Glycoconjugate Journal 15, 895–904 (1998)

Enzymatic 4-*O*-acetylation of *N*-acetylneuraminic acid in guinea-pig liver

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Sialic acids from the liver and serum of guinea-pig are composed of *N*-acetylneuraminic acid (Neu5Ac; 85% and 61%, respectively), *N*-acetyl-4-*O*-acetylneuraminic acid (Neu4,5Ac$_2$; 10% and 32%, respectively) and *N*-glycolylneuraminic acid (Neu5Gc; 5% and 7%, respectively), besides traces of *N*-glycolyl-4-*O*-acetylneuraminic acid in serum. The analysis was carried out using thin-layer chromatography, high-performance liquid chromatography, electron impact ionization mass spectrometry, and different enzymes (sialidase, sialate esterase, and sialate-pyruvate lyase after hydrolysis and purification of the sialic acids by ion-exchange chromatography). We showed that this *O*-acetylation of sialic acids is due to the activity of an acetyl-coenzyme A:sialate-4-*O*-acetyltransferase (EC 2.3.1.44), which occurs together with sialyltransferase activity in Golgi-enriched membrane fractions of guinea-pig liver. The enzyme operates optimally at 30°C in 70 mM potassium phosphate buffer at pH 6.7 and in the presence of 90 mM KCl with an apparent K_M for AcCoA of 0.6 1μM and a V_{max} of 20 pmol/mg protein x min. The enzyme is inhibited by coenzyme A in a mixed-competitive manner (K_i = 4.2 μM), as well as by *para*-chloromercuribenzoate, $MnCl_2$, saponin and Triton X-100.

Keywords: **Sialate-4-*O*-acetyltransferase, analytical techniques, Golgi-membranes, guinea-pig liver, sialic acid**

Abbreviations: **AcCoA, acetyl coenzyme A; AUS, *Arthrobacter ureafaciens* sialidase; BSA, bovine serum albumin; CoA, coenzyme A; DFP, diisopropyl-fluorophosphate; DMB, 1,2-diamino-4,5-methylenedioxybenzene; EDTA, ethylenediamine tetraacetic acid; EI-MS, electron impact ionisation-mass spectrometry; GC, gas chromatography; GC-MS, gas chromatography – mass spectrometry. HPLC, high-performance liquid chromatography; MU-Ac, methylumbelliferyl acetate; Neu2en5Ac, *N*-acetyl-2-deoxy-2,3-didehydro-neuraminic acid; Neu4,5Ac$_2$, *N*-acetyl-4-*O*-acetylneuraminic acid; Neu4Ac5Gc, 4-*O*-acetyl-*N*-glycolylneuraminic acid; Neu5,9Ac$_2$, *N*-acetyl-9-*O*-acetylneuraminic acid; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; 4-OAT, sialate-4-OAT, sialate-4-*O*-acetyltransferase; *p*-CMB, *para*-chloromercuribenzoate; PCA, perchloric acid; R_{Neu5Ac}, relative retention time referred to Neu5Ac; R_f, relative migration rate; Sia, sialic acid(s); TLC, thin-layer chromatography.**

Introduction

Sialic acids (Sia) comprise a family of about forty carbohydrates derived from the unstable 5-amino-3, 5-dideoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid, commonly designated as neuraminic acid [1, 2]. The striking variety of Sia is much influenced by *O*-acetyl esters that have been found in positions 4, 7, 8, and 9, combined or alone. Such *O*-acetylated Sia are frequently found in higher animals and in some bacterial glycoconjugates [1–5]. The probably richest source of different Sia *O*-acetylated at the glycerol side-chain is the bovine submandibular gland [6]. In contrast to *O*-acetylation at C-7 to C-9, the occurrence of 4-*O*-acetylated Sia seems to be limited to fewer animal species and has been found so far in a Japanese dace [7], a South American pit viper [8], the Australian monotreme Echidna, the horse and donkey [summarized in Refs. 2, 4], as well as in guinea-pig [9]. In the latter animal, Sia, as a component of α_2-macroglobulin, was identified by ^{1}H-NMR-spectroscopy.

An outstanding feature of these Sia is their resistance toward enzymes of Sia catabolism, like most sialidases, sialate lyases, and sialate esterases [1–3, 10]. Since the diverse biological and pathophysiological roles of Sia in general and of *O*-acetylated derivatives in special are more and more recognized [1–5, 11], interest in the biosynthesis of these substances is increasing. The transfer of *O*-acetyl groups to the various positions in Sia is the result of the activity of at least two enzymes, the acetyl-CoA:sialate-4-*O*-acetyltransferase (4-OAT, EC 2.3.1.44) and the acetyl-CoA:sialate-7(9)-*O*-acetyltransferase (EC 2.3.1.45) [1–5]. To our present knowledge, based on studies with rat liver [12, 13] and bovine submandibular gland [14], *O*-acetyla-

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tion of Sia occurs after the transfer of Sia onto growing glycoconjugates in the trans-Golgi compartment [1–3, 15].

First evidence for the existence of 4-OAT was obtained in studies with intact cells of surviving slices of horse submandibular glands [16]. Based on the discovery of *N*-acetyl-4-*O*-acetylneuraminic acid (Neu4,5Ac_2) in α_2-macroglobulin of guinea-pig serum [9], we decided to investigate the acetylation at the 4-hydroxyl position of Sia in guinea-pig liver and to reveal the subcellular localization of the respective enzyme. It was the aim to confirm the occurrence of Neu4,5Ac_2 in the guinea-pig and to investigate the nature of the various Sia occurring in this animal by using chromatographic, mass spectrometric, and enzymatic techniques. With these tools, it was possible to analyze the reaction products from the enzymatic *O*-acetylation in microquantities and to gain insight into some basic properties of the membrane-bound sialate-4-*O*-acetyltransferase.

Materials and methods

Animals, chemicals and enzymes

Guinea-pigs of either sex and different age were supplied by the *Institut für Immunologie*, Christian-Albrechts-Universität, Kiel.

All chemicals were of analytical grade except those for the high-performance liquid chromatography (HPLC) eluates which were of gradient grade. If not denoted otherwise, thin-layer chromatography (TLC) plates (cellulose, 20 × 20 cm), reversed phase columns (RP18, Lichrospher 100, particle size 5 µm), HPLC solvents, and other chemicals were obtained from Merck (Darmstadt, Germany). Ion-exchange resins, Dowex 2 × 8 (200–400 mesh) and Dowex 50W × 8 (20–50 mesh) were purchased from Pharmacia Biosystems (Freiburg, Germany). 1,2-Diamino-4,5-methylenedioxybenzene (DMB) was obtained from Dojindo Laboratories (Tokyo, Japan). Acetyl coenzyme (AcCoA), Coenzyme A (CoA), diisopropyl-fluorophosphate (DFP), ethylenediamine tetraacetic acid (EDTA) and saponin were purchased from Sigma (Deisenhofen, Germany). Radioactive compounds were obtained from Amersham (Braunschweig, Germany). Neu4,5Ac_2 as a standard Sia was either prepared from guinea-pig serum by propionic acid hydrolysis [17] or from horse submandibular glands [18]. *N*-acetyl-9-*O*-acetylneuraminic acid (Neu5,9Ac_2) was prepared accordingly from bovine submandibular glands. Asialofetuin was prepared from fetuin (Sigma) by H_2SO_4 hydrolysis at 80° C for 1 h followed by exhaustive dialysis and lyophilization. *N*-Acetyl-2-deoxy-2,3-didehydro-neuraminic acid (Neu2en5Ac) was purchased from Boehringer Mannheim (Mannheim, Germany). Scintillation-cocktail (rotiszint eco plus) was from Carl Roth GmbH (Karlsruhe, Germany).

A suspension of influenza C-virus, used as source of sialate-9-*O*-acetylesterase, was kindly provided by Prof. Dr. Georg Herrler, *Institut für Virologie, Tierärztliche Hochschule*, Hannover, Germany. Sialate-pyruvate lyase from *Clostridium perfringens* and sialidase from *Arthrobacter ureafaciens* (AUS) were purchased from Sigma and Boehringer Mannheim, respectively.

Isolation of sialic acids

Sia were released by propionic acid according to Mawhinney and Chance [17]. Ten ml guinea-pig serum were adjusted with 4 M propionic acid to pH 2.3 and heated at 80°C for 3 h in a tightly closed vessel. After exhaustive dialysis over night in the cold, the dialysate was lyophilized. A second hydrolysis with HCl at pH 1 was carried out with the remaining content of the dialysis tube followed by dialysis. In most cases, Sia of the combined lyophilisates were purified by ion-exchange chromatography described in detail in [18].

Quantification of sialic acids

Sia were quantified by using either the Fe^{3+}/orcinol/HCl-reagent [18] or by integrating the HPLC-peaks (see below); both methods were in correlation to a *N*-acetylneuraminic acid (Neu5Ac) standard curve.

De-*O*-acetylation of sialic acids

In some experiments, *O*-acetylated Sia were saponified in 0.1 M NaOH for 15 min at room temperature, followed by neutralization of the mixture with an equivalent amount of 0.1 M HCl, before the following analytical procedures were carried out.

High-performance liquid chromatography of sialic acids

Sia were predominantly analyzed using a modified method of that described by Hara *et al.* [19]. Purified and lyophilized Sia samples of more than 10 pmol each were dissolved in 10 µl of 2 M acetic acid. After the addition of 49 µl DMB reagent, the mixture was shaken vigorously and heated for 1 h at 56°C in the darkness. Separation was carried out on a RP18-column (250 × 5 mm i.d., particle size 5 µm) using methanol/acetonitril/water (7:9:84 by vol) in an isocratic mode (1 ml/min) for elution and detection of the 3-Sia-substituted 6,7-methylene dioxy-2(1H)-quinoxalinones at an excitation wavelength of 373 nm and an emission wavelength of 448 nm.

Alternatively, analysis by this method was carried out directly after hydrolysis and lyophilization. However, previous purification by ion-exchange chromatography [18] should be preferred, since it is likely that impurities interfere with the Sia-specific peak.

Thin-layer chromatography of sialic acids

This analysis was carried out on cellulose plates [18]. Since the resolution of Sia is by far less than that of the HPLC-method, this analytical tool was mainly used to analyze radioactively labeled Sia. Among three TLC-systems applied (SYSTEM 1 = 1-butanol/acetic acid/water (64:50:18, by vol), SYSTEM 2 = 1-butanol/1-propanol/0.1 M HCl (1:2:1) [18], SYSTEM 3 = 1-butanol/pyridine/water (6:4:3) [20]), the best resolution was achieved by using the butanol/propanol/HCl-mixture as solvent. The plates were prerun twice in this system before the application of Sia, and after development were stained either by the Fe^{3+}/orcinol/HCl-reagent or in the case of radioactively labeled compounds detected by an automatic TLC-linear-analyzer (Automatic TLC-linear Analyzer, LB 284, Berthold, Wildbad, Germany). Radioactive Sia were co-chromatographed with Sia standards.

For Sia analysis of radioactively labeled Golgi-membranes (see below) on TLC, membrane pellets were sonicated gently for 3 s in 200 µl of 2 M propionic acid and hydrolyzed at 80°C for 4 h. The mixtures were cooled on ice and centrifuged for 10 min at 100 000 × **g** and 4°C. After collection of the supernatants, the pellets were sonicated in 100 µl of 0.1 M HCl, heated at 80°C for 50 min and centrifuged. The supernatants from the two hydrolysis steps were lyophilized separately. Both lyophilisates were combined in a total volume of 1 ml ice-cold water and rinsed through 1 ml Dowex 50W × 8 (20–50 mesh, protonated form), followed by washing with 5 ml water in the cold [18]. The eluate was then applied to 0.5 ml Dowex 2 × 8 (200-400 mesh, formate form). After washing this anion-exchange column with 5 ml water, Sia were eluted with 10 ml of 0.8 M formic acid and lyophilized. The residue was taken up in a minimum volume of water and applied to cellulose TLC plates for development in the above mentioned solvents, co-chromatographed with standards. The radioactively labeled Sia were detected with the radio-TLC-scanner. By integration of the resulting peaks and correlation to the protein concentration of each fraction, the relative amounts of radioactive Sia and, correspondingly, the enzyme activity were determined.

For detection of the radioactivity remaining after the two hydrolyses in the Golgi-membranes, the pellets were heated in 400 µl 1 M NaOH for 15 min at 80°C, cooled on ice for 10 min, and neutralized with 200 µl of ice-cold 2 M HCl. Five hundred fifty µl of the mixture were counted with 1.5 ml of szintillation cocktail. Total radioactivity incorporated was determined by hydrolyzing the washed pellet directly in 400 µl of 1 M NaOH as described above.

Enzymatic sialic acid analyses

A further proof for the existence of 4-*O*-acetylated Sia was the application of enzymes that do not react or react only weakly on free or glycosidically bound Neu4,5Ac_2, as summarized in Reuter and Schauer [21].

Sia isolated from serum (12 µg) or from radioactively labeled Golgi-membranes of guinea-pig liver were incubated with a suspension of influenza C-viruses containing 4 mU sialate-9-*O*-acetylesterase (referred to methylumbelliferyl acetate [MU-Ac]) in 50 µl of 0.1 M Tris/HCl-buffer of pH 7.4 for 1 h at 37°C [22] or with 1 U sialate-pyruvate lyase from *Clostridium perfringens* in 50 µl 50 mM phosphate buffer of pH 7.2 for 2.5 h at 37°C. Serum (25 µl) from guinea-pig was incubated with 15 mU sialidase from AUS in 100 µl 100 mM sodium acetate buffer of pH 5 for 2 h at 37°C [18, 21]. AUS sialidase and influenza C-viruses were applied to radioactively labeled Golgi-membrane fractions and to isolated Sia from this source, respectively, whereas Sia from Golgi-membranes were treated by sialate-pyruvate lyase.

Mass spectrometry of sialic acids

For mass spectrometry, purified Sia from guinea-pig serum were converted to their pertrimethylsilylated ester/ether derivatives and applied to a GC-system coupled with EI-MS and analyzed according to a fragmentation scheme by Kamerling *et al.* [23]. These analyses were kindly performed by Professor Dr. J. P. Kamerling at the *Bijvoet Center for Biomolecular Research*, *Department of Bio-Organic Chemistry*, Utrecht, The Netherlands.

Golgi-membrane preparation

Fractions of subcellular membranes were isolated from guinea-pig liver by discontinuous saccharose gradient centrifugation, following a procedure described by Lepers *et al.* [24] with slight modifications. For this purpose, 10 g of guinea-pig liver were homogenized in a Potter-Elvehjem homogenizer (Braun, Melsungen, Germany) with three strokes in a volume of 24 ml ice-cold 0.01 M Tris/HCl buffer of pH 7.4 containing 0.25 M saccharose. The crude homogenate was adjusted to 1.4 M saccharose by the addition of 1.3 volumes of the same buffer containing 2.3 M saccharose. Three ml of the 2.3 M saccharose buffer were filled into a Beckman SW28 tube and subsequently overlaid with 3 ml of 1.6 M saccharose buffer, 9 ml of the homogenate in 1.4 M saccharose, 9 ml of 1.2 M saccharose buffer, and finally with 9 ml of 0.8 saccharose buffer. Six gradients prepared in this way were centrifuged for 90 min at 90 000 × **g** in a Beckman SW28 rotor. The turbid bands at the 0.8 M/1.2 M saccharose interface representing Golgi-enriched membranes (fraction A) were harvested in a minimum volume by a Pasteur-pipette, combined and washed in 200 ml of 0.01 M Tris/HCl buffer (pH 7.4), filled into 4 Beckman 45Ti tubes and centrifuged 30 min at 100 000 × **g**. The four pellets were each suspended in 1 ml 70 mM potassium phosphate buffer (pH 6.7) and centrifuged in 4 Beckman TLA45 tubes for 5 min at 100 000 × **g**. The supernatants

were discarded and the pellets frozen and stored at −80°C until use. The membrane fractions accumulated at the other interfaces (B-D) were treated correspondingly.

Proteins were quantified according to the method of Lowry as modified by Peterson [25]. As a marker enzyme for the Golgi-enriched fraction, sialyltransferase activities of the different subcellular fractions were tested following Vandamme *et al.* [26]. The sialyltransferase as typical Golgi-marker was regarded as sufficient proof in accordance with [24]. For this purpose the membrane fractions A, B, C, and D corresponding to 322 μg, 1732 μg, 1438 μg, and 1508 μg protein, respectively, were rehomogenized in 100 μl 0.1 M sodium cacodylate buffer of pH 6.5 containing 0.1 % BSA, 0.2 M galactose, 1 mM Neu2en5Ac, 1.67% Triton X-100, and 200 μg asialofetuin as exogenous acceptor and incubated for 6 h with CMP-Neu5-[^{14}C]Ac (0.1 μCi, 24 μM) in a total volume of 250 μl. The reaction was stopped on ice, and the tubes were centrifuged for 15 min at 500 × **g** and 15 μl of the supernatant spotted on a cellulose TLC plate. The plate was run in 95% ethanol/0.1 M NH_4OAc (7:3 by vol) and, after drying, scanned by the TLC-linear analyzer. Sialylated glycoproteins remained at the start, whereas the other radioactive compounds such as residual CMP-Neu5Ac and Neu5Ac had migrated, so that the radioactivity detected at the starting line served as the degree of glycoprotein sialylation. Correlation of this radioactivity to the protein amount of the membrane fraction incubated gave the specific sialyltransferase activity.

Sialate-4-*O*-acetyltransferase assay

This assay was based on the *O*-acetylation of endogenous substrates of the subcellular membrane fractions from guinea-pig liver, principally using the method described by Varki and Diaz for Sia *O*-acetylation in rat liver [27]. Subcellular membranes (20-100 μg protein each) were incubated with [^{3}H]AcCoA (0.07 μCi, 6 μM) in 200 μl 70 mM potassium phosphate buffer, pH 6.7, containing 90 mM KCl, usually for 5 min at 30°C. In the case of other conditions used, these are indicated. The reaction was stopped by the addition of 800 μl perchloric acid (PCA) in a final concentration of 4%. After 40 min on ice, the mixtures were centrifuged at 100 000 × **g** at 4°C for 10 min. While the supernatants were carefully removed and discarded, the pellets were sonicated for 3 s in 100 μl ice-cold water with a minimum of energy and filled up with 1 ml of ice-cold water. The resuspensions were shaken vigorously for 5 s and again centrifuged under the above conditions. Supernatants were discarded and the washed pellets used for Sia analyses or the determination of incorporated radioactivity by scintillation counting after dissolving the membranes in 400 μl of 1 M NaOH as described above.

To detect a soluble form of the enzyme, a particle-free 100 000 × **g** supernatant was prepared from guinea-pig liver homogenate (prepared from 10 g liver and 20 ml of the above phosphate-buffer). Two hundred μl of this supernatant were incubated with 300 μM fetuin-bound Sia. The enzyme assay was carried out as described above.

Factors influencing the sialate-4-*O*-acetyltransferase activity

To test the influence of bivalent cations (Mg^{2+}, Ca^{2+}, and Mn^{2+}, 2 mM each), EDTA (2 mM), *para*-chloromercuribenzoate (*p*-CMB) (1 mM), Triton-X 100 (0 − 0.05%), an saponin (0 − 0.2%) on enzyme activity, Golgi -enriched fractions were incubated as described, and the compounds of interest added in the indicated concentrations.

Nonspecific esterase activity, which may destroy sialate ester groups, was investigated in the Golgi-fraction using MU-Ac. Sia liberate from guinea-pig serum (which contains predominantly Neu4,5Ac_2 as *O*-acetylated Sia) were used to investigate the specificity of the esterase reaction by HPLC, following the procedure described by Reuter and Schauer [18, 21].

To optimize the enzyme activity with regard to potassium phosphate concentration, Golgi-membranes of the same protein concentration were incubated with different buffer concentrations in the range from 0 – 120 mM at pH 6.7, whereas the optimal KCl concentration was investigated by adjusting the optimal potassium phosphate buffer to different KCl concentrations in the range from 0 – 250 mM. To determine the optimal pH of the enzymatic reaction, equal amounts of Golgi-membranes were homogenized in 100 μl 20 mM potassium phosphate buffer of pH-values between 5 and 7.7, containing 150 mM KCl and were incubated in 200 μl of the respective buffer. The optimal temperature was investigated by incubating Golgi-membranes in 150 mM KCl-containing 20 mM potassium phosphate buffer (pH 6.7) at temperatures from 0°C to 56°C.

The dependence of the enzyme reaction on the protein concentration was studied by incubating membrane proteins between 30 and 240 μg under optimal conditions.

Kinetic parameters of the sialate-4-*O*-acetyltransferase activity

The K_M-value for AcCoA was calculated according to Lineweaver-Burk after incubating Golgi-membranes containing 23 μg protein each with different concentrations of AcCoA under optimal conditions (70 mM potassium phosphate, pH 6.7, 90 mM KCl at 30°C). CoA was investigated for inhibitory potency by incubation of Golgi-membranes containing 27 μg protein each at three different AcCoA concentrations (1, 2, and 5 μM) with different concentrations of CoA (0, 2, and 5 μM) under optimal conditions. Plotting the reciprocal velocities versus reciprocal substrate concentrations led to three linear graphs crossing in one point, thus giving the K_i-value.

All experiments described in this methodical section were carried out at least twice.

Results

Nature of sialic acids in guinea-pig

As a basis for the enzymatic investigations, the existence of 4-*O*-acetylated Sia had to be proven not only in guinea-pig serum, where it had been demonstrated earlier in α_2-macroglobulin by Hanaoka *et al.* [9], but predominantly in guinea-pig liver and Golgi-vesicles from this tissue, the assumed origin of this kind of Sia modification. This was possible in all three materials by the application of fluorimetric HPLC in comparison with Sia standards and by including saponification steps, as well as by gas chromatography–mass spectrometry (GC-MS) and treating the samples with different enzymes. The enzyme-based analysis was of great advantage in identifying the minute Sia quantities formed in the *O*-acetylation experiments described below. As shown in Figure 1a, three kinds of Sia exist in guinea-pig serum. Two of them could be identified as *N*-glycolylneuraminic acid (Neu5Gc) (R_{Neu5Ac} [relative retention time referred to Neu5Ac] = 0.8) and Neu5Ac (R_{Neu5Ac} = 1 by definition). The third peak was identified as Neu4,5Ac$_2$ (R_{Neu5Ac} = 1.69) by comparison with a standard enriched in this Sia from horse submandibular glands. As expected, this Sia was sensitive to de-*O*-acetylation by treatment with 0.1 M NaOH; its disappearence in favor of Neu5Ac is shown in Figure 1b.

Since the resolution of Neu4,5Ac$_2$ from HPLC and TLC compared with Neu5,9Ac$_2$ is quite similar in the systems used, we could not unequivocally exclude the existence of Neu5,9Ac$_2$-residues in this Sia peak. It was possible, however, to discriminate between these Sia isomers by the use of three different enzymes known to catabolize free or glycosidically bound Sia [21]. Influenza C-viruses contain the receptor-destroying enzyme sialate-9-*O*-acetylesterase that can de-*O*-acetylate Neu4,5Ac$_2$ in only 3% yield relative to Neu5,9Ac$_2$ [22]. The *O*-acetylated Sia from guinea-pig serum was not affected by treatment with influenza C-viruses, in contrast to Neu5,9Ac$_2$ from bovine submandibular glands incubated as a control, which was de-*O*-acetylated approximately 75%. This experiment is a strong proof for the Neu4,5Ac$_2$ nature of the esterified guinea-pig Sia.

A second enzyme that catabolizes free Sia is sialate-pyruvate lyase, which cleaves Sia in a retro-aldol-reaction into *N*- and *O*-acyl-D-mannosamines and pyruvate. However, 4-*O*-acetylated Sia are not substrates [21]. Correspondingly, the assumed Neu4,5Ac$_2$ peak remained unaffected in contrast to the not *O*-acetylated Sia of the sample, which disappeared almost completely (Figure 2). This confirms its assumed nature and also excludes the presence of other *O*-acetylated Sia species in guinea-pig serum and in liver Golgi-membranes.

It is known that sialidases are strongly influenced by the degree and position of *O*-acetyl groups, as well as by the

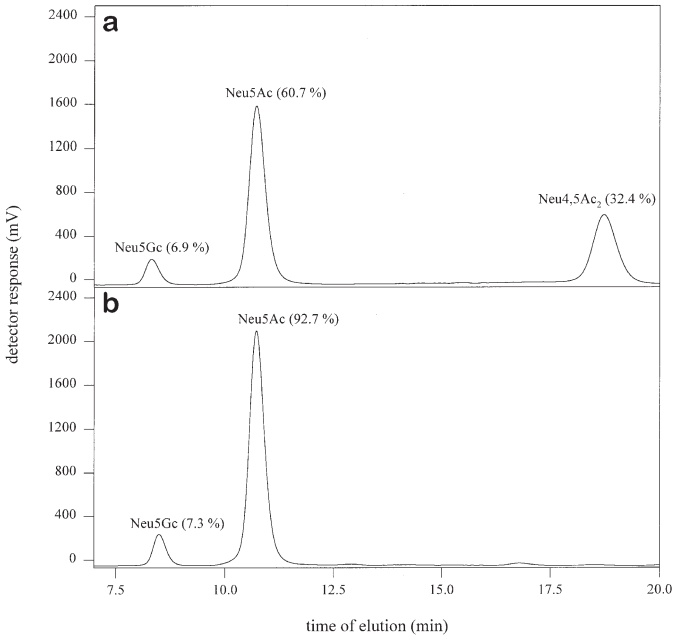

Figure 1. Fluorimetric detection of Sia isolated from guinea-pig serum (a) before saponification and (b) after saponification with 0.1 M NaOH. The relative Sia amounts are indicated. For experimental details see, Materials and Methods. Note that in equivalence with the disappearance of the Neu4,5Ac$_2$ peak after saponification, the Neu5Ac peak grew from 61% to 93% of total Sia.

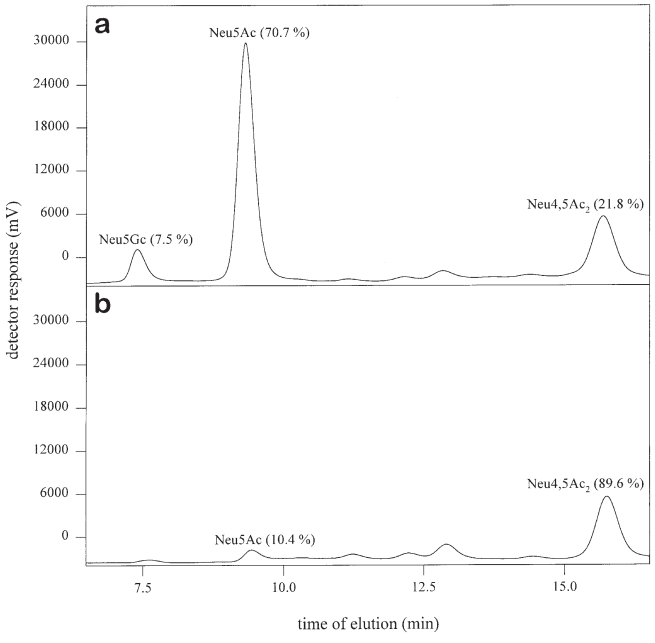

Figure 2. Treatment of purified Sia from guinea-pig serum with sialate-pyruvate lyase (a) before and (b) after enzyme treatment. The resistance of the Neu4,5Ac$_2$ peak toward this enzyme in contrast to Neu5Gc and Neu5Ac is in favor of its assumed structure [21].

linkage of Sia to glycoconjugates [10, 21]. We chose this enzyme from *Arthrobacter ureafaciens*, since it is the most unspecific sialidase known. Nevertheless, it does not recognize 4-*O*-acetylated Sia as a substrate. As shown in Figure 3a, AUS was not able to release *O*-acetylated Sia from guinea-pig serum glycoproteins, although this Sia was present in the samples, as shown by its liberation by propionic acid (Figure 3c).

Although these analytical techniques applied so far gave convincing results, unequivocal proof for the existence of Neu4,5Ac_2 in guinea-pig came from GC-MS analysis of pertrimethylsilylated samples of carefully purified Sia mixtures from serum. The characteristic fragment ions from the *O*-acetylated Sia are shown in the Table 1, thus confirming its nature as Neu4,5Ac_2. Additionally, traces of 4-*O*-acetyl-*N*-glycolylneuraminic acid (Neu4Ac5Gc) were found, which was not detected by the previously used analytical methods.

Table 1. Mass spectrometry of Sia isolated from guinea-pig serum

Fragment ions	*A*	*B*	*C*	*D*	*E*	*F*	*G*
Neu5Ac	726	624	536	356	375	205	173
Neu5Gc	814	712	624	444	375	205	261
Neu4,5Ac_2	696	594	506	356	None	205	143
Neu4Ac5Gc	ND	ND	594	444	None	205	ND
	(784)	(682)					(231)

ND = not detected
The characteristic fragment ions of the Sia were obtained after pertrimethyl silylation in combination with gas-liquid-chromatography [23]. All m/z values have been detected with the exception of Neu4Ac5Gc, for which only the fragments C, D, and F were found. Expected values for the fragment ions A, B, and G [23] are indicated in brackets.

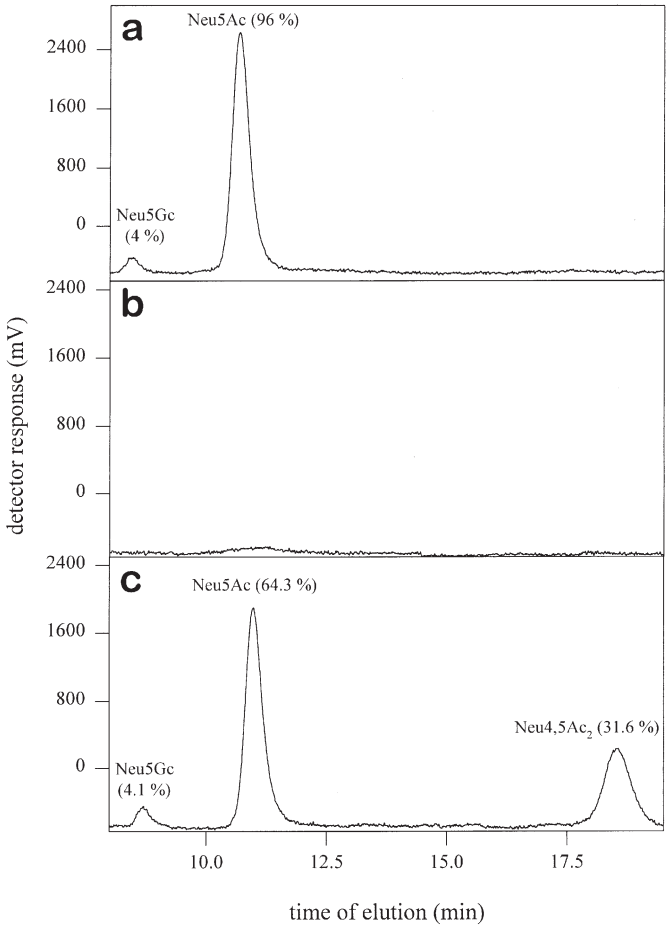

Figure 3. Incubation of guinea-pig serum with sialidase and two additional control reactions. (a) Incubation of serum with AUS, (b) control assay without AUS, and (c) hydrolysis of the same serum sample with propionic acid. The resistance of the third peak of Figure 4c corresponding to originally glycoprotein-bound *O*-acetylated Sia toward sialidase is in favor of its Neu4,5Ac_2 nature. This serum sample contained the typical amount of about 32% glycosidically bound Neu4,5Ac_2.

Based on these results obtained with Sia from guinea-pig serum, we analyzed the Sia of total guinea-pig liver and the Golgi-fraction isolated from this tissue by HPLC after propionic acid hydrolysis. Both materials contain the same kind of Sia but at different ratios: Neu5Ac (total liver 85%, Golgi-vesicles 78%) is found in highest yields, followed by Neu4,5Ac_2 (liver 10%, Golgi-vesicles 18%) and Neu5Gc (liver 5%, Golgi-vesicles 4%). The content of Sia in guinea-pig liver homogenate was about 1.5 nmol/mg protein, and in Golgi-vesicles the amount turned out to be 80-fold higher with 120 nmol/mg protein. Comparably, serum contained a medium Sia quantity (38 nmol Sia/mg protein), but the highest relative amount of Neu4,5Ac_2 (61% Neu5Ac, 32% Neu4,5Ac_2, and 7% Neu5Gc).

Enzymatic sialic acid 4-*O*-acetylation

Based on the observation that Neu4,5Ac_2 was present in the guinea-pigs used for this study in significant amounts and with analytical tools in hands allowing the analysis of minute quantities of 4-*O*-acetylated Sia, we investigated the fundamental enzymatic *O*-acetylation reaction of Sia in guinea-pig liver and tried to get insight into the subcellular site of this process. After separation of different subcellular membrane fractions from guinea-pig liver, using a saccharose discontinuous density gradient, the 4-*O*-acetylation of endogenous substrates in the different fractions was tested by incubation of the particulate fractions with Ac-CoA and measurement of the radioactivity incorporated. Figure 4a shows that the highest amount of radioactivity per mg protein was incorporated into the Golgi-enriched fraction, thus allowing the conclusion that the 4-OAT in guinea-pig liver is a membrane-bound enzyme. No 4-OAT activity was found in the particle-free supernatant of the homogenate, as was tested with fetuin. This glycoprotein was shown to be a substrate for the solubilized 4-OAT

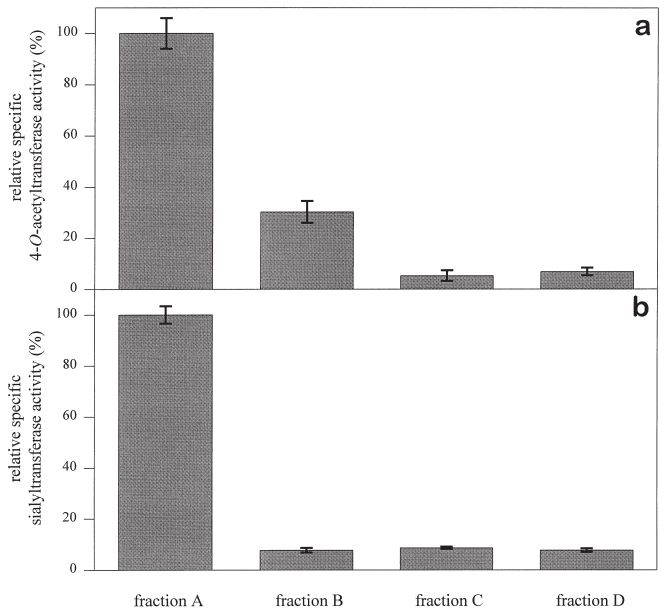

Figure 4. Subcellular location of sialate 4-*O*-acetyltransferase and sialytransferase activities in guinea-pig liver. The distribution of activities of the sialate-4-*O*-acetyltransferase (a) and sialyltransferase (b) over the membrane fractions A-D obtained by discontinuous saccharose density gradient centrifugation were carried out [24]. The highest measured amounts of [^{3}H]acetate/mg protein incorporated into Sia (896 pmol/min × mg protein) (a) as well as of transferred Sia (2.15 nmol/min × mg protein) (b) were each set to 100%. The experiments were carried out twice.

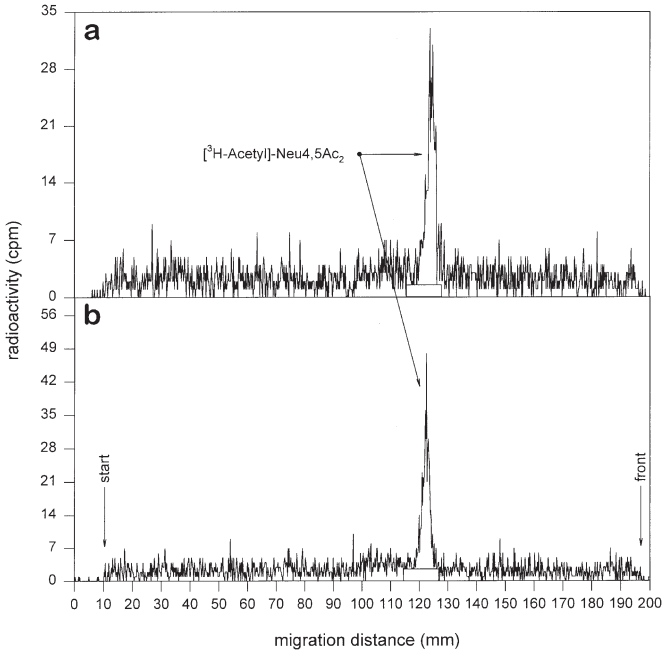

Figure 5. Radio-TLC on cellulose of purified, radioactively labeled Sia isolated from guinea-pig liver Golgi-membrane fractions. Only one labeled Sia ($R_{fSystem2}$ = 0.61) was obtained (a) that was resistant to saponification with influenza C-virus esterase (b). Authentic Neu4,5Ac$_2$; comigrates with the radioactive Sia: The rectangles demonstrate the position of orcinol staining. As described in the section *Enzymatic sialic acid analyses*, Sia from bovine submandibular gland mucus glycoprotein were treated in a parallel experiment leading to 75% de-*O*-acetylation of Neu5,9Ac$_2$ under the conditions used.

(Iwersen, M., Schmid, H. & Schauer, R., *et al.*, unpublished results). The 4-OAT is co-localized with sialyltransferase activity in the Golgi-vesicles, as can be seen from Figure 4b. This also confirms the prevalence of Golgi-membranes in fraction A.

On TLC-analyses in three different solvent systems, the acid-released and purified radioactively labeled Sia residues comigrated with the stained band of authentic Neu4,5Ac$_2$ [$R_{f\ SYSTEM\ 1}$ (relative migration rate) = 0.51, $R_{f\ SYSTEM\ 2}$ = 0.61 (Figure 5a), $R_{f\ SYSTEM\ 3}$ = 0.27]. To confirm the position of the *O*-acetyl groups at C-4, Sia liberated from radioactively labeled guinea-pig liver Golgi-membranes were incubated with influenza C-viruses and applied to TLC-plates after their purification. The inability of the esterase to saponify the ester group (Figure 5b) agrees with experiments made with standards (see above) and confirmed that the only enzymatic *O*-acetylation of Sia in Golgi-enriched fractions from guinea-pig liver was due to an enzyme transferring acetyl groups to the hydroxyl at C-4 of Sia. The observation that AUS did not release incorporated radioactivity from Golgi-membranes also contributed to the identification of the neo-*O*-acetylated Sia as Neu4,5Ac$_2$.

Since the synthesis of [^{3}H]Neu4,5Ac$_2$ contributes to the major part of incorporated radioactivity, we continued to characterize the insoluble, Golgi-bound 4-*O*-acetyltransferase activity by this assay using endogenous substrates. The enzyme operates optimally at 30°C and pH 6.7, with broad optima for these two parameters, as well as in 70 mM potassium phosphate buffer in the presence of 90 mM KCl (Figures 6a to 6d). The radioactivity was taken up at a linear rate within the first 5 min of incubation and then led gradually into a plateau after about 30 min. No significant increase of enzyme activity was detectable by the addition of further AcCoA after 30 min of incubation, showing that the enzymatic system is already saturated by its cosubstrate under these conditions of a 10-fold excess of AcCoA regarding the K_M-value. Experiments with variable amounts of AcCoA and a constant quantity of endogenous substrate resulted in the determination of an app K_M of 0.6 µM for AcCoA and the maximal velocity V_{max} of 19.9 pmol/min × mg protein (Figure 7). The enzyme reaction was inhibited by CoA in a mixed competitive type, revealing a K_i-value of about 4.2 µM (Figure 8). We could further demonstrate that the uptake of radioactivity into Golgi-membranes was dependent on the protein concentration and linear in the range from 30 to 240 µg protein under the assay conditions. Ammonium acetate optimally stimulated the activity at 100 mM, whereas concentrations above 150 mM inhibited it. Furthermore, significant inhibitory effects on the *O*-acetyl-

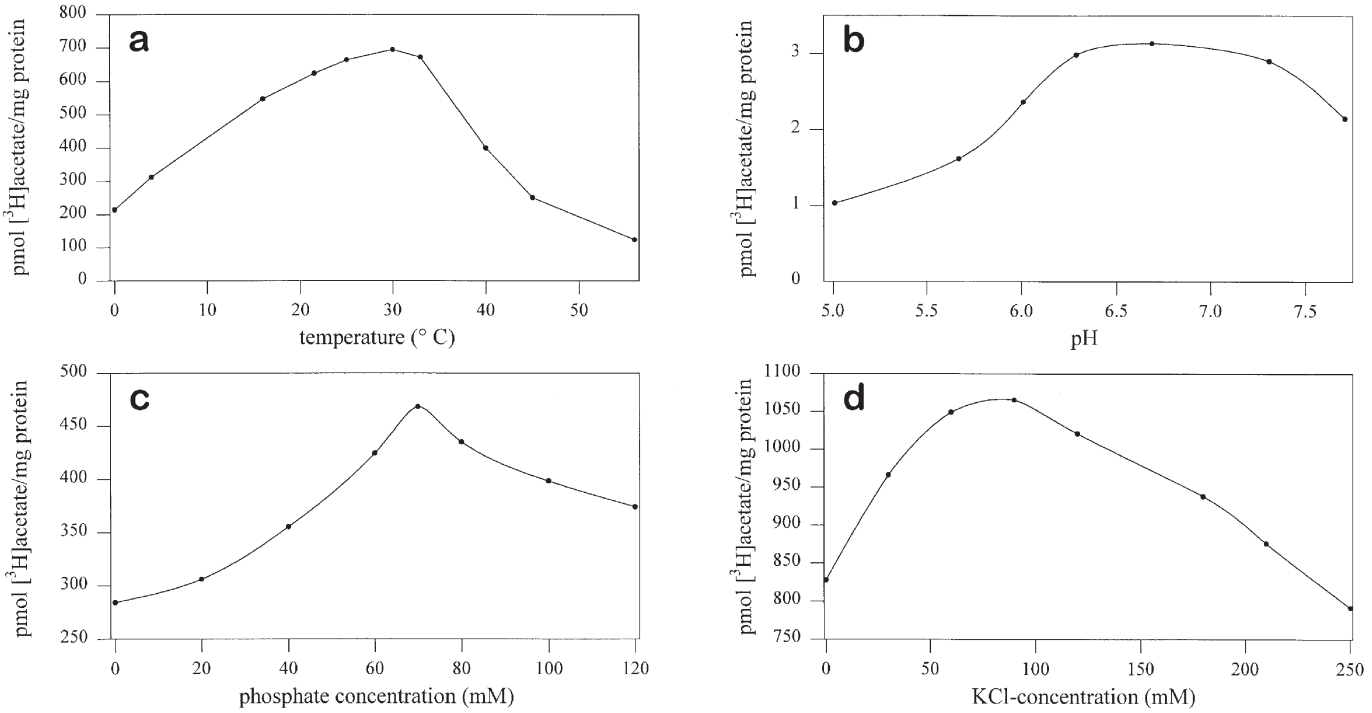

Figure 6. The optimal conditions for radioactive labeling of endogenous Sia by AcCoA in Golgi-membranes from guinea-pig liver. The dependence on the temperature (a), pH (b), phosphate buffer concentration at pH 6.7 (c), and KCl concentration (d) of the acetyl transfer is shown. In (a), (c), and (d) the incubation occurred at pH 6.7; in (a) and (b) 20 mM phosphate buffer was used containing 150 mM KCl, whereas in (c) KCl was omitted; (d) was carried out in optimal potassium phosphate buffer. The incubation temperature was 30°C (b–d).

transferase activity were observed with *p*-CMB (85% inhibition at 1 mM), $MnCl_2$ (20% inhibition at 2 mM), saponin (60% inhibition at 0.015%), and Triton X-100 (50% inhibition at 0.015%). Interestingly, 0.002% Triton X-100 had a weak activating effect (10%).

Since the yield of 4-*O*-acetylation may have been decreased by the presence of sialate-4-*O*-acetylesterase activity in the Golgi-enriched fractions, experiments were carried out in the presence and absence of the esterase inhibitor DFP. Such esterase activity seems to be absent, because only negligible differences were visible. Accordingly, no such esterase activity could be shown by incubating the membrane fractions with MU-Ac or free $Neu4,5Ac_2$; no significant hydrolysis of these esters was observed.

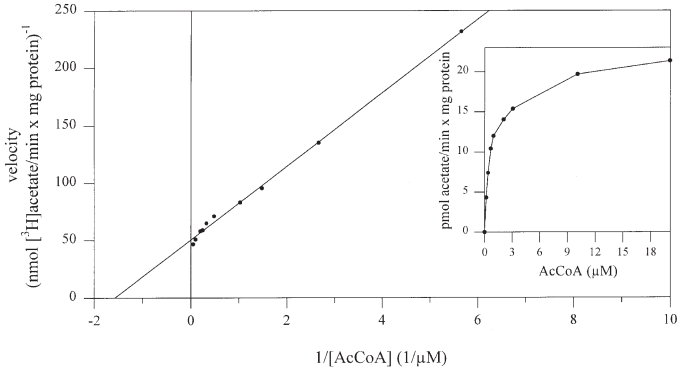

Figure 7. Investigation of Michaelis-Menten kinetics for AcCoA of the transfer of acetyl groups to Golgi-membrane-bound acceptors from guinea-pig liver. According to the plots shown, the K_M for the incorporation of acetate was 0.6 µM and the V_{max}-value was 19.9 pmol/min × mg protein.

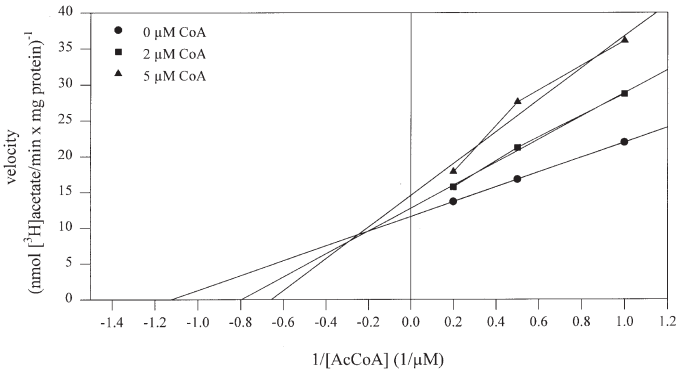

Figure 8. Inhibition of the transfer of acetyl groups from AcCoA to Golgi-membrane-bound acceptors by CoA. The conditions of incubation were the same as in Figure 9. The inhibitory constant K_i of 4.2 µM was calculated.

Discussion

We report for the first time on the investigation of Sia from serum and liver of guinea-pig by reversed phase HPLC of the corresponding fluorescent derivatives and by GC-MS analysis after pertrimethyl silylation. The occurrence of Neu4,5Ac$_2$ besides of Neu5Ac had been detected before in the α_2-macroglobulin of this animal by NMR-spectroscopy [9]. In serum, we furthermore found Neu5Gc and traces of Neu4Ac5Gc.

As an additional tool to discriminate 4-*O*-acetylation from other *O*-acetyl derivatives of Sia, we applied sialidase to glycosidically bound Sia and sialate-pyruvate lyase, as well as influenza C-virus esterase to free Sia, thus confirming the occurrence of Neu4,5Ac$_2$ in guinea-pig, because this type of Sia does not serve as substrate for these enzymes [10, 21,22]. This enzymatic approach is of great value for the analysis of trace amounts of *O*-acetylated Sia and makes it extremely suitable for the identification of radioactive species from biosynthetic studies.

Since more than 30% *O*-acetylation of Neu5Ac at C-4 was observed in guinea-pig serum, these animals allow the investigation of the AcCoA:sialate-4-*O*-acetyltransferase on a subcellular level. Earlier attempts to demonstrate a 4-OAT activity were made by incubation of surviving slices of horse submandibular glands with radioactive precursors, which showed a vivid synthesis of 4-*O*-acetylated Sia [16]. However, experiments to characterize and localize this enzyme incorporating acetate into the pyranose ring of this monosaccharide have not yet been carried out. The 4-OAT in guinea-pig liver was found to be localized in the Golgi-membranes obtained after discontinuous saccharose gradient centrifugation carried out [24], together with sialytransferase activity. This agrees with studies on 7(9)-*O*-acetyltransferase(s) in bovine submandibular glands [28, 14] and in rat liver [27, 29], where the enzyme activities were also shown to be localized in the Golgi-membrane fractions. Co-chromatography of the radioactively labeled neo-*O*-acetylated Sia released from endogenous substrates by acid on TLC-plates, together with authentic Neu4,5Ac$_2$, as well as the susceptibility to saponification and the resistance toward sialidase, lyase, and esterase, proved its nature as Neu4,5Ac$_2$. Characterization of the membrane-bound enzyme surprisingly revealed identity with the rat liver system regarding the K_M for AcCoA (0.6 μM in both guinea-pig and rat liver Golgi-membranes) and V_{max} (19.9 pmol/min × mg protein in guinea-pig liver Golgi-membranes and 21.7 pmol/min × mg protein in rat liver Golgi-membranes), whereas the K_i-values differed (4.2 μM for the guinea-pig and 26.3 μM for the rat system) but were still in the same order of magnitude and followed the same type of inhibition. Compared with the bovine system [14], these parameters are also in the same range, and the V_{max}-values are even identical. Furthermore, the data from other experiments such as the time-dependent uptake of acetate into membrane-bound acceptors or inhibition of the same reaction by CoA or *p*-CMB show similarities in both animals, whereas optimal conditions for incubation such as temperature, pH, buffer, and salt concentration differ from each other.

The experimental data are not yet sufficient to decide whether a transmembrane enzyme complex consisting of an AcCoA transporter, a hydrolase, and the actual *O*-acetyltransferase supposed to occur in the rat liver system [12] also operates in guinea-pig liver or whether another pathway exists. All attempts to completely purify or clone one of the sialate-*O*-acetyltransferases have failed so far. However, an increase of *O*-acetylation of Sia in CHO cells after the transfection of a cDNA encoding the lactosamine-specific sialyltransferase ST6Gal I has been reported [30]. This has also been observed in COS cells after the transfection of a cDNA clone encoding a part of the bacterial P3 plasmid or encoding the rat vitamine D-binding protein after the stable transfection of G_{D3} synthase (ST8Sia I) and ST6Gal I, respectively, to generate acceptor molecules for *O*-acetylation [31]. Additional experiments have to be performed to clarify these findings, but it is primarily important to solubilize and purify the enzyme. Only cDNA of the putative AcCoA transporter isolated from COS-1 cells [32] and cDNA from CST cells encoding the expression of a protein involved in the biosynthesis of 9-*O*-acetyl HG_{D3} after transfection of cDNAs from rat brain by a mammalian expression vector [33] have been cloned. Work is in progress to purify and further characterize the 4-OAT from guinea-pig liver.

Acknowledgments

Dr. Valérie Vandamme-Feldhaus was recipient of a postdoctoral fellowship from the *Deutsche Forschungsgemeinschaft (DFG)*, Bonn, Germany. Thanks are due to *Professor Dr. Georg Herrler*, *Institut für Virologie*, *Tierärztliche Hochschule*, Hannover, Germany, who kindly provided the influenza C-viruses; *Professor Dr. J.P. Kamerling*, and *Dr. C. Versluis*, *Department of Bio-Organic Chemistry*, *Bijvoet Center for Biomolecular Research*, *Utrecht University*, Utrecht, the Netherlands, for GC-MS analyses; and Dipl.-Ing agr. *Hiltrud Schmid* for expert technical assistance. This work was supported by the *Fonds der Chemischen Industrie*, Frankfurt, the *Mizutani Foundation for Glycoscience,* Tokyo, and the *Sialic Acids Society,* Kiel.

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Received 4 March 1998; revised and accepted 27 April 1998