

Synthesis of *N*-acetylneuraminic acid and of CMP-*N*-acetylneuraminic acid in the rat liver cell

Wijnholt FERWERDA, Corrie M. BLOK and Johanna VAN RINSUM
*Department of Medical Chemistry, Vrije Universiteit, Van der Boechorststraat 7,
NL-1007 MC Amsterdam, The Netherlands*

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Adult male rats, under starving and normal conditions, were injected intravenously with *N*-acetyl[³H]mannosamine and after various time intervals the specific radioactivities of free *N*-acetylneuraminic acid (NeuAc) and CMP-*N*-acetylneuraminic acid were determined in the liver. The specific radioactivity of free NeuAc was high even within 20 s after injection; the maximum was reached between 7 and 10 min. The specific radioactivity of CMP-NeuAc showed a lag phase of approx. 1 min. Thereafter it increased quickly and rose above the specific radioactivity of free NeuAc, reaching a maximum about 20 min after injection. These results point to a channelling of the newly synthesized NeuAc molecules into a special compartment, from which they are preferentially used by the enzyme CMP-sialic acid synthetase. It is suggested that the cytosolic enzyme *N*-acetylneuraminic acid 9-phosphate phosphatase is working in concert with the nuclear localized enzyme CMP-*N*-acetylneuraminic acid synthetase. Incorporation of radioactive sialic acid into sialoglycoproteins in liver occurred 2 min after injection, and after 10 min bound radioactivity began to appear in the circulation, indicating a transport time of 8 min of sialoglycoproteins from the point of attachment of sialic acid to the point of excretion.

Sialic acid plays an important role in biological phenomena, being the negatively charged terminal sugar of many glycoproteins. Sialic acid is thought to be synthesized in its non-modified form, NeuAc, by a series of enzymes in the cytosol (for reviews, see Warren, 1972; McGuire, 1976). Recently we were able to confirm the cytosolic localization of some precursor-forming enzymes (Van Rinsum *et al.*, 1983). The enzyme that activates NeuAc, CMP-NeuAc synthetase, is localized in the nucleus (Kean, 1970; Gielen *et al.*, 1970, 1971; Van den Eijnden, 1973; Van Dijk *et al.*, 1973; Coates *et al.*, 1980), whereas the transfer of NeuAc to various glycoprotein and glycolipid acceptors takes place in the Golgi apparatus (for reviews, see Schachter, 1974; Schachter & Roseman, 1980). Modifying reactions, such as *O*-acylation and *N*-hydroxylation, can occur in the Golgi region and perhaps in the cytosol (Corfield *et al.*, 1976; Buscher *et al.*, 1977). In

addition, a CMP-NeuAc hydrolase exists, which is predominantly found in the plasma membrane (Kean & Bighouse, 1974; Van Dijk *et al.*, 1976, 1977). The significance of this distribution at a topographical level is unknown, but it might indicate a high degree of regulation of sialic acid metabolism. Assuming that CMP-NeuAc synthetase is functionally active in the nucleus (Coates *et al.*, 1980), the question arises as to how this synthetase is interrelated with the cytosolic NeuAc-forming enzymes. Studying the metabolism of sialic acid in rat brain, after intracranial injection of the specific precursor *N*-[³H]acetyl-*D*-mannosamine, we observed a higher specific radioactivity of CMP-NeuAc than of free NeuAc, in the first few hours after the pulse (Ferwerda *et al.*, 1981*a*). This result points to a channelling of the newly synthesized NeuAc molecules to a special compartment, where they will be used directly by CMP-NeuAc synthetase. We have now extended our studies to rat liver to investigate whether this phenomenon is general or specific to the brain. In addition, we have measured the incorporation of radioactivity into bound NeuAc

Abbreviations used: NeuAc, *N*-acetylneuraminic acid; ManNAc, *N*-acetyl-*D*-mannosamine; h.p.l.c., high-pressure liquid chromatography.

and the time between incorporation and excretion. Part of this work has been presented previously (Ferwerda *et al.*, 1981*b*).

Materials and methods

N-Acetyl[4-¹⁴C]neuraminic acid (sp. radioactivity 57 Ci/mol), CMP-*N*-acetyl[4-¹⁴C]neuraminic acid (sp. radioactivity 1–2 Ci/mol) and *N*-acetyl-D-[³H]-mannosamine [G-³H; sp. radioactivity 2807 Ci/mol; or 6(n)-³H, sp. radioactivity 19 000 Ci/mol] were obtained from New England Nuclear, Boston, MA, U.S.A. (During our experiments the delivery of the ManNAc with lower specific radioactivity was stopped.) NeuAc was purchased from Koch–Light Laboratories, Colnbrook, Bucks., U.K.; 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid was from Boehringer-Mannheim G.m.b.H., Mannheim, Germany, and Dowex AG1 (X8) was from Bio-Rad, Richmond, CA, U.S.A.

All other chemicals were of analytical grade.

Animals

Male Wistar rats of 2–3 months old (weight 190–220 g) were used. The rearing conditions were: light from 08:00 h to 20:00 h, temperature 18–20°C, food from 7:00 h to 9:00 h and water *ad libitum*. The rats easily adapted to this schedule, although their growth was somewhat retarded. On the day of injection no food was given. These rats are referred to as starved rats. Rats fed *ad libitum* (weight 220–240 g) are designated as normal rats.

The rats were injected intravenously between 09:00 h and 11:00 h with 0.5 ml of phosphate-buffered saline, pH 7.4, containing 100 μ Ci of [³H]ManNAc. They were killed by immersing the whole animal in liquid N₂, whereafter the bodies were stored at –40°C. The livers and sometimes the heart and large blood vessels were removed in a frozen state. Therefore the frozen animal was cut into pieces with the aid of a guillotine and the organs were rapidly dissected out in the cold to prevent thawing, and stored in liquid N₂ until use. To check the effect of the killing procedure on the results, some animals were killed by decapitation, the livers were rapidly removed and stored in liquid N₂.

Isolation and purification of free NeuAc and of CMP-NeuAc

The procedure used was a modification of the method described previously (Ferwerda *et al.*, 1981*a*). Briefly, the frozen livers were weighed and, avoiding thawing, immediately homogenized in 9 vol. of cold 75% (v/v) ethanol, sonicated for 1 min and centrifuged at 105 000 g for 1 h, at 0–4°C in an MSE-50 centrifuge (8 × 50 ml angle rotor). After evaporation of the ethanol the extracted material was dialysed at 0°C against 250 ml of 5 mM-Teb

(triethylammonium bicarbonate) buffer, pH 7.8, containing 1 ml of toluene/litre to prevent bacterial growth. The diffusate was evaporated, the material solubilized in a few millilitres of the same buffer and transferred on to a Dowex AG1 (X8; HCO₃⁻ form; 100–200 mesh) column (100 cm × 0.5 cm). Elution was achieved with a linear concentration gradient of 0.005–0.25 M-Teb buffer. The radioactivity of the fractions was determined and the various peaks were pooled and concentrated by evaporation. The positions of the different metabolites on the column were checked with radioactive standards. Fractions containing free NeuAc were pooled, the content of NeuAc and the radioactivity were determined in triplicate and the specific radioactivity was calculated. This material still contained some radioactive impurities. To correct for this, h.p.l.c. was performed on an analytical scale by the method of Bergh *et al.* (1981). Normally, 70–80% of the radioactivity appeared to coincide with NeuAc and the specific radioactivity was corrected accordingly. With exogenous [¹⁴C]NeuAc, it was shown that the impurities were formed in part during the purification procedure, probably during evaporation in Teb buffer, which can lead to a pH of approx. 9.

The CMP-NeuAc-containing material was hydrolysed under very mild conditions (1 h at 80°C, in bidistilled water acidified with formic acid to pH 4–5) and rechromatographed on a small Dowex AG1 (X8; HCO₃⁻ form; 100–200 mesh) column (5 cm × 0.5 cm), eluted stepwise with Teb buffer. Fractions containing NeuAc, eluted at a concentration of 40 mM-Teb buffer, were pooled, evaporated and the radioactivity and the content of NeuAc were determined in triplicate. The specific radioactivity could be calculated after analysis with h.p.l.c. as described above; 80–90% of the radioactivity coincided with NeuAc. Some 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid was always found, which is formed during evaporation of CMP-NeuAc under mild alkaline conditions (Beau *et al.*, 1978).

The concentrations of free NeuAc and of CMP-NeuAc *in vivo* were measured with the isotope-dilution technique using ¹⁴C-labelled standards. The purification procedure was the same as described above. To determine whether hydrolysis of bound NeuAc easily occurred some measurements were performed after homogenization of the liver in distilled water.

Isolation and purification of bound NeuAc

A suitable portion was taken from the liver pellets, obtained after centrifugation of the homogenates (see above), and washed by dispersion and centrifugation in 70% ethanol. The NeuAc was released using 0.05 M-H₂SO₄ and purified as described previously (Ferwerda *et al.*, 1981*a*). Small Dowex AG1 (X8; HCO₃⁻ form; 100–200 mesh) columns

(5 cm × 0.5 cm) were used for purification and were eluted stepwise with Teb buffer.

Serum was obtained from the heart and large blood vessels after thawing, clotting and centrifugation. Some haemolysis could not be avoided. The serum was extensively dialysed at 0°C against distilled water to eliminate low-molecular-weight radioactive compounds. Subsequently, the serum was hydrolysed and the NeuAc was purified as described above. The specific radioactivity of the purified NeuAc was calculated after determination of the content of NeuAc and of the radioactivity.

Analytical techniques

Teb-stock buffer was prepared by bubbling CO₂ into a 1.25 M solution of triethylamine in a closed vessel under constant stirring at 4°C until the pH was 7.8. The various concentrations were prepared by dilution. Sialic acid was measured by the thio-barbituric acid method (Warren, 1959). Radioactivity was counted in a Berthold liquid-scintillation counter (model BF 8000) equipped with an automatic external standard for calculation of d.p.m.

Results and discussion

The metabolism of rats fed only 2 h a day became typical for starving conditions. The total body weight remained almost the same, no fat deposits were formed and the liver weight (4–5 g) was approximately half that of normal rats. With such rats the biological variation was reduced, resulting in a better reproducibility. However, to exclude the possibility that starvation introduced misleading results we also investigated a series of rats fed *ad libitum*.

The quantity of [³H]ManNAc injected was 36 nmol (sp. radioactivity 2807 Ci/mol) or 5.3 nmol (sp. radioactivity 19 000 Ci/mol). The livers of starved rats as well as of normal rats absorbed very little of the injected ManNAc. After correction for the radioactivity present in blood it could be calculated that from the 36 or 5.3 nmol injected, between 1 and 2% was absorbed by the liver. This corresponds to maximally 0.7 and 0.1 nmol respectively of ManNAc, a negligible quantity compared with the total amount of metabolites under investigation (Table 1). It appeared that injection of 5.3 nmol with the higher specific activity resulted in somewhat higher specific radioactivity of the metabolites than injection of 36 nmol with the lower specific radioactivity. Most of the injected radioactivity was rapidly cleared from the circulation by the kidneys. After 10 min over 50% had already been excreted. Harms & Reutter (1974) reported an absorption of approx. 3% by the liver of normal male Buffalo rats independent of the injected quantity. However, they did not correct for the blood content of liver.

The values shown in Table 1 were obtained after killing the animals in liquid N₂ and homogenization in ethanol. It appeared that homogenization in water resulted in 2-fold higher values for free NeuAc, whereas the values found for CMP-NeuAc remained the same. Probably some bound NeuAc can be split off in water by the enzyme sialidase, even in the cold, as has been reported for rat brain (Irwin *et al.*, 1973). Killing by the liquid-N₂ method or by decapitation resulted in the same values. We decided to use the liquid-N₂ method to stop the metabolic processes as abruptly as possible. A few seconds after immersing the animal in liquid N₂ the liver will be frozen. So time corrections can be avoided, perhaps with the exception of periods shorter than 1 min.

The reliability of the procedure is very important in view of the results described below. The effect of bound NeuAc split off during the procedure, especially, can introduce misleading results. No indications exist that in our procedure such a hydrolysis occurred. In addition, frozen liver freeze-dried on a cold plate and homogenized as dry powder in cold ethanol gave fully comparable results (results not shown).

The specific radioactivities of free NeuAc and of CMP-NeuAc were determined at various time intervals. A few minutes after injection the specific radioactivity of free NeuAc was high. The maximum was reached after 7–10 min, and then it declined slowly. During the first few minutes the specific radioactivity of CMP-NeuAc was low, but it increased quickly and rose above the specific radioactivity of free NeuAc, remaining higher for more than 2 h. The maximum was reached between 15 and 20 min. The specific radioactivities found for normal rats were lower than those of starved rats, but the profiles of the curves were similar (Fig. 1). As we used the ManNAc batch with low specific radioactivity (2807 Ci/mol) for starved rats and the batch with high specific radioactivity (19 000 Ci/mol) for normal rats the observed differences between starved and normal rats should be more pronounced. From our experiments it became clear that the livers of starved rats have a greater absorption capacity for ManNAc than the livers of normal rats. The livers

Table 1. Concentration of free NeuAc and of CMP-NeuAc in rat liver

Values are expressed as means ± s.e.m. The numbers of experiments are given in parentheses.

	Concentration (nmol/g wet wt.)	
	Starved rats	Normal rats
Free NeuAc	80 ± 6 (6)	94 ± 9 (7)
CMP-NeuAc	30 ± 1 (8)	34 ± 2 (4)

of starved rats absorbed even more than twice as much per g wet wt. A greater absorption capacity by hepatic cells during starving conditions was also observed by Summerfield (1982) for the uptake of agalacto-orosomucoid.

On the basis of our observations the conclusion can be drawn that in liver, as in brain, newly synthesized NeuAc molecules are channelled to CMP-NeuAc synthetase. To determine whether the newly synthesized NeuAc molecules are directly transferred to the synthetase or are directed into a special compartment (the substrate pool of the synthetase), we measured the specific radioactivities of NeuAc and of CMP-NeuAc in a series of starved rats very shortly after injection. If a special compartment exists the specific radioactivity of CMP-NeuAc should show a lag phase relative to the specific radioactivity of NeuAc; the duration of the lag phase gives an indication of the size of the substrate pool. The specific radioactivity of free NeuAc was high from the first test point (20s), and thereafter it increased further. The specific radioactivity of CMP-NeuAc showed a lag phase of 40–60s, after which time it increased quickly (Fig. 1, inset). This result points to the existence of a special pool of newly synthesized NeuAc molecules. A half-life of CMP-NeuAc shorter than 20 min can be calculated from the decrease in specific radioactivity between 30 and 50 min (Fig. 1) in the liver of starved rats. An exact determination is not possible because incorporation of radioactivity into

CMP-NeuAc still continues during this time. From this half-life and the pool size of CMP-NeuAc (Table 1) it can be calculated that the synthesis rate of CMP-NeuAc is greater than 1.0 nmol/min per g wet wt. A lag phase of 1 min points to a pool of NeuAc for synthesis of more than 1.0 nmol/g wet wt.

The difference in lag phase between CMP-NeuAc and NeuAc should be used for an exact calculation, but for NeuAc no lag phase could be determined. In addition, after 1 min the incorporation of radioactivity into CMP-NeuAc started, but the pool for synthesis may only be partly renewed. However, it is clear that the pool for synthesis is small, maximally a few percent of the total amount of free NeuAc in the liver.

As mentioned in the introduction, CMP-NeuAc synthetase is localized in the nucleus. Experiments with nucleus-free cells (Coates *et al.*, 1980) give support to the idea that CMP-NeuAc synthetase is also functionally active in the nucleus. The channelling of newly synthesized NeuAc molecules can be explained by the assumption that the last enzyme in NeuAc biosynthesis, NeuAc 9-phosphatase, delivers its product into the special pool, in this case the nucleus. The subcellular localization of the NeuAc 9-phosphatase has never been determined. No indications exist that this enzyme is nuclear-bound; on the contrary the results of the investigations point to a cytosolic localization (Warren & Felsenfeld, 1962; Jourdan *et al.*, 1964; Watson *et al.*, 1966). However, cytosol-localized

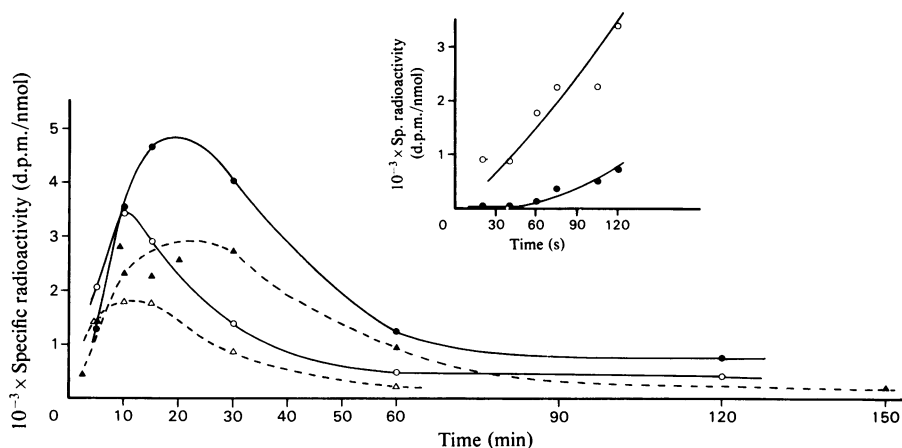


Fig. 1. Time course of the specific radioactivities of CMP-NeuAc and of free NeuAc in rat liver after a single injection of 100 μ Ci of [3 H]ManNAc

Rats were killed at various time intervals after injection by immersing the whole animal in liquid N₂. The livers were removed in a frozen state and CMP-NeuAc and free NeuAc were isolated and purified as described in the Materials and methods section. Starved rats: ●, CMP-NeuAc; ○, free NeuAc; sp. radioactivity of the injected [3 H]ManNAc was 2807 Ci/mol (inset: sp. radioactivity 19000 Ci/mol). Normal rats: ▲, CMP-NeuAc; △, free NeuAc; sp. radioactivity of the injected [3 H]ManNAc was 19000 Ci/mol. The indicated times in the main Figure and in the inset are the periods between injection and immersion in liquid N₂.

enzymes need not be freely diffusible, but are often functionally ordered within the cell. Interaction with structural components can occur, sometimes resulting in modified catalytic activities (for review, see Masters, 1978). In the case of NeuAc-synthesis it might be that NeuAc 9-phosphatase is adsorbed on the nuclear membrane in its active state, resulting in the delivery of newly synthesized NeuAc into the special pool. We realize that this hypothesis needs experimental support. One important objective is to prove that CMP-NeuAc synthetase is really functionally active in the nucleus and does not exert its action elsewhere. It is noteworthy that in liver a relatively high concentration of 300–400 nmol of UDP-GlcNAc/g wet wt. exists (Molnar *et al.*, 1964; Bates *et al.*, 1966; Harms *et al.*, 1973), whereas the concentrations of ManNAc, ManNAc 6-phosphate and NeuAc 9-phosphate are low or below the detectable level (Voisey & Winterburn, 1982; W. Ferwerda, C. M. Blok & J. Van Rinsum, unpublished work). This might indicate that a highly ordered system exists of the enzymes UDP-GlcNAc 2-epimerase, ManNAc kinase, NeuAc 9-phosphate synthetase and NeuAc 9-phosphatase working in concert with UDP-GlcNAc as starting substrate and resulting in the synthesis of NeuAc.

The activated sugar, CMP-NeuAc, has to be transported to the Golgi vesicles. This can occur through the lumen of the contiguous membrane system of the rough and smooth endoplasmic reticulum, as has been suggested by Hanover &

Lennarz (1981) or via the cytosol. Experiments with isolated Golgi vesicles give support to the latter possibility (Carey *et al.*, 1980; Carey & Hirschberg, 1981). This transport of CMP-NeuAc from the place of synthesis to the place of incorporation seems to occur promptly. Approx. 1 min after the appearance of radioactivity in CMP-NeuAc, incorporation into bound NeuAc of the liver could be detected (Fig. 2). Some difference was found between normal and starved rats, but it is questionable whether this difference is significant. Between 9 and 10 min after injection, radioactivity began to appear in the circulating sialoglycoproteins. So the transport of sialoglycoproteins from the point of attachment of the sialic acid molecules to the point of excretion took 7–8 min. In this respect the livers of starved rats behaved the same as normal rats. With radioactive glucosamine as precursor an incorporation plus excretion time of approx. 15 min and 25 min has been reported for α_1 -acid glycoprotein and transferrin respectively (Schreiber *et al.*, 1979). However, glucosamine serves as a precursor of several sugars and the radioactivity is incorporated at an earlier stage.

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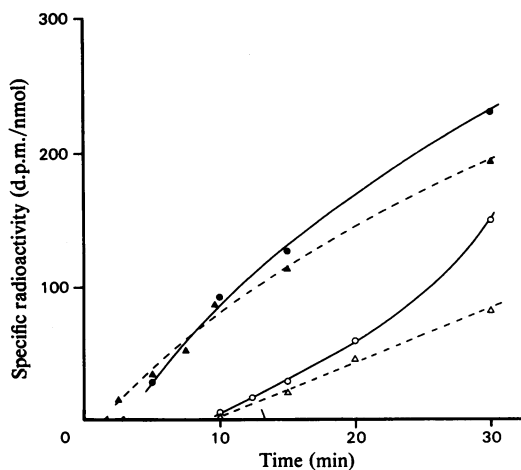


Fig. 2. Time course of the specific radioactivities of bound sialic acid from sialoglycoproteins of rat liver and of serum after a single injection of 100 μ Ci of [3 H]ManNAc

Starved rats: ●, liver; ○, serum. Normal rats: ▲, liver; △, serum. Sp. radioactivity of the injected [3 H]ManNAc was 19 000 Ci/mol.

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