After injection of 1500 IU of heparin and narcotization with sodium pentobarbital, the abdomen was opened and the liver was perfused via the portal vein with cold phosphate-buffered saline, pH 7.4, containing 0.9% (w/v) sodium citrate. The kidneys were only partly perfused by this method. The outstreaming (diluted) blood was collected and the blood cells were removed by centrifugation. After perfusion, the liver and the kidneys were removed. For determination of total bound NeuAc in liver and kidney, rats were killed by decapitation and the organs were removed. All material was stored at -40°C until use.

Isolation and Purification of the Various Compounds

All soluble metabolites were isolated from homogenates of the tissues or the slices in cold 75% (by vol) ethanol and further purified as described by Ferwerda *et al.* [11]. For

the determination of the concentration of free NeuAc, CMP-NeuAc and UDP-GlcNAc, [^{3}H]-labelled standards were added, where appropriate. Most radioactive products, other than NeuAc and CMP-NeuAc, were further characterized by high-voltage electrophoresis [6].

Purification of UDP-GlcNAc was achieved on Dowex AG1-X8 (HCO_3^- form; 100-200 mesh) column (4.0 cm × 0.5 cm) stepwise eluted with increasing concentrations of triethylammonium bicarbonate (Teb) buffer, pH 7.8 [5]. For additional purification of UDP-GlcNAc, HPLC was performed using a Perkin-Elmer Series 2 liquid-chromatograph, equipped with a Rheodyne 7125 injection valve and with a Perkin-Elmer LC-75 spectrophotometer. Separation was performed on a pre-packed Partisil-SAX (10 μm, Bètron Scientific, The Netherlands) column (4 mm × 250 mm). Elution was carried out by a modification of the procedure as described by Fleischer [12] using a constant mixture of 7 mM K_2HPO_4, pH 4.0 (A) and 25 mM K_2HPO_4, pH 4.5 containing 0.5 M KCl (B) (98/2, by vol) for 10 min, followed by a linear gradient of increasing solvent B at a rate of 3% per min. The flow rate of the solvent mixtures was maintained at 2 ml/min. The concentration of UDP-GlcNAc was calculated from the peak areas of known concentrations of UDP-GlcNAc solutions. Fractions of the effluent (2 ml) were collected and counted for radioactivity. In this chromatographic system UDP-GlcNAc co-migrated with UDP-GalNAc. No further attempts were made to separate these two compounds. UDP-GlcNAc and CMP-NeuAc concentrations were also determined in homogenates prepared for the enzyme determinations, to measure the possible hydrolysis of these metabolites in the buffered sucrose medium.

For determination of the specific radioactivities of bound NeuAc, it was split off with 0.05 M H_2SO_4 and purified as described by Ferwerda *et al.* [11]. Liver and kidney were first homogenized in 75% (by vol) ethanol and from the pellets, obtained after centrifugation and washing, appropriate amounts were taken. Diluted plasma was first extensively dialysed against distilled water at 0°C to eliminate radioactive low molecular weight compounds, then the retentate was used directly for acid hydrolysis. The total amount of bound NeuAc in liver and kidney was determined by mixing equal amounts of a 10% (w/v) homogenate and 0.1 M H_2SO_4 with [^{3}H]NeuAc as internal standard. Hydrolysis and purification was as described above.

Enzyme Assays

The rats were decapitated and the tissues were rapidly removed and chilled. All subsequent procedures were conducted at 4°C. The tissues were minced and washed with 0.25 M sucrose in water. Suspensions of the tissues in 2 vol of a solution containing 0.25 M sucrose and 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (Hepes), pH 7.5, were homogenized in a motor-driven glass/Teflon Potter-Elvehjem type homogenizer (clearance 0.25 mm) for 2 × 5 sec at 1 000 rev./min. The homogenates were centrifuged at 180 000 × *g* for 60 min yielding the soluble cell fractions. All incubations were carried out at 37°C for 30 min, immediately after the preparation of the soluble cell fractions. Control incubation mixtures lacked the tissue fractions. The reactions were stopped by heating at 100°C for 3 min. The incubation mixture was centrifuged and the precipitate was washed three times with water. Isolation of the products from the combined supernatants was achieved on Dowex AG1-X8 HCO_3^- form; 100-200 mesh) co-

lumn (4.0 cm × 0.5 cm). stepwise eluted with increasing concentrations of Teb buffer, pH 7.8, as previously described [5]. The products were further characterized by high-voltage electrophoresis [6].

UDP-GlcNAc 2-epimerase. The incubation mixture consisted of 0.1 M Tris-HCl buffer, pH 7.4, 0.5 mM UDP-[^{3}H]GlcNAc (sp. act. 2 Ci/mol), 2 mM $MgCl_2$, 20 mM AMP and 50 μl of soluble cell fraction (0.5-1.0 mg of protein) in a total volume of 120 μl. The enzyme activity was calculated from the radioactivity found in the ManNAc region after high-voltage electrophoresis of the water eluate of the Dowex AG1 column. A correction was made for the dilution of UDP-[^{3}H]GlcNAc by endogenous UDP-GlcNAc.

ManNAc Kinase. The composition of the incubation mixture was: 0.1 M Tris-HCl buffer, pH 8.0, 1.9 mM [^{14}C]ManNAc (sp. act. 10 Ci/mol), 15 mM ATP, 15 mM $MgCl_2$, 25 mM NaF, 1.9 mM GlcNAc and 50 μl of soluble cell fraction (0.5-1.0 mg of protein) in a total volume of 120 μl. The enzyme activity was calculated from the radioactivity found in the ManNAc region after high-voltage electrophoresis of the dephosphorylated compounds obtained in the 6-phosphate fraction, eluted from the Dowex AG1 column.

Analytical Methods

Protein was determined by the method of Lowry *et al.* [13], with bovine serum albumin in 0.25 M sucrose as standard. Sialic acid was determined as described by Warren [14]. Radioactivity was counted in a Berthold liquid-scintillation counter (BF 8000) equipped with an automatic external standard for calculation of the dpm.

Results and Discussion

Turnover of Bound N-Acetylneuraminic Acid

The total amount of bound NeuAc, measured with the isotope dilution technique in liver and kidney was 1.17 ± 0.06 and 2.14 ± 0.02 μmol/g wet weight respectively (average ± SEM. of three rats). Liver also synthesizes 85% of the plasma glycoproteins [15-17]. The plasma content of rats, weighing 200 g, is approximately 7.5 ml [18,19], containing 2.9 μmol bound NeuAc per ml [20]. Plasma contains only one third of the total amount of plasma proteins in the whole body [21], so liver is responsible for the synthesis of 55.5 μmol of bound NeuAc in plasma glycoproteins, i.e. 7.4 μmol/g wet weight by a liver weight of 7-8 g.

Fig. 1 shows the decrease of specific radioactivities of bound NeuAc in liver, plasma and kidney after the injection of [^{14}C]ManNAc. The average turnover rate for bound NeuAc determined from the data shown in Fig. 1 in liver is 1.5 days, which is slightly slower than the half-life of 26-33 h for protein-bound NeuAc in single rat plasma-membrane glycoproteins [22,23]. In plasma we determined an average turnover rate of 1.5 days for bound NeuAc (Fig. 1), which is in the same range as the half-lives of 50 h, 36 h and 31 h for three different plasma glycoproteins, as reported by Schreiber *et al.* [21]. From the half-lives of bound NeuAc in liver and plasma we calculated a synthesis rate for NeuAc of 2.7 nmol/min/g wet weight for liver. Such calculations neglect a possible reutilization of

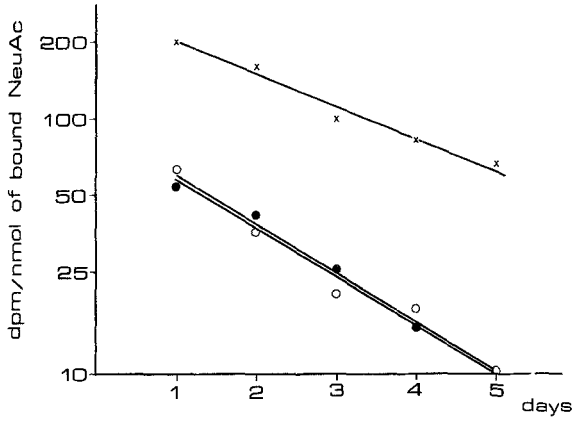

Figure 1. Time course of the specific radioactivities of bound NeuAc in rat liver, plasma and kidney after a single intraperitoneal injection of [^{3}H]ManNAc. Bound NeuAc was isolated and purified as described in the Materials and Methods section. Indicated are the days on which the rats were killed after the injection of [^{3}H]ManNAc. ○, liver; ●, plasma; ×, kidney.

NeuAc. The average half-life of bound NeuAc in kidney is 2.4 days (Fig. 1), which is in the same range as the half-lives of 16-72 h reported for total protein in kidney [24-28]. From the half-life of bound NeuAc and the total amount of NeuAc a synthesis rate for NeuAc of 0.4 nmol/min/g wet weight for kidney was calculated. In calculating these synthesis rates of NeuAc no correction was made for the blood content of the tissues. Such a correction would affect the calculated values only little, because the synthesis rate of NeuAc in liver is predominantly determined by the synthesis of NeuAc bound to plasma glycoproteins and in kidney the amount of bound NeuAc is approximately the same as the amount of bound NeuAc in plasma glycoproteins.

Incubation of Tissues Slices with [^{14}C]ManNAc

To determine whether kidney could use ManNAc as a substrate for NeuAc biosynthesis, we incubated kidney slices with [^{14}C]ManNAc and for comparison, liver slices. In kidney slices as well as in liver slices approximately 2.5% of the [^{14}C]ManNAc present in the medium was absorbed and metabolized in 2 h. Besides bound [^{14}C]NeuAc, the intermediates [^{14}C]ManNAc-6-phosphate, [^{14}C]NeuAc-9-phosphate and CMP-[^{14}C]NeuAc were formed (Table 1). In kidney slices more [^{14}C]GlcNAc and its derivatives were formed than in liver slices, although the activity of GlcNAc 2-epimerase in liver and in kidney is the same when measured *in vitro* [5].

In kidney slices an unknown product was detected, which behaved slightly more acidically than NeuAc on Dowex AG 1. Efforts to identify this product failed. The amount of NeuAc, synthesized from exogenous [^{14}C]ManNAc, calculated from the sum of dpm in

Table 1: Radioactively-labelled products after incubation of rat liver and kidney slices with [^{3}H]ManNAc. The nmoles of labelled products formed were calculated from the radioactivity of the products and the specific radioactivity of [^{3}H]ManNAc (15 Ci/mol).

The concentrations (nmol/g wet weight ± SEM) of NeuAc and of CMP-NeuAc *in vivo* in liver are 94 ± 9 (7 rats) and 34 ± 2 (4 rats) respectively (data taken from ref. 11) and in kidney 39 ± 2 (6 rats) and 48 ± 4 (6 rats) respectively as measured by the isotope-dilution technique.

	nmoles of labelled product/g wet weight formed in 2 h	
Product	Liver	Kidney
GlcNAc	18.0	55.6
GlcNAc-6-phosphate/GlcNAc-1-phosphate	2.6	5.2
UDP-GlcNAc	3.3	8.5
ManNAc-6-phosphate	8.6	4.8
NeuAc-9-phosphate	1.1	1.1
NeuAc	107.8	33.6
CMP-NeuAc	5.6	8.9
Bound NeuAc	10.2	0.6
Unidentified product	0	21.8

[^{14}C]NeuAc-9-phosphate, [^{14}C]NeuAc, CMP-[^{14}C]NeuAc and bound [^{14}C]NeuAc is 1.0 and 0.4 nmol/min/g wet weight for liver and kidney slices respectively. These values do not represent the real synthesis rates, because the contribution of endogenous ManNAc, resulting from ManNAc-forming enzymes was not measured. Furthermore, comparing the pool sizes *in vivo* (legend to Table 1) with the nmoles of labelled product in liver as well as in kidney slices, indicates that a disruption of the flux of [^{14}C]NeuAc into CMP-[^{14}C]NeuAc and bound [^{14}C]NeuAc in the slices had taken place. However, the values are indicative of a synthesis rate in the range of nmol/min/g wet weight and support evidence that ManNAc is converted into NeuAc in kidney tissue.

Turnover of CMP-NeuAc in Vivo

Intravenously injected [^{14}C]ManNAc was absorbed by the liver and the kidney and metabolized into [^{14}C]NeuAc, which was incorporated into glycoconjugates. The intermediates [^{14}C]ManNAc-6-phosphate, [^{14}C]NeuAc and CMP-[^{14}C]NeuAc were found in the liver and kidney. In kidney an unknown [^{14}C]-labelled product was formed, predominantly in the first minutes after the injection. This product showed the same characteristics as the unidentified product found after incubation of kidney slices with ManNAc. This product was never found after incubation of kidney extracts with ManNAc.

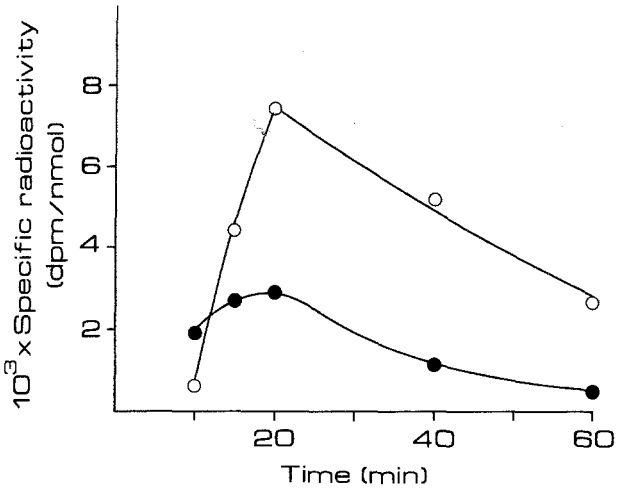

Figure 2. Time course of the specific radioactivities of free NeuAc and CMP-NeuAc in rat kidney after a single intravenous injection of [^{14}C]ManNAc. Free NeuAc and CMP-NeuAc were isolated and purified as described in the Materials and Methods section. The indicated times are the periods between the injection of [^{14}C]ManNAc and immersing the rats in liquid nitrogen. ●, NeuAc; ○, CMP-NeuAc.

Fig. 2 shows the specific radioactivities of free NeuAc and of CMP-NeuAc in kidney at various time intervals after the injection of [^{14}C]ManNAc. The maximal specific radioactivity of CMP-NeuAc became higher than that of free NeuAc, suggesting that in kidney newly synthesized NeuAc molecules are channelled to CMP-NeuAc synthase. This channelling was also observed in brain [29] and in liver [11]. In kidney the maximal specific radioactivities of NeuAc and CMP-NeuAc are more than twice as high as in liver. From the decrease in specific radioactivity of CMP-NeuAc between 20 and 50 min after the injection a half-life of CMP-NeuAc of 28 min was determined for kidney. The real half-life of CMP-NeuAc is slightly shorter, because during this time incorporation of radioactivity into CMP-NeuAc still continues. Using the pool size of CMP-NeuAc (legend to Table 1) it can be calculated that in kidney the synthesis rate of CMP-NeuAc is at least 1.2 nmol /min/g wet weight, which is approximately the same as was found for liver [11]. When the same experiments were performed with starved rats (rearing conditions as described by Ferwerda *et al.* [11] the half-life of CMP-NeuAc was 25 min and the pool size of CMP-NeuAc was 31 ± 3 nmol/g wet weight, resulting in a synthesis rate of CMP-NeuAc in kidney of 0.9 nmol/min/g wet weight, which is not significantly lower than was found in kidney of normal rats. If CMP-NeuAc hydrolase [30-32] is functionally active *in vivo* the synthesis rate of CMP-NeuAc will be greater than the incorporation rate of NeuAc.

Table 2: Activity of ManNAc kinase and UDP-GlcNAc 2-epimerase in the soluble cell fraction of rat liver and kidney. Enzyme activities are given in mU/mg of protein and in mU/g wet weight and are expressed as means ± SEM of 3 separate experiments. For liver the enzyme activities were previously determined using other incubation conditions [5].

Enzyme	Liver	Kidney
ManNAc kinase		
mU/mg protein	5.2 ± 1.7	0.020 ± 0.002
mU/g wet weight	283 ± 40	1.19 ± 0.07
UDP-GlcNAc 2-epimerase		
mU/mg protein	2.5 ± 0.8	0.0050 ± 0.0004
mU/g wet weight	136 ± 18	0.30 ± 0.05

Determination of the UDP-GlcNAc 2-Epimerase and ManNAc Kinase Activities in Vitro

In previous studies the activity of ManNAc kinase in kidney was below the detectable level, which was 10 nmol/min/g wet weight [5]. As described above the synthesis rate of NeuAc from ManNAc *in vivo* is much lower. To determined the corresponding low enzyme activity *in vitro* we used in this study ManNAc with a higher specific radioactivity and more concentrated tissue fractions. GlcNAc was added to the incubation mixture to suppress GlcNAc 2-epimerase activity as was previously shown [5]. However, GlcNAc did not completely suppress the GlcNAc 2-epimerase activity under the assay conditions used in the present study (results not shown). With this adapted assay we found in kidney a ManNAc kinase activity of 1.19 nmol/min/g wet weight (Table 2). When the ManNAc kinase activity in liver was measured with this adapted assay, 40% of the substrate was phosphorylated after incubation for 30 min, indicating that the adapted assay conditions were not optimal to determine liver ManNAc kinase activity. When measured under optimal conditions the ManNAc kinase activity in liver is 283 ± 40 nmol/min /g wet weight [5].

Because kidney can use ManNAc as a substrate for NeuAc biosynthesis *in vivo* and ManNAc kinase was detected *in vitro* a logical consequence was to look for UDP-GlcNAc 2-epimerase activity in kidney. If we used an assay system adapted in the same way as for ManNAc kinase, we could measure a UDP-GlcNAc 2-epimerase activity of 0.30 nmol/min/g wet weight in kidney (Table 2). In this assay concentrated tissue extracts were used, so it was important to determine whether the substrate UDP-[^{3}H]GlcNAc was diluted by endogenous UDP-GlcNAc. The UDP-GlcNAc (+ UDP-GalNAc) pool sizes, determined by the isotope-dilution technique were 357 ± 96 nmol/g wet weight and 107 ± 5 nmol/g wet weight for liver and kidney, respectively. However, during homogenization and centrifugation of both tissues, approximately 70% of the endogenous UDP-GlcNAc (+ UDP-GalNAc)was hydrolysed. Only the UDP-GlcNAc 2-epimerase activity in

Table 3: Synthesis rate of NeuAc or CMP-NeuAc in rat liver and kidney calculated by three different methods. The synthesis rates were calculated from: I, the half-life of CMP-NeuAc after injection of [^{3}H]ManNAc; II, total NeuAc and the turnover of sialoglycoconjugates; III, the lowest enzyme activity as measured *in vitro*.

	Synthesis rate (nmol/min/g wet weight)	
Method	Liver	Kidney
I	1.0[a]	1.2
II	2.7	0.4
III	36[b]	0.3

[a] Data taken from Ferwerda *et al.* [9]
[b] NeuAc-9-phosphate synthase activity [6]

kidney needed a small correction for the dilution of UDP-[^{3}H]GlcNAc by the residual endogenous UDP-GlcNAc (not corrected for UDP-GalNAc content). As for ManNAc kinase, the adapted assay conditions were not optimal for the determination of UDP-GlcNAc 2-epimerase activity in liver. When measured under optimal conditions the UDP-GlcNAc 2-epimerase activity in liver was 136 ± 18 nmol/min/g wet weight [5].

In kidney the UDP-GlcNAc 2-epimerase activity is low as compared to the ManNAc kinase activity (Table 2) and other enzymes involved in the pathway of NeuAc [5,6]. However, when using concentrated tissue extracts endogenous CMP-NeuAc was detected in the incubation mixture, which inhibits the epimerase activity [33-35]. In contrast to UDP-GlcNAc, only 1-2% of the endogenous CMP-NeuAc is hydrolysed during homogenization and centrifugation of the tissues. From the pool sizes of CMP-NeuAc (legend to Table 1) it could be calculated that the concentration of CMP-NeuAc in the incubation mixture was approximately 10 μM, which is sufficient to cause some inhibition of liver UDP-GlcNAc 2-epimerase [35]. When the adapted assay conditions were used to determine the UDPGlcNAc 2-epimerase and ManNAc kinase activity in spleen, we found 2.1 ± 0.1 and 2.6 ± 0.5 nmol/min/g wet weight respectively. For brain these activities were 0.21 ± 0.02 and 0.6 ± 0.1 nmol/min/g wet weight respectively. The UDP-GlcNAc 2-epimerase activity in both tissues was not corrected for dilution of the substrate by endogenous UDP-GlcNAc.

The major objective of this study was to investigate the biosynthetic pathway of NeuAc in kidney, because in previous experiments we could not detect UDP-GlcNAc 2-epimerase and ManNAc kinase activity *in vitro* [5]. From our turnover studies and incubation of tissue slices it could be calculated that, in kidney, NeuAc could be synthesized from ManNAc in a rate comparable to that in liver. With the adapted assays both UDP-GlcNAc 2-epimerase and ManNAc kinase activity could now be established. In this respect the activity of UDP-GlcNAc 2-epimerase is rather low (Table 2), while for the presence of this enzyme no support is obtained from our experiments *in vivo* and slices experiment. ManNAc can also be formed by epimerization of GlcNAc [36]. From our experiments we cannot exclude that ManNAc is synthesized from GlcNAc, but precautions were taken

to prevent the formation of GlcNAc from UDP-GlcNAc and the conditions for GlcNAc 2-epimerase were not optimal. Therefore, we concluded that in the UDP-GlcNAc 2-epimerase assay, ManNAc was directly formed from UDP-GlcNAc. This means that UDP-GlcNAc can be a precursor for NeuAc biosynthesis and the pathway of NeuAc biosynthesis in kidney is the same as in liver. For spleen and brain a similar pathway is suggested. The synthesis rate of NeuAc from ManNAc in liver *in vivo* (Table 3) is in the same range as the activity of 3 nmol/min/g wet weight for GlcN-6-phosphate synthase *in vivo* [37,38] and the transfer rate of *N*-acetylhexosamine from UDP-GlcNAc into sialoglycoproteins is 2.6-2.9 nmol/min/g wet weight [39]. However, it must be considered that UDP-GlcNAc is not only the precursor for NeuAc, but it can also be utilized for the transfer of GlcNAc and *N*-acetylgalactosamine into glycoconjugates. When the NeuAc (precursor-) forming enzymes were assayed in soluble cell fractions from liver under optimal conditions [5,6], NeuAc-9-phosphate synthase showed the lowest activity. The maximal potential rate for NeuAc biosynthesis obtained by extrapolation of this enzyme activity to the activity *in vivo* is 36 nmol/min/g wet weight, which is much greater than the actual synthesis rate of NeuAc *in vivo* (Table 3). This indicates that normally in liver only 10% or less of the maximal synthesis capacity is expressed. GlcN-6-phosphate synthase operates in liver also at 10% of its maximal rate (7,38].

From these results it appears that in contrast to kidney the regulation of NeuAc biosynthesis is exceptionally extensive in liver. This could be related to the specialized function of the liver, i.e. the biosynthesis of plasma glycoproteins. Probably such a regulation mechanism also exists in other sialoglycoprotein-secreting tissues and not, or to a lesser extent, in non-sialoglycoprotein secreting tissues.

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