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Indications for the enzymatic synthesis of 9-*O*-lactoyl-*N*-acetylneuraminic acid in equine liver

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Fractionation of horse liver homogenate by centrifugation into heavy membranes at 10 000 × g, microsomal fraction at 105 000 × g, and the supernatant revealed sialate 9-*O*-lactoyltransferase activity only in the latter fraction. For the enzyme assay, the various fractions were incubated with ^{14}C labelled CMP-*N*-acetylneuraminic acid, *N*-acetylneuraminic acid and glycoconjugate-bound *N*-acetylneuraminic acid. Lactoylation was identified in three different TLC systems after acid hydrolysis and purification of the sialic acids in the incubation mixtures. Enzyme activity was found only in the supernatant fraction. Glycoconjugate-bound *N*-acetylneuraminic acid was the best substrate tested, although some lactoylation was also found when using CMP-*N*-acetylneuraminic acid.

Keywords: 9-*O*-Lactoyl-*N*-acetylneuraminic acid, equine liver, enzymatic synthesis, sialic acid

Introduction

Sialic acids comprise a group of more than 20 derivatives of neuraminic acid, most of them being *O*-acetylated at positions 4, 7, 8 and/or 9 [1]. Lactoylation at C-9 has been found only in few sources, i.e., in human serum, urine and saliva [2], in gastric [3] and nasal mucin [4], in horse plasma [5] and submandibular mucin [6], in bovine submandibular gland mucin [7], and in trout liver (unpublished).

The biological function and metabolism of this type of sialic acid modification is unknown. It has been found only that the enzymes of the sialic acid catabolism, sialidase, sialate *O*-acetyl esterase and sialate lyase, that act on *N*-acetylneuraminic acid (Neu5Ac) are either inactive or only significantly less active on *N*-acetyl-9-*O*-lactoylneuraminic acid (Neu5Ac9Lt) [8, 9]. In the present study, evidence for an enzymatic synthesis of this compound in horse liver will be presented.

Materials and methods

Unless otherwise stated, all procedures were carried out at 4 °C, and the chemicals were from E. Merck or Sigma and were of the highest purity available. A sialic acid mixture containing 80% *N*-acetylneuraminic acid, 15% Neu5Ac9Lt, and 5% Neu5,9Ac_2 purified from human nasal mucin [4] and Neu4,5Ac_2 purified from equine submandibular mucin [6] were used as TLC standards.

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Fractionation of equine liver homogenates

Horse liver (10 g), obtained freshly from a local slaughterhouse and stored on ice or frozen at −70 °C until processed, was minced and homogenized by 30 strokes with a Potter–Elvehjem homogenizer in 40 ml 50 mM sodium phosphate buffer, pH 7.2. The homogenate was centrifuged at 10 000 × g for 20 min to obtain a pellet of heavy membranes (M1). The microsomal membranes (M2) were collected by centrifugation of the supernatant at 105 000 × g for 1 h. The supernatant (S1) was taken off. Both membrane containing sediments (M1 and M2) were resuspended in 10 ml 50 mM sodium phosphate buffer, pH 7.2, for enzyme assays.

Sialylation of equine microsomal glycoconjugates with [^{14}C]Neu5Ac

Fresh horse liver (20 g) was homogenized and centrifuged as described above but with 80 ml of 20 mM Tris-HCl buffer, pH 7.2, containing 0.25 M sucrose, 1 mM EDTA, and 8 mM magnesium acetate. The microsomal fraction, resuspended in 20 ml water, was adjusted to pH 1.0 with 1 N HCl and desialylated by incubation at 80 °C for 50 min. After thorough dialysis for 36 h against three times 1.5 l water at 4 °C, the content of the dialysis bag was centrifuged at 10 000 × g for 30 min. The supernatant was lyophilized and resuspended in 5 ml of 35 mM sodium cacodylate buffer, pH 6.0, containing 0.5% Triton CF54. From this solution containing desialylated soluble glycoproteins, 2 ml were added to 7 ml of a freshly prepared suspension of microsomal membranes in the same buffer supplemented

with 74 kBq cytidine-5′-monophospho-*N*-acetyl[4,5,6,7,8,9-^{14}C]neuraminic acid (CMP-[^{14}C]Neu5Ac, New England Nuclear, Dreieich, Germany) and incubated at 37 °C for 3 h for sialylation by endogenous sialyl transferases. The suspension was cleared by centrifugation at 100 000 × g for 1 h and the supernatant thoroughly dialysed for 36 h against three times 1 l water. The non-dialysable material was lyophilized, resuspended in 20 mM Tris-HCl buffer, pH 7.2, and the sialylated glycoproteins were purified by chromatography on a 95 × 1.5 cm column of Sephadex G 25 (Pharmacia, Freiburg, Germany) with 5 mM Tris-HCl, pH 7.2, as eluent. The fractions of the first peak of radioactivity, eluting at the void volume, were combined, lyophilized, and redissolved in 0.2 ml water. Residual peaks of radioactivity eluted at the volumes of CMP-Neu5Ac and Neu5Ac, respectively.

Incubation conditions

To 2 ml of the fractions M1, M2 or S1 were added 2.5 mM ATP, 2 mM magnesium acetate [10] and one of the following substrates: 1.25 µmol (3.3 kBq) *N*-acetyl[4,5,6,7,8,9-^{14}C]-neuraminic acid ([^{14}C]Neu5Ac, Amersham, Braunschweig, Germany), 1.3 µmol (3.3 kBq) CMP-[^{14}C]Neu5Ac, or 1.7 kBq microsomal glycoconjugates, [^{14}C]sialylated as described above. The incubations were carried out at 25 °C for 1 h in a shaking water bath.

Purification of sialic acids

The incubations were stopped and sialic acids cleaved from glycoconjugates either by adjustment of the reaction mixtures to pH 2.0 with 2 N formic acid and heating for 1 h at 70 °C [11] or by adjustment to pH 2.5 with 2 N acetic acid and incubation at 80 °C for 3 h [12]. In both cases the samples were cooled on ice thereafter and the pH adjusted to 5.0 with 1 M Na_2CO_3. The solution was applied to a column (0.7 cm × 5 cm) with Dowex 2 X8 (100–200 mesh, $HCOO^-$ form). After rinsing with 10 ml water, bound sialic acids were eluted with 10 ml 1 N formic acid and 6 ml 1.5 N formic acid. The combined eluates were frozen at −70 °C, lyophilized and resuspended in 0.5 ml water. The amount of [^{14}C]sialic acids isolated was estimated by liquid scintillation counting with 5 µl of the samples. The remaining solution was concentrated by lyophilization and resuspended in water at 5000 cpm per 10 µl.

Radio thin-layer chromatographic analysis of reaction products

Three TLC systems were used for the analysis of the purified reaction products.

System A consisted of glass-backed cellulose high performance thin-layer chromatography (HPTLC) plates (Merck, Darmstadt, Germany) pre-run and developed with the solvent system n-propanol:n-butanol:0.1 M HCl (2:1:1 by vol).

For system B, samples were applied to HPTLC glass plates with 0.25 mm silica gel 60 (Merck, Darmstadt, Germany) and developed with a solvent [13] consisting of ethanol:pyridine:n-butanol:water:acetic acid (100:10:10:30:3 by vol).

As system C, HPTLC with cellulose on glass plates was used with the solvent system n-butanol:pyridine:water (6:4:3 by vol) [14].

Bands of radioactivity on the plates were detected using a linear analyser LB 284 (Berthold, Wildbad, Germany) with computerized peak integration. Co-chromatographed sialic acid standards were visualized with the orcinol:Fe^{3+}:HCl reagent [11].

Identification of reaction products by alkaline and esterase treatment

For analytical de-*O*-acylation, the sialic acids purified as described above were subjected to alkaline hydrolysis by incubation for 30 min at pH 12 and 4 °C [11]. Thereafter, the samples were neutralized with 1 N HCl and directly applied to the TLC plates.

For incubation with sialate *O*-acetylesterase from bovine brain [15], 15 µl of purified sialic acids were adjusted to pH 7.5 with 15 µl 50 mM Tris. To 10 µl aliquots were added either 10 µl sialate *O*-acetylesterase (0.255 mU as determined with methylumbelliferyl acetate [15]), 10 µl esterase denatured at 100 °C for 10 min, or 10 µl water, respectively. After incubation for 1 h at 37 °C the samples were analysed with the HPTLC system A.

Results and discussion

Identification of 9-O-lactoyl-N-acetylneuraminic acid

Sialic acids obtained from the incubation of the supernatant fraction S1 with CMP-[^{14}C]Neu5Ac after hydrolysis co-migrated with the *N*-acetylneuraminic acid ($R_F = 0.45$) and the Neu5Ac9Lt ($R_F = 0.56$) standards in HPTLC system A (Fig. 1(a)). Between 2 and 25% of the radioactivity were found at the Neu5Ac9Lt position. This variation, measured with supernatants prepared from different liver samples, is comparable to that observed when investigating the sialic acid *O*-acetylation in rat colon [16] and in bovine submandibular gland [17]. No peak was detectable co-migrating with Neu4,5Ac_2, a sialic acid that is present in equine liver [18], or with Neu5,9Ac_2. After alkaline hydrolysis, the peak at the Neu5Ac9Lt position was no longer present (Fig. 1(b)); only the peak co-migrating with Neu5Ac was still detectable. This result excludes *O*-acylated derivatives of *N*-glycoloylneuraminic acid (Neu5Gc), that had been found in equine blood plasma [5], as products of the incubation, since a peak migrating at the appropriate position was not observed. The substance with $R_F = 0.56$ is distinguishable from 4,8-anhydroneuraminic acid, a previously described molecule from acid hydrolysates of collocalia mucoid [19] and of equine serum [20], as this would migrate with Neu5Ac in system A [19]. Furthermore,

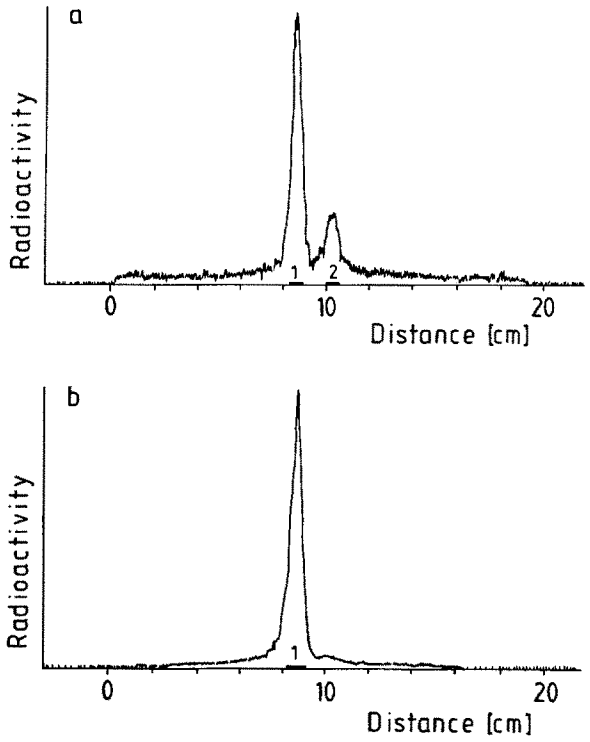

Figure 1. (a) Radio-TLC analysis with system A of sialic acids purified after acid hydrolysis of the products of incubation of the 105 000 × g supernatant S1 of an equine liver homogenate with CMP-[^{14}C]Neu5Ac; lane (b) sialic acids of lane (a) after alkaline hydrolysis. The bars 1 and 2 indicate the position of co-chromatographed *N*-acetylneuraminic acid and Neu5Ac9Lt standards, respectively.

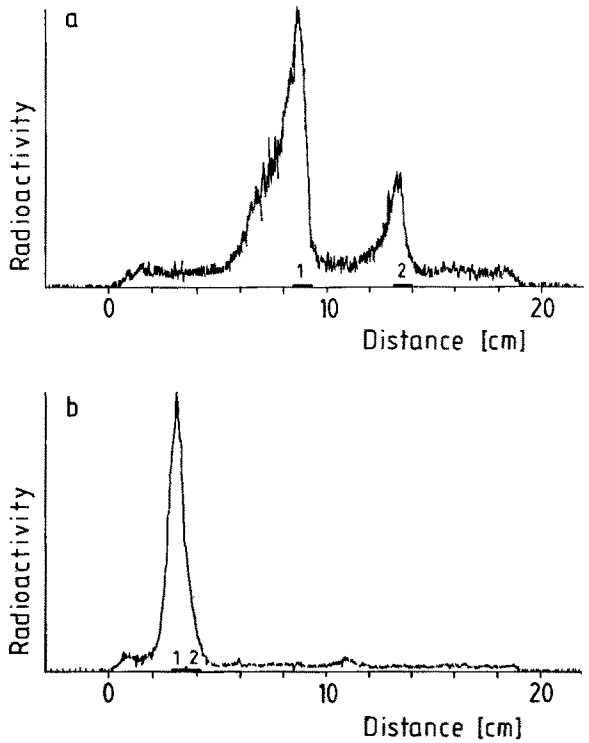

Figure 2. Radio-TLC analysis of sialic acids prepared as described for Fig. 1a, but analysed with (a) HPTLC system B and (b) system C. The bars 1 and 2 indicate the migration positions of *N*-acetylneuraminic acid and Neu5Ac9Lt standards, respectively.

the anhydro compound should not be hydrolysed with alkali. In TLC system B, two peaks of radioactivity were observed, the slower one running with Neu5Ac and the faster one with Neu5Ac9Lt (Fig. 2(a)). In system C, only one peak appeared with a faster moving shoulder co-migrating with authentic Neu5Ac9Lt (Fig. 2(b)). No band was observed at the position of the faster migrating Neu4,5Ac$_2$.

Incubation of the purified sialic acids with sialate *O*-acetylesterase from bovine brain, an enzyme known to hydrolyse the *O*-acetyl groups of Neu5,9Ac$_2$ and, at lower speed, also of Neu4,5Ac$_2$ but not of Neu5Ac9Lt [15], did not result in any decrease of the peak intensity at $R_F = 0.56$ on HPTLC system A relative to the Neu5Ac peak when compared to incubations without or with heat-denatured esterase, supporting the identification of this substance as Neu5Ac9Lt.

Characterization of the enzymatic activity

The lactolylating activity with CMP-[^{14}C]Neu5Ac was only detectable with the 105 000 × g supernatant S1 but not with the heavy membranes M1 or with the microsomal fraction M2. A cytosolic localization would be in contrast to that of the side chain O-acetylating activity in rat liver that resides in Golgi vesicles [10, 21]. Homogenization of fresh and especially of frozen liver with 50 mM sodium phosphate buffer, however, will probably release soluble enzymes from these compartments. These enzymes will then be found in the supernatant where they may catalyse the formation of glycosidically bound Neu5Ac9Lt.

Incubating CMP-[^{14}C]Neu5Ac with the 105 000 × g supernatant S1 that had been kept at 100 °C for 10 min did not yield radioactivity at the Neu5Ac9Lt position, indicating that the lactoylation reaction is enzyme catalysed. The possibility of a nonenzymatic reaction of lactic acid with *N*-acetylneuraminic acid, CMP-Neu5Ac, or sialoglycoconjugates during the incubation or the purification steps was tested by adding 1 mM L-lactate to the incubation mixture. No increased [^{14}C]Neu5Ac9Lt production was detectable. This result also excludes that lactate is the co-substrate of the enzymatic reaction. Experiments with lactic acid supporting these results have already been described by Schauer *et al.* [7], who did not find Neu5Ac9Lt when incubating 7.5 mM lactate with [^{14}C]Neu5Ac at pH 7.4 and 37 °C for 24 h and at pH 2.0 and 100 °C for 1 h and by Reuter *et al.* [4], who incubated lactate with free Neu5Ac and with collocalia mucin for several hours without detecting Neu5Ac9Lt thereafter.

Glycoconjugates with bound [^{14}C]Neu5Ac were synthesized with desialylated microsomal glycoconjugates and

microsomal sialyl transferases in the presence of detergent [22]. It can be ruled out that the sialo-glycoconjugates produced were already partially lactoylated because no lactoylating activity was found in the microsomal fraction employed for their synthesis (see above).

When the supernatant S1 was incubated with [^{14}C]Neu5Ac, no radioactivity co-migrated with the Neu5Ac9Lt band. In the experiment with glycoconjugate-bound [^{14}C]Neu5Ac 10% of the radioactivity was detected at the Neu5Ac9Lt position. In comparison only 5% was converted to this product when an equal amount of CMP-[^{14}C]Neu5Ac was used as acceptor.

From these experiments we conclude that glycoconjugate-bound sialic acid is the best substrate of those tested for 9-*O*-lactoylation. On the basis of the result with CMP-[^{14}C]Neu5Ac, it cannot be excluded that CMP-Neu5Ac is a substrate for the lactoyltransferase, too, but it is more likely that it first serves as substrate for a sialyltransferase, and is lactoylated only after transfer of *N*-acetylneuraminic acid on to soluble glycoconjugates in the supernatant S1. Thus, the lower degree of lactoylation found with CMP-Neu5Ac when compared with sialoglycoconjugates is most probably due to the relatively low activity of endogenous sialyltransferases or to low concentrations of sialic acid acceptors in the supernatant fraction.

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