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Subcellular localization and tissue distribution of sialic acid precursor-forming enzymes

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The enzymes UDP-*N*-acetylglucosamine pyrophosphorylase, UDP-*N*-acetylglucosamine 2-epimerase, *N*-acetylmannosamine kinase, *N*-acetylglucosamine kinase and *N*-acetylglucosamine 2-epimerase, which are involved in the metabolism of *N*-acetylneuraminic acid, were studied in rat with regard to their subcellular localization and tissue distribution. The subcellular distribution studies in liver indicated that the enzymes are localized in the soluble cell fraction. In other tissues the comparison of enzyme activities in homogenates with that in high-speed supernatants led to a similar conclusion. UDP-*N*-acetylglucosamine pyrophosphorylase, *N*-acetylglucosamine kinase and *N*-acetylglucosamine 2-epimerase were detected in almost all tissues studied. UDP-*N*-acetylglucosamine 2-epimerase and *N*-acetylmannosamine kinase, two enzymes considered to be key enzymes in the *N*-acetylneuraminic acid biosynthesis, were detected only in sialoglycoprotein-secreting tissues, i.e. liver, salivary gland and intestinal mucosa. The low activity of the key enzymes in other tissues suggests that the biosynthetic pathway of *N*-acetylneuraminic acid is not the same in various tissues.

N-Acetylneuraminic acid (NeuAc) is an important constituent of the carbohydrate chain of many glycoproteins and glycolipids. The biosynthesis of NeuAc has been studied extensively *in vivo* and *in vitro* (for reviews, see Warren, 1972; McGuire, 1976). The enzymes participating in the metabolism of NeuAc appear to be compartmentalized in the cell. The synthesis of NeuAc from UDP-GlcNAc is thought to be cytosol-mediated. The NeuAc-activating enzyme, CMP-NeuAc synthetase (CTP:*N*-acetylneuraminic acid cytidylyltransferase, EC 2.7.7.43), has been found in the nucleus of many tissues (Kean, 1970; Gielen *et al.*, 1970, 1971; Van den Eijnden, 1973; Van Dijk *et al.*, 1973; Coates *et al.*, 1980). CMP-NeuAc appears to be used in the Golgi system, since the sialyltransferases are mainly present in this organelle (Schachter, 1974; Carey & Hirschberg, 1980, 1981; Creek & Morré, 1981). This compartmentalization may be part of a regulation mechanism. The cytosolic localization of the NeuAc precursor-forming enzymes is mainly based on its easy extractability with buffers and salt solutions. However, to our knowledge, a detailed study of the subcellular localization of NeuAc precursor-forming enzymes has never been published, and a connection between these enzymes and the nuclear CMP-NeuAc synthetase has not been investigated. Furthermore, the generally accepted pathway for the biosynthesis of NeuAc from UDP-GlcNAc (Fig. 1) has only been established for rat liver. In other tissues the activity of various enzymes has never been reported, whereas the existence of some enzymes is questionable (McGuire, 1976).

As shown in Fig. 1, two pathways are available for the synthesis of NeuAc. The significance of these routes is not known, since only few comparative studies have been carried out. The present study demonstrates the cytosolic localization of UDP-GlcNAc pyrophosphorylase (UTP:2-acetamido-2-deoxy-D-glucose 1-phosphate uridylyltransferase, EC 2.7.7.23), UDP-GlcNAc 2-epimerase (UDP-2-acetamido-2-deoxy-D-glucose 2-epimerase, EC 5.1.3.14), ManNAc kinase (ATP:2-acylamino-2-deoxy-D-mannose 6-phosphotransferase, EC 2.7.1.60), GlcNAc kinase (ATP:2-acetamido-2-deoxy-D-glucose 6-phosphotransferase, EC 2.7.1.59) and GlcNAc 2-epimerase (2-acylamido-2-deoxy-D-glucose 2-epimerase, EC 5.1.3.8) in rat liver. Moreover, the activities of these enzymes in 11 other tissues are presented, showing considerable variations in metabolic capacity.

Part of this work has been presented in preliminary form (Van Rinsum *et al.*, 1981).

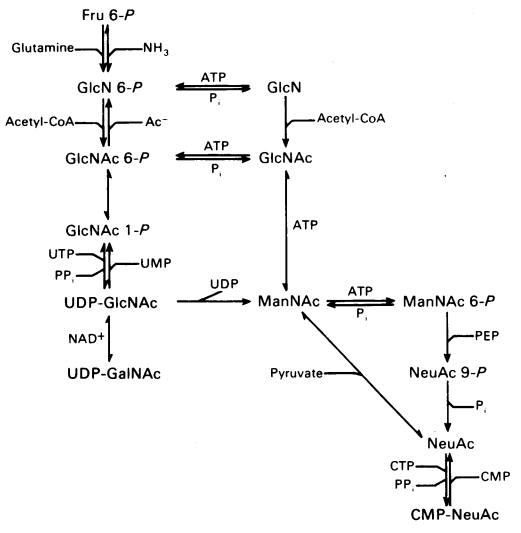

Fig. 1. *Biosynthetic pathway for NeuAc in mammals (Warren, 1972)*

Non-standard abbreviations used: 6-*P*, 6-phosphate; 1-*P*, 1-phosphate; PEP, phosphoenolpyruvate; Ac^-, acetate.

Materials and methods

The following materials were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.: UDP-*N*-acetylglucosamine, *N*-acetylglucosamine, *N*-acetylmannosamine, ATP (disodium salt) and acid phosphatase from potatoes. CTP (disodium salt) was obtained from Merck, Darmstadt, Germany, and 5'-AMP from Boehringer Mannheim G.m.b.H., Mannheim, Germany. *N*-Acetylneuraminic acid was purchased from Koch–Light Laboratories, Colnbrook, Bucks., U.K. *N*-Acetyl-D-[1-^{14}C]glucosamine (sp. radioactivity 60.8 Ci/mol) and *N*-acetyl-D-[U-^{14}C]mannosamine (sp. radioactivity 254 Ci/mol) were products from The Radiochemical Centre, Amersham, Bucks., U.K. UDP-*N*-acetyl-D-[1-^{14}C]glucosamine (sp. radioactivity 250 Ci/mol) and *N*-acetyl-D-[6(n)-^{3}H]mannosamine (sp. radioactivity 19000 Ci/mol) were obtained from New England Nuclear, Boston, MA, U.S.A. *N*-[^{14}C]Acetylneuraminic acid (sp. radioactivity 6 Ci/mol) was prepared by the method of Warren & Glick (1966). Dowex AG 1 (X8; 100–200 mesh; Cl^- form) was purchased from Bio-Rad, Richmond, CA, U.S.A., and was converted into the HCO_3^- form as recommended by the manufacturer. All other chemicals were obtained from commercial sources and were of analytical grade. Adult male rats (Wistar strain), weighing 175–200 g, were used. The rats were starved for 24 h before use. They were killed by decapitation, always between 09:00 h and 10:00 h.

Subcellular fractionation of rat liver

The livers of the killed rats were rapidly removed and chilled. All subsequent procedures were conducted at 4°C. The livers were minced with scissors, washed with 0.25 M-sucrose in water and suspended in 9 vol. of a solution containing 0.25 M-sucrose and 10 mM-4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (Hepes), pH 7.5. The suspension was homogenized in a motor-driven glass/Teflon Potter–Elvehjem type homogenizer (clearance 0.25 mm) for 3 × 5 s at 1000 rev./min. The crude homogenate was filtered through nylon gauze (440 μm) and centrifuged at 90 *g* for 5 min. The pellet was discarded. The supernatant, which is the cell-free homogenate, was centrifuged at 1000 *g* for 5 min, yielding a pellet and a supernatant S_1. The pellet was rehomogenized in 9 vol. of 2.2 M-sucrose containing 5 mM-Hepes, pH 7.5, and 3 mM-$MgCl_2$, and was centrifuged at 40000 *g* for 60 min in a swing-out rotor. The pellet was washed in 0.25 M-sucrose containing 5 mM-Hepes, pH 7.5, and 3 mM-$MgCl_2$ (1000 *g* for 5 min) yielding pellet P_1. P_1 was resuspended in 1 vol. of the same solution.

The supernatant S_1 was centrifuged subsequently at 40000, 200000 and 10800000 *g*-min, yielding the pellets P_2, P_3 and P_4 respectively and the final supernatant S_4. The pellets were washed in 0.25 M-sucrose containing 10 mM-Hepes, pH 7.5, and 1 mM-EDTA. The washes were discarded and the pellets were resuspended in 4 vol. of 0.25 M-sucrose in water.

The composition of the subcellular rat liver fractions was checked by the distribution of DNA and RNA (Van den Eijnden *et al.*, 1972) and the activity of lactate dehydrogenase (EC 1.1.1.27), 5'-nucleotidase (EC 3.1.3.5), glucose 6-phosphatase (EC 3.1.3.9), β-glucosidase (EC 3.2.1.21) (Lisman *et al.*, 1979) and succinate dehydrogenase (EC 1.3.99.1) (Pennington, 1961) in four separate experiments.

Preparation of tissue homogenates and soluble cell fractions

The rats were decapitated and the various tissues were rapidly removed and chilled. All subsequent procedures were conducted at 4°C. Blood was collected in a 1.5% sodium citrate solution, blood cells were spun down and are designated as erythrocytes. The submaxillary gland with the embedded sublingual gland were removed and investigated together and are referred to as salivary gland. The small intestine was rinsed out with 0.15 M-NaCl and the mucosa was scraped off. The musculus biceps femoris was removed.

The tissues were minced with scissors and washed with 0.25 M-sucrose in water. All tissues were suspended in 9 vol. of a solution containing 0.25 M-sucrose and 10 mM-Hepes, pH 7.5. The suspensions

were homogenized in a motor-driven glass/Teflon Potter–Elvehjem-type homogenizer (clearance 0.10mm) for 3 × 5s at 1000rev./min. The crude homogenates were filtered through nylon gauze (440μm) and centrifuged at 180000**g** for 60min yielding the tissue soluble cell fractions.

Enzyme assays

Incubations were carried out immediately after the preparation of the various subcellular or tissue fractions. All incubations were carried out at 37°C for 30min. The reactions were stopped by heating for 3min at 100°C. An incubation mixture was centrifuged, the precipitate was washed three times with water and the combined supernatants were applied to a Dowex Ag 1 (X8; 100–200 mesh; HCO_3^- form) column (4.0cm × 0.5cm) to separate the products (see under 'Separation of products and substrates'). One unit of enzyme activity was defined as the amount of protein catalysing the formation of 1μmol of product per min at 37°C.

UDP-GlcNAc pyrophosphorylase

The assay system consisted of 0.1M-Tris/HCl, pH7.4, 2.0mM-UDP-[^{14}C]GlcNAc (sp. radioactivity 0.23Ci/mol), 25mM-sodium pyrophosphate, 2mM-$MgCl_2$, 20mM-AMP and 50μl of tissue fraction (0.1–0.2mg of protein) in a total volume of 120μl. AMP suppressed the endogenous UDP-GlcNAc pyrophosphatase activity (Pattabiraman *et al.*, 1964). We used two controls, one lacking the tissue fraction and one lacking sodium pyrophosphate. The latter was used to make a correction for the residual activity of UDP-GlcNAc pyrophosphatase. The products formed were [^{14}C]GlcNAc 1-phosphate, partly isomerized by an endogenous mutase to [^{14}C]GlcNAc 6-phosphate, [^{14}C]GlcNAc derived from the phosphate esters after the action of endogenous phosphatases and [^{14}C]ManNAc formed by the action of endogenous UDP-GlcNAc 2-epimerase on UDP-[^{14}C]GlcNAc.

The first eluate of the Dowex AG 1 column, containing [^{14}C]ManNAc and [^{14}C]GlcNAc, was freeze-dried and the residue was subjected to high-voltage electrophoresis to separate these components (see below). The UDP-GlcNAc pyrophosphorylase activity was calculated from the sum of counts found in the phosphate esters and in GlcNAc.

UDP-GlcNAc 2-epimerase

The assay was the same as that described for UDP-GlcNAc pyrophosphorylase, only lacking sodium pyrophosphate. Control incubation mixtures lacked the tissue fraction or contained heat-denatured tissue fraction. The separation of the products was the same as described for the UDP-GlcNAc pyrophosphorylase assay. After high-voltage electrophoresis the radioactivity found in the ManNAc region was determined, and used for the calculation of the UDP-GlcNAc 2-epimerase activity.

ManNAc kinase and GlcNAc kinase

We measured simultaneously the ManNAc kinase and GlcNAc kinase activity. The incubation system consisted of 0.1M-Tris/HCl, pH8.0, 5mM-[^{14}C]GlcNAc (sp. radioactivity 0.075Ci/mol), 5mM-[^{3}H]ManNAc (sp. radioactivity 0.15Ci/mol), 15mM-ATP, 15mM-$MgCl_2$, 25mM-NaF and 50μl of tissue fraction (0.1–0.2mg of protein) in a total volume of 120μl. NaF suppressed the phosphatase activity present in the crude tissue fractions (Maxwell *et al.*, 1962). Control incubation mixtures lacked the tissue fraction, or contained heat-denatured tissue fraction. The ManNAc kinase and GlcNAc kinase activity was calculated from the ^{3}H label and ^{14}C label respectively in the 6-phosphate ester fraction after Dowex passage. A correction was made for the 1% of [^{3}H]GlcNAc present in the [^{3}H]ManNAc batch we used.

GlcNAc 2-epimerase

The incubation system consisted of 0.1M-Tris/HCl, pH8.0, 5mM-[^{14}C]ManNAc (sp. radioactivity 0.075Ci/mol), 15mM-ATP, 15mM-$MgCl_2$, 25mM-NaF and 50μl of tissue fraction (0.1–0.2mg of protein) in a total volume of 120μl. The water eluent of the Dowex column was freeze-dried and subjected to high-voltage electrophoresis to separate [^{14}C]ManNAc and [^{14}C]GlcNAc. The 6-phosphate ester fraction was evaporated, the 6-phosphates were dephosphorylated and subjected to high-voltage electrophoresis (Kundig *et al.*, 1966). The GlcNAc 2-epimerase activity was calculated from the sum of the [^{14}C]GlcNAc and [^{14}C]GlcNAc 6-phosphate radioactivity. A correction was made for the 2.5% of [^{14}C]GlcNAc present in the [^{14}C]ManNAc batch we used.

CMP-NeuAc synthetase

The incubation conditions used for the determination of the CMP-NeuAc synthetase activity were the same as described by Van den Eijnden *et al.* (1972), with [^{14}C]NeuAc (sp. radioactivity 0.045Ci/mol) as substrate. The CMP-NeuAc synthetase activity was calculated from the CMP-[^{14}C]NeuAc radioactivity.

Separation of products and substrates

Separation of the various compounds was achieved on Dowex AG 1 as described previously (Ferwerda *et al.*, 1981*a*), using small columns (4.0cm × 0.5cm) with discontinuous salt elution. The supernatant of an incubation mixture was applied to the column followed by 10ml of water

and a wash of 10ml of 5mM-triethylammonium bicarbonate (Teb) buffer, pH7.8. All subsequent elutions and washings were carried out with 10ml of Teb buffer, pH7.8. The Teb buffers were prepared from a stock Teb solution, which was adjusted to pH7.8 by passing CO_2 over the solution in a closed vessel with constant stirring at 4°C. The Teb concentrations by which elution was achieved were: for NeuAc, 40mM; for monophosphate esters, 100mM; for CMP-NeuAc, 150mM; for UDP-GlcNAc, 300mM; and for NeuAc 9-phosphate, 1000mM. Neutral compounds, such as ManNAc and GlcNAc, were found in the water eluate. To minimize errors resulting from 'tailing' effects, an extra washing step was often introduced after the elution of a compound.

High-voltage electrophoresis was performed on Whatman 3MM paper strips in a 1% sodium tetraborate solution at 5–10°C with 65V/cm for 60min, and with Bromophenol Blue as a marker. After electrophoresis, the strip was cut into 1cm pieces and the radioactivity was counted (R_F of GlcNAc, −0.07; R_F of ManNAc, 0.50) (Jourdian & Roseman, 1962).

Analytical methods

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin solutions in 0.25M-sucrose as standards. Radioactivity was determined with a Berthold liquid-scintillation counter (BF 8000), equipped with an automatic external standard for calculation of the disintegrations/min. The scintillation fluid used was Maxifluor A (Baker Chemicals BV, Deventer, Holland). Efficient counting of the cut paper strips was achieved by adding 1ml of water before the addition of the scintillation fluid.

Results and discussion

Enzyme assays

When the activity of an enzyme, which is part of a biosynthetic pathway, is determined in a crude tissue fraction, it sometimes implies that the substrate is also a substrate for another endogenous enzyme. On the other hand, formed products can be substrates for other enzymes present in the crude tissue fractions. In the past, the NeuAc precursor-forming enzyme activities have mainly been determined by colorimetric assays, by which the effects of interfering enzymes are difficult to establish.

In our assays we tried to suppress the hydrolysis of substrate and/or products by endogenous pyrophosphatases, ATPases and non-specific phosphatases by using special inhibitors. Furthermore, we used radioactive substrates, so all formed products were detected, separated and analysed. When possible, we carried out control experiments to detect residual activities of interfering enzymes.

UDP-GlcNAc is a precursor for NeuAc as well as a precursor for GlcNAc transfer to glycoconjugates. UDP-GlcNAc pyrophosphorylase catalyses the reversible reaction by which UDP-GlcNAc is formed (Strominger & Smith, 1959). We determined the enzyme activity by measuring the pyrophosphorolytic cleavage of UDP-GlcNAc. The residual pyrophosphatase activity was 0.1–0.6m-units/mg of protein for homogenates. When soluble cell fractions were tested no residual pyrophosphatase activity was detected.

UDP-GlcNAc 2-epimerase probably possesses a key position in NeuAc biosynthesis, because it catalyses the irreversible formation of ManNAc, and its sensitivity to feedback inhibition by CMP-NeuAc (Kornfeld *et al.*, 1964; Sommar & Ellis, 1972; Kikuchi *et al.*, 1973).

The hydrolysis of the substrate UDP-GlcNAc by endogenous pyrophosphatase would yield GlcNAc 1-phosphate, which in turn is a substrate for endogenous phosphatases yielding GlcNAc. By subsequent action of endogenous GlcNAc 2-epimerase on GlcNAc yielding ManNAc, false positive results would be obtained. The pyrophosphatase activity was suppressed by AMP; the residual activity was the same as that described above.

Besides the UDP-GlcNAc 2-epimerase activity we measured the GlcNAc 2-epimerase activity to determine whether the alternative pathway of NeuAc biosynthesis, as has been suggested by Datta (1970), is possible in various tissues. The enzyme was assayed in the presence of ATP, which exerts a stimulatory effect on the enzyme (Ghosh & Roseman, 1965*b*; Datta, 1970). To suppress ATPase activity, present in the crude fractions, NaF was added. Without NaF we found a slightly lower activity of GlcNAc 2-epimerase (results not shown).

The ManNAc kinase assay is complex, because of the epimerization of the substrate ManNAc to GlcNAc by GlcNAc 2-epimerase, followed by GlcNAc kinase action yielding GlcNAc 6-phosphate. GlcNAc 6-phosphate can only be distinguished from ManNAc 6-phosphate after treatment with phosphatase and subsequent separation by high-voltage electrophoresis (Kundig *et al.*, 1966). In our kinase assay we suppressed the epimerization of ManNAc by the addition of GlcNAc. One might expect that the epimerization is not completely suppressed, resulting in values for ManNAc kinase that are somewhat too high, especially in tissues with low ManNAc kinase activity and high GlcNAc kinase activity. Determination of the ManNAc kinase activities in soluble cell fractions of various rat tissues by the assay described by Kundig *et al.* (1966) and by our assay resulted in the same activities (results not shown). It was concluded that the

suppression of GlcNAc 2-epimerase activity by GlcNAc in our assay was sufficient, and also that ManNAc kinase was not inhibited by GlcNAc or by GlcNAc 6-phosphate.

Using [^{14}C]GlcNAc and [^{3}H]ManNAc in our assay we determined the kinase activities in a time-saving test. Although the role of GlcNAc kinase in NeuAc biosynthesis is indirect, we wanted to compare the ManNAc and GlcNAc kinase activities and could check the incubation conditions especially in respect to endogenous phosphatase activity. Phosphatase action is not only on the products ManNAc 6-phosphate and GlcNAc 6-phosphate, but also on the substrate ATP. The formation of ADP could inhibit the kinase activity (Allen & Walker, 1980*b*). We suppressed the phosphatase activity by NaF (Maxwell *et al.*, 1962). When liver homogenates were tested in the absence of NaF, both ManNAc and GlcNAc kinase activities were four times lower. However, the recovery of the kinase activities in soluble cell fractions from corresponding homogenates exceeded 100%, suggesting that the phosphatase activity in homogenates was not completely inhibited. For liver soluble cell fractions, the effect of NaF was negligible, indicating the virtual absence of phosphatase activity from these fractions (results not shown).

Subcellular localization

The distribution of the various biochemical markers (Table 1) suggests that pellet P_1 consisted mainly of nuclei with hardly any plasma-membrane contamination. Pellet P_2 contained mitochondria with some plasma-membrane and nuclear contamination. Pellet P_3 contained the lysosomes and some rough endoplasmic reticulum. Rough and smooth endoplasmatic reticulum were present in pellet P_4. The supernatant S_4 was almost devoid of subcellular particles. Our aim was to prepare particulate fractions almost devoid of plasma membranes, which contain hydrolysing-enzyme activities. This was achieved by washing the pellets. Because of the lability of some NeuAc precursor-forming enzymes, we required a rapid method, so the washes were not characterized. However, 39% of the protein was recovered in these washes, which explains the low recoveries of markers in the fractions.

From the results shown in Fig. 2 it appears that the NeuAc precursor-forming enzymes are localized in the soluble cell fraction. This fraction contained 96% or more of the total enzyme activity recovered. The nuclear fraction contained 2% or less.

UDP-GlcNAc pyrophosphorylase is a cytosolic enzyme (Fig. 2), as has been shown for other sugar-nucleotide synthetases (Coates *et al.*, 1980). In contrast, CMP-NeuAc synthetase activity was predominantly localized in the nuclear fraction, which is in agreement with the results reported by others (Kean, 1970; Gielen *et al.*, 1970, 1971; Van den Eijnden, 1973; Van Dijk *et al.*, 1973; Coates *et al.*, 1980). No CMP-NeuAc synthetase activity was detected in the soluble cell fraction. This might be due to our mild homogenization technique resulting in few broken nuclei or to inactivation of the non-nucleus-bound enzyme by sucrose (Van den Eijnden, 1973). We did not dialyse our fractions, because sucrose did not influence our radioactive assay. Other authors, who used the thiobarbituric acid assay, had to remove the sucrose, which interferes with this colorimetric assay.

Ferwerda *et al.* (1981*a*,*b*) injected rats with [^{3}H]ManNAc and found that the specific radioactivity of CMP-NeuAc in brain and liver was higher than that of NeuAc in the first hours after

Table 1. *Percentage distribution of marker enzyme activities, nucleic acids and protein in subcellular fractions of rat liver*
Homogenates were fractionated as described in the text. Values are expressed as means ± S.E.M. of four to six separate experiments in which the markers were determined in duplicate. Recoveries represent the sum of the concentrations of a marker in the fractions relative to the concentration in the crude homogenate. Percentage distributions are given for each of the markers in a fraction as percentage of the total in the five fractions.

Marker	Recovery (%)	Distribution (%)				
		P_1	P_2	P_3	P_4	S_4
DNA	82 ± 8	67 ± 8	17 ± 2	7 ± 3	5 ± 4	3 ± 3
5′-Nucleotidase	58 ± 9	11 ± 4	38 ± 9	17 ± 3	27 ± 7	7 ± 1
Succinate dehydrogenase	49 ± 3	2 ± 1	85 ± 1	3 ± 1	3 ± 1	7 ± 1
β-Glucosidase	44 ± 14	7 ± 1	15 ± 2	55 ± 10	14 ± 5	9 ± 3
RNA	75 ± 11	10 ± 2	9 ± 2	13 ± 2	41 ± 3	27 ± 3
Glucose 6-phosphatase	54 ± 7	9 ± 2	7 ± 2	25 ± 2	57 ± 5	4 ± 2
Lactate dehydrogenase	79 ± 9	0	1 ± 1	3 ± 1	14 ± 3	82 ± 3
Protein	56 ± 4	3 ± 1	31 ± 3	4 ± 1	11 ± 2	51 ± 4

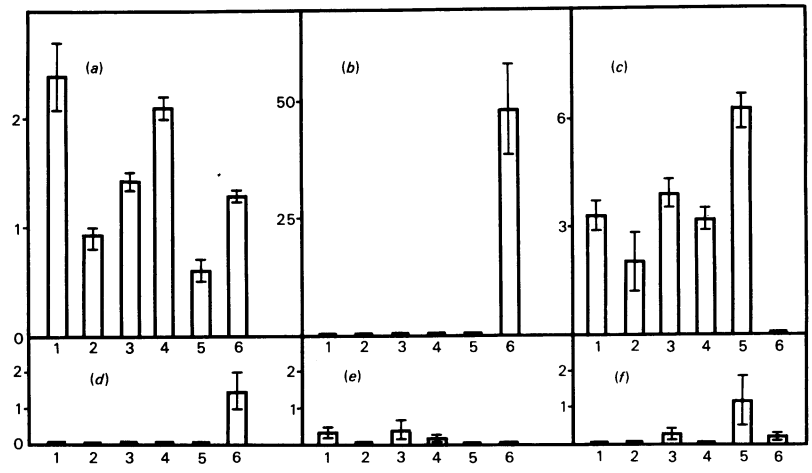

Fig. 2. *Distribution of some NeuAc precursor-forming enzymes and CMP-NeuAc synthetase in subcellular rat liver fractions prepared by differential centrifugation*

(*a*) The cell-free homogenate; (*b*) nuclei (P_1); (*c*) soluble cell fraction (S_4); (*d*) mitochondria (P_2); (*e*) lysosomes and rough endoplasmatic reticulum (P_3); (*f*) microsomes (P_4). The numbers on the abscissae indicate the enzymes: 1, UDP-GlcNAc pyrophosphorylase; 2, UDP-GlcNAc 2-epimerase; 3, ManNAc kinase; 4, GlcNAc kinase; 5, GlcNAc 2-epimerase; 6, CMP-NeuAc synthetase. The numbers on the ordinates indicate the enzyme activities: (*a*), m-units/mg of protein; (*b*)–(*f*), specific activities of the subcellular fractions relative to the cell-free homogenate. Values are expressed as means ± S.E.M. (represented by the bars) of three separate experiments.

Table 2. *Specific activity of some NeuAc precursor-forming enzymes in the soluble cell fraction of various rat tissues*

For experimental details see the Materials and methods section. Enzyme activities are given in m-units/mg of protein and are expressed as means ± S.E.M. of three separate experiments, except for the thyroid gland. Total enzyme activities recovered in the soluble cell fractions from the corresponding homogenates are given in parentheses and are expressed as percentages of homogenate values. Activities preceded by < are below the detectable level.

Tissue	UDP-GlcNAc pyrophosphorylase	UDP-GlcNAc 2-epimerase	ManNAc kinase	GlcNAc kinase	GlcNAc 2-epimerase
Liver	8.4 ± 2.3 (112)	2.5 ± 0.8 (99)	5.2 ± 1.7 (123)	5·9 ± 1.2 (134)	2.3 ± 0.6 (102)
Kidney	5.7 ± 0.5 (102)	< 0.1	< 0.2	14.3 ± 0.5 (267)	2.3 ± 0.3 (165)
Spleen	3.8 ± 0.4 (78)	< 0.1	0.3 ± 0.1 (281)	6.0 ± 0.9 (744)	1.4 ± 0.2 (63)
Brain	6.9 ± 0.4 (92)	< 0.1	< 0.2	5.5 ± 0.4 (190)	1.1 ± 0.2 (60)
Lung	7.6 ± 2.0 (106)	< 0.1	< 0.2	4.9 ± 1.2 (315)	0.4 ± 0.2 (45)
Muscle	2.5 ± 0.3 (87)	< 0.1	< 0.2	0.4 ± 0.3 (32)	< 0.3
Erythrocytes	0.3 ± 0.2 (63)	< 0.1	< 0.2	0.6 ± 0.5 (143)	< 0.3
Intestinal mucosa	15.3 ± 0.7 (138)	0.3 ± 0.1 (33)	0.9 ± 0.2 (146)	12.0 ± 1.4 (443)	0.4 ± 0.2 (34)
Salivary gland	22.7 ± 2.1 (100)	0.7 ± 0.1 (32)	3.5 ± 0.8 (260)	9.8 ± 1.3 (693)	1.7 ± 0.3 (105)
Thymus	6.7 ± 2.0 (200)	< 0.1	< 0.2	4.1 ± 0.4 (243)	1.5 ± 0.1 (58)
Pancreas	13.1 ± 2.2 (106)	< 0.1	< 0.2	7.1 ± 2.7 (172)	0.9 ± 0.5 (145)
Thyroid gland	5.3 ; 7.7 (99)	< 0.1	< 0.2	6.0 ; 3.8 (109)	0.8 ; 1.2 (47)

injection. From these results it was suggested that two NeuAc pools exist, one pool representing newly synthesized NeuAc and another pool of NeuAc derived from catabolized sialoglycoconjugates. The newly synthesized NeuAc may be channelled into the nucleus, where the CMP-NeuAc synthetase is localized. In view of these suggestions it is possible that the cytosolic NeuAc precursor-forming enzymes, although soluble after homogenization, are organized in the cytosol in connection with the nucleus.

Tissue distribution

The distribution of NeuAc precursor-forming enzymes in various rat tissues is presented in Table 2.

All tissues contained UDP-GlcNAc pyrophos-

phorylase and GlcNAc kinase activity. The UDP-GlcNAc pyrophosphorylase activity in the soluble cell fraction of rat liver is of the same order of magnitude as the activity in a crude calf liver extract (Strominger & Smith, 1959). For rat brain the enzyme activity is four times higher than reported for a crude sheep brain extract (Pattabiraman & Bachhawat, 1961).

GlcNAc kinase activity has been detected in various rat tissues (Leloir *et al.*, 1958; Kikuchi & Tsuiki, 1979; Allen & Walker, 1980*a*,*b*; Allen *et al.*, 1980). The activities in crude tissue fractions detected by various authors show much variation, possibly due to different assays. However, rat kidney showed the highest activity. Our results (Table 1) are in agreement with these observations.

In view of the recoveries of UDP-GlcNAc pyrophosphorylase and GlcNAc kinase activity in the soluble cell fractions from the corresponding homogenates (Table 2), a cytosolic localization of these enzymes in the various tissues is likely.

The activity of UDP-GlcNAc 2-epimerase was relatively high in liver; moderate activity was detected in salivary gland and intestinal mucosa (Table 2). This enzyme has mainly been investigated in normal liver, hepatomas and regenerating rat liver (Comb & Roseman, 1958; Kornfeld *et al.*, 1964; Spivak & Roseman, 1966; Salo & Fletcher, 1970; Kikuchi & Tsuiki, 1971, 1979; Sommar & Ellis, 1972; Kikuchi *et al.*, 1973; Harms *et al.*, 1973; Okamoto & Akamatsu, 1980). Only Kikuchi & Tsuiki (1979) presented a value for the enzyme activity in rat kidney. Our results are in good agreement with the results presented in the literature for liver and kidney. The virtual absence of this enzyme from all other tissues investigated is noteworthy. These low activities are not due to epimerization of the product ManNAc, yielding GlcNAc, because after testing soluble cell fractions of these tissues, neither ManNAc nor GlcNAc was detected.

The ManNAc kinase activity is relatively high in liver and salivary gland, low in intestinal mucosa and below the detectable level in other tissues. ManNAc kinase has mainly been studied in liver and salivary gland (Ghosh & Roseman, 1961; Warren & Felsenfeld, 1961, 1962; Kundig *et al.*, 1966; Kikuchi & Tsuiki, 1971; Okamoto & Akamatsu, 1980). Only Kundig *et al.* (1966) determined ManNAc kinase in a number of tissues, including rat tissues. Our results are comparable with those reported by them. When no UDP-GlcNAc 2-epimerase or ManNAc kinase activity was detected in the soluble cell fractions (Table 2) the activities in the corresponding tissue homogenates were also undetectable (results not shown). Therefore, these enzyme activities are not associated with membranes in these tissues. A cytosolic localization of these enzymes, if present, seems likely, in spite of the low recoveries found for UDP-GlcNAc 2-epimerase. This is probably due to inactivation of the enzyme, which is known to be extremely unstable (Salo & Fletcher, 1970; Sommar & Ellis, 1972). Stabilizing agents, such as uridine or dithiothreitol (Sommar & Ellis, 1972), were not added during preparation of the fractions. It is noteworthy that glycoprotein-secreting tissues especially contain UDP-GlcNAc 2-epimerase and ManNAc kinase activity in detectable amounts. Moreover, the activities of these two enzymes seem to be correlated.

The enzyme activities were determined on 24h-starved rats. As a consequence, the capacity of synthesizing glycoproteins could have been decreased. Pilot experiments on fed rats indeed showed a slight increase of UDP-GlcNAc 2-epimerase and ManNAc kinase in liver and intestinal mucosa (results not shown). However, in kidney, spleen, brain and pancreas of the fed rats the enzyme activities were still below the detectable level.

The low activity of UDP-GlcNAc 2-epimerase and ManNAc kinase in non-secreting tissues may be due to a low level of NeuAc biosynthesis, which might be sufficient for these tissues. Another possibility is that the low activity found for UDP-GlcNAc 2-epimerase and ManNAc kinase is caused by an unknown inhibitor or a greater extent of instability of these two enzymes in non-secreting tissues. A third possibility is that the biosynthetic pathway of NeuAc in non-secreting tissues differs from the generally accepted scheme (Fig. 1) and GlcNAc 2-epimerase possesses a role in NeuAc biosynthesis. As a consequence, the feedback inhibition by CMP-NeuAc towards UDP-GlcNAc 2-epimerase is avoided and a regulation mechanism is lost. Although all tissues, except muscle and erythrocytes, showed GlcNAc 2-epimerase activity (Table 2), the next step in NeuAc biosynthesis probably is not the phosphorylation of ManNAc. Pathways in which ManNAc kinase is not involved are the condensation of ManNAc and phosphoenolpyruvate, occurring in bacteria (Blacklow & Warren, 1962), or the reversible reaction catalysed by NeuAc aldolase (Comb & Roseman, 1960; Brunetti *et al.*, 1962). Direct epimerization of GlcNAc 6-phosphate to ManNAc 6-phosphate has only been reported to occur in bacteria (Ghosh & Roseman, 1965*a*).

In our kinase assay 1% or less of the radioactive label was eluted from the Dowex AG 1 column with 1000mM-Teb buffer. Testing liver soluble cell fractions, the 1000mM-Teb eluent consisted of NeuAc 9-phosphate. However, when kidney soluble cell fractions were tested, the 1000mM-Teb eluent contained an unknown product, which was not identical with NeuAc 9-phosphate (results not shown).

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