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SPECIFICITY OF PORCINE LIVER Galβ(1 → 3)GalNAc-R α(2 → 3) SIALYLTRANSFERASE

SIALYLATION OF MUCIN-TYPE ACCEPTORS AND GANGLIOSIDE G_{M1} IN VITRO

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Porcine liver microsomes are capable of transferring sialic acid from CMP-NeuAc to [^{14}C]galactosylated ovine submaxillary asialo-mucin, porcine submaxillary asialo/afuco-mucin and ganglioside G_{M1} The specificity of the porcine liver sialyltransferase (CMP-*N*-acetylneuraminate D-galactosyl-glycoprotein *N*-acetylneuraminyltransferase, EC 2 4 99 1) towards the first acceptor, [^{14}C]Gal-GalNAc-protein, was investigated by means of methylation studies on the oligosaccharide chains cleft-off from the sialylated product glycoprotein by β-elimination under reductive conditions It appeared that sialic acid was transferred solely to position C-3 of galactose residues on Galβ(1 → 3)GalNAc disaccharide units Transfer to GalNAc residues was completely absent Competition experiments and heat inactivation studies suggested that the same enzyme also converts ganglioside G_{M1} to ganglioside G_{D1a} Therefore, this porcine liver sialyltransferase can be designated as a Galβ(1 → 3)GalNAc-R α(2 → 3) sialyltransferase

Introduction

To date, many glycoproteins of different organic origins are known, which contain *O*-glycosidically linked oligosaccharide chains of the following structure NeuAcα(2 → 3)Galβ(1 → 3)[NeuAcα(2 → 6)]-GalNAc [1] Accordingly, it is often expected that the two sialyltransferases required for the synthesis of this structure occur in the respective tissues

With regard to a hepatic sialylation of *O*-glycosidically linked chains, it is known that porcine liver contains a sialyltransferase which introduces sialic acid into ovine submaxillary asialo-mucin [2] It has previously been suggested that this transfer was to *N*-acetylgalactosamine residues in a position other than C-6 [2] Recently, however, we showed that porcine liver in fact is incapable of transferring sialic acid to *N*-acetylgalactosamine and that the above-mentioned incorporation was due to the sialylation of galactose residues on Galβ(1 → 3)GalNAc units,

Abbreviations NeuAc, *N*-acetylneuraminic acid, NeuAcOH, reduced *N*-acetylneuraminic acid, Gal, galactose, GalOH, galactitol, GalNAc, *N*-acetylgalactosamine, GalNAcOH, *N*-acetylgalactosaminitol, Glc, glucose, CMP-NeuAc, cytidine-5'-monophospho-*N*-acetylneuraminic acid, UDP-Gal, uridine-5'-diphospho-galactose, ganglioside G_{M1}, Galβ(1 → 3)-GalNAcβ(1 → 4)[NeuAcα(2 → 3)]Galβ(1 → 4)Glc-Cer, ganglioside G_{D1a}, NeuAcα(2 → 3)Galβ(1 → 3)GalNAc-β(1 → 4)[NeuAcα(2 → 3)]Galβ(1 → 4)Glc-Cer, ganglioside G_{D1b}, Galβ(1 → 3)GalNAcβ(1 → 4)[NeuAcα(2 → 8)-NeuAcα(2 → 3)]Galβ(1 → 4)Glc-Cer All sugars are in the pyranose form and are of the D configuration The prefixes asialo- and asialo/afuco- refer to the removal of sialic acid and/or fucose by mild acid hydrolysis

Supplementary data to this article are deposited with, and can be obtained from, Elsevier/North-Holland Biomedical Press B V , BBA Data Deposition, P O Box 1345, 1000 BN Amsterdam, The Netherlands Reference should be made to No BBA/DD/182/69307/660/161–169 The supplementary information includes a table of the numerical values of the data from Fig 4

which occur as minor chains on ovine submaxillary asialo-mucin [3] The type of linkage still remained to be established

This study was undertaken to investigate the specificity of the Galβ(1 → 3)GalNAc-protein sialyltransferase of porcine liver by means of detailed studies on the structure formed in vitro using a more appropriate mucin-type acceptor, containing Gal-GalNAc sequences to a far greater extent than occurring in ovine submaxillary asialo-mucin For this purpose, in vitro [^{14}C]galactosylated ovine submaxillary asialo-mucin [4] was used In addition, experiments were carried out to determine whether this sialyltransferase could also be involved in the conversion of ganglioside G_{M1} to G_{D1a} This work has been presented in a preliminary form [5]

Materials and Methods

Materials Porcine liver was obtained from a local slaughterhouse The tissue was transported to the laboratory on ice and stored frozen at −20°C until use Ovine submaxillary mucin was a kind gift from Dr P A Roukema, Vrije Universiteit, Amsterdam Porcine submaxillary mucin was prepared according to de Salegui and Plonska [6] The mucin was desialylated and defucosylated by mild acid hydrolysis for 1 h in 0 1 M trifluoroacetic acid at 100°C Ganglioside G_{M1} was extracted from formalin-fixed human brain [7] and partially purified by chromatography on DEAE-Sephadex [8] G_{D1a} and G_{D1b} were kindly donated by Dr Berra, Milano Cutscum (iso-octylphenoxypolyoxyethanol) was purchased from Fisher Scientific Co CMP-[^{14}C]NeuAc (1 68 Ci/mol) was obtained from New England Nuclear Corp and NaB^3H_4 (293 Ci/mol) from The Radiochemical Centre, Amersham Unlabeled CMP-NeuAc was prepared as described previously [9] Partially methylated galactose standards were a kind gift of Dr P Stoffyn, E K Shriver Center, Waltham, MA Neuraminidase type VI (*Clostridium perfringens*) and sialyllactose from bovine colostrum were obtained from Sigma NeuAcOH, GalNAcOH and GalOH were prepared by $NaBH_4$ reduction of NeuAc, GalNAc and Gal, respectively The preparation of NeuAcα(2 → 6)-GalNAcOH [10] and Galβ(1 → 3)GalNAcO^{3}H [4] was described before NeuAcα(2 → 3)Galβ(1 → 3)-GalNAcOH and NeuAcα(2 → 3)Galβ(1 → 3)[NeuAcα(2 → 6)]GalNAcOH were prepared from fetuin [11] Analytical grade resins were from BioRad Other materials were of the highest purity available from commercial sources and were used without further purification

Preparation of a cell-free porcine liver fraction as sialyltransferase source All operations were performed at 0–4°C Minced porcine liver (6 5 g) was homogenized in 32 ml 0 25 M sucrose at pH 7 4 using a Potter-Elvehjem system with Teflon pestle rotating at 1 400 rev /min for 2 min The homogenate was centrifuged at 1 000 × *g* for 10 min The pellet was discarded and the supernatant was centrifuged at 100 000 × *g* for 60 min The pellet was resuspended in 3 5 ml 0 25 M sucrose, giving a cell-free porcine liver fraction as sialyltransferase source at a protein concentration of 40 mg/ml The suspension was stored at −20°C until use

Preparation of [^{14}C]Gal-GalNAc-protein [^{14}C]-Galβ(1 → 3)GalNac-protein was prepared from ovine submaxillary asialo-mucin The mucin was incubated with porcine submaxillary gland microsomes as source for galactosyltransferase, UDP-[^{14}C]Gal and Triton X-100 at pH 6 0 for 24 h More than 48% of the available GalNAc monosaccharide side chains were converted to Galβ(1 → 3)GalNAc chains Details of the procedure as well as the subsequent purification of this acceptor are given elsewhere [4]

Sialylation of [^{14}C]Gal-GalNAc-protein The incubation mixture contained 6 mg acceptor Galβ(1 → 3)GalNAc-protein (3 μmol [^{14}C]Gal, 0 0348 Ci/mol), 3 μmol CMP-NauAc, 6 5 μl Cutscum, 30 μmol Tris-maleate (pH 6 7) and liver cell-free preparation (23 mg of protein) in a total volume of 600 μl To prevent microbial growth, 10 μl toluene was added After 24 h at 37°C, the incubation mixture was diluted to 14 ml with 0 15 M NaCl (pH 7 0) and centrifuged at 100 000 × *g* for 60 min to sediment the microsomes The supernatant containing the sialylated glycoprotein was lyophilized Detergent was removed by extracting the residue three times with 1 ml ethanol Low molecular weight substances were removed from the sialylated glycoprotein by gel filtration on a column of Bio-Gel P-4 as described previously [4]

Isolation of reduced oligosaccharides from sialylated [^{14}C]Gal-GalNAc-protein Oligosaccharide chains were cleft off by β-elimination under reductive

conditions The glycoprotein was incubated in 5 ml 0 1 M NaOH containing 1 M NaB^3H_4 (0 606 Ci/mol) for 72 h at 37°C The excess of NaB^3H_4 was destroyed by the addition of 4 M acetic acid at 0°C until pH 6 was reached Cations were removed by passing the mixture over a column (1 6 × 8 cm) of Dowex 50-X2 H^+, 100–200 mesh at 0°C, which was washed with 4 bed vol 0 01 M formic acid The combined eluate and wash were lyophilized Boric acid was removed as methylborate by five additions and evaporations of methanol containing 5% (v/v) acetic acid Reduced acidic oligosaccharides were separated from neutral chains by passing the mixture over a column (0 6 × 6 cm) of Dowex 1-X2 acetate, 100–200 mesh, equilibrated with water Neutral oligosaccharides were washed from the column with 4 bed vol water Reduced acidic oligosaccharides were then eluted with 8 ml 1 M pyridine acetate (pH 5 4) and subsequently fractionated on a calibrated column (1 6 × 200 cm) of Bio-Gel P-4, 200–400 mesh in 0 05 M pyridine acetate (pH 5 4) eluted with the same buffer at a flow rate of 10 ml/h In a similar way, the neutral oligosaccharides were further resolved on a column (1 6 × 200 cm) of Bio-Gel P-2, 200–400 mesh Appropriate fractions were pooled and lyophilized

Chromatography and electrophoresis of oligosaccharides Thin-layer chromatography (TLC) of sialylated oligosaccharides, NeuAc and NeuAcOH was performed on Silica gel 60 plates (Merck) employing solvent A ethylacetate/1-butylacetate/acetic acid/water (2 1 2 1, v/v), and on cellulose plates (Merck) with solvent B 1-butanol/ethanol/acetic acid/water (10 8 2 3, v/v) Both solvents were used under continuous flow for 17 h in a jar 15 cm in height Visualization was done with resorcinol-hydrochloric acid [12] or periodate-resorcinol [13] Neutral oligosaccharides were chromatographed on Silica gel 60 plates in solvent C ethylacetate/pyridine/water (10 4 3, v/v) for 2 h and in solvent D ethylacetate/1-propanol/2-propanol/water (8 5 1 1, v/v) for 7 h under continuous flow [14] Reference compounds were located with aniline phthalate or periodate-benzidine ^{14}C-labeled compounds were detected by autoradiography and ^3H-labeled materials by fluorography [15] High voltage electrophoresis of reduced monosaccharides was performed on Whatman 3 MM paper strips in 1% sodium tetraborate at 5–10°C at 65 V/cm for 60 min Reference compounds were revealed with periodate-benzidine Gas chromatography of reduced neutral disaccahrides was done after direct trimethylsilylation as described previously [4]

Methylation analysis Methylation and subsequent isolation of the methylated oligosaccharides were performed as described previously [16] The methylated samples were hydrolyzed in 400 μl 2 M trifluoroacetic acid for 1 h at 115°C After the addition of 2 ml ethanol, the mixtures were evaporated under a nitrogen stream The residues were redissolved in 50 μl methanol and aliquots were spotted on Silica gel 60 plates and run in solvent system E acetone/4 5 M NH_4OH (500 9, v/v) with appropriate tri- and tetramethylgalactose references [17] Detection of reference compounds was done with aniline phthalate

Neuraminidase digestion of sialylated products The reduced acidic trisaccharide obtained from the sialylated glycoprotein by β-elimination and subsequent gel filtration was digested with neuraminidase The reaction mixture contained in 100 μl 180 nmol reduced trisaccharide, 0 9 I U *Cl perfringens* neuraminidase (EC 3 2 1 18) and 10 μmol sodium acetate buffer, pH 5 0 After incubation for 3 h at 37°C, the mixture was diluted with 1 ml water and applied to a column (0 6 × 6 cm) of Dowex 1-X8 acetate, 100–200 mesh The desialylated oligosaccharide was washed from the column with 4 bed vol water and further purified by passage over a column (1 6 × 40 cm) of Bio-Gel P-4, 100–200 mesh, equilibrated and eluted with 0 05 M ammonium acetate (pH 5 4) Fractions containing ^{14}C radioactivity were pooled, lyophilized and characterized by TLC in solvent systems C and D

The reduced acidic disaccharide was desialylated in a similar way The incubation mixture contained 33 nmol disaccharide, 0 8 I U neuraminidase and 10 μmol sodium acetate buffer (pH 5 0) in a total volume of 100 μl After incubation for 3 h at 37°C, the resulting neutral monosaccharide was isolated and purified as indicated above, and subsequently analyzed by high voltage electrophoresis

Sialyltransferase assay A modified version of the sialyltransferase assay of Grimes and Robbins [18] was used to measure the separate sialic acid incorpo-

ration into glycolipid and glycoprotein after both acceptors have been sialylated simultaneously The glycoprotein acceptor used was porcine submaxillary asialo/afuco-mucin, containing 319 nmol theoretical acceptor sites (calculated as available galactose) per mg protein The glycolipid acceptor was ganglioside G_{M1} The standard incubation mixtures contained 0 126 μmol CMP-[^{14}C]NeuAc (0 792 Ci/mol), 0 65 μl Cutscum, 4 μmol Tris-maleate (pH 6 7) various amounts of asialo/afuco-mucin (ranging from 0 08 to 1 6 μmol theoretical acceptor sites) and/or various amounts of ganglioside G_{M1} (ranging from 0 12 to 0 8 μmol) and porcine liver cell-free preparation (410 μg of protein) in a total volume of 80 μl After incubation for 1 h at 37°C, the reaction was stopped by cooling in ice and the addition of 2 9 ml of an ice-cold solution of 0 5 M HCl containing 1% phosphotungstic acid (w/v) The tubes were centrifuged and the resulting pellets were washed twice with 2 ml of the same solution Subsequently, the pellets were extracted once with 4 ml methanol/ether (1 1, v/v) and twice with 2 ml ethanol/ether (1 1, v/v) The organic extracts were collected in counting vials and, after evaporation of the solvent by mild warming, radioactivity was assyed by liquid scintillation The extracted pellets were dissolved in 200 μl Soluene 350 (Packard) and counted Radioactivity extractable into the organic solvent originates from glycolipid, whereas radioactivity remaining in the pellet is present in glycoprotein, as was confirmed by control experiments with both acceptors separately An endogenous acceptor control incubation lacking exogenous glycoprotein and glycolipid acceptor was included for each set of assays and the result was subtracted from the incorporation in the presence of exogenous acceptor

Results

Sialylation of [^{14}C]Gal-GalNAc-protein

[^{14}C]Galactosylated ovine submaximillary asialomucin as well as porcine submaxillary asialo/afucomucin, which glycoproteins both contain Galβ(1 → 3)GalNAc disaccharide chains, appear to be good acceptors for the porcine liver sialyltransferase investigated in this study At a concentration of 5 mM theoretical acceptor sites, the rate of incorporation into the latter glycoprotein amounted to 13 nmol/h per mg protein When [^{14}C]Gal-GalNAc-protein was used as an acceptor in the large scale incubation, 81% of the initial ^{14}C radioactivity was recovered after centrifugation and gel filtration as high molecular weight material After β-elimination of this sialylated glycoprotein product in the presence of NaB^3H_4 and anion-exchange chromatography of the resulting oligosaccharide material, 1 04 μmol ^{14}C- and ^{3}H-containing neutral chains and 0 85 μmol acidic oligosaccharides were recovered, indicating that about 45% of the available [^{14}C]Gal-GalNAc disaccharide units of the glycoprotein were sialylated

Fractionation and characterization of reduced acidic and neutral oligosaccharides

The reduced acidic oligosaccharide fraction, which was recovered after the anion-exchange step, was fractionated on Bio-Gel P-4 This resulted in the separation of two ^{14}C- and ^{3}H-labeled peaks in addition to a peak containing ^{3}H radioactivity only (Fig 1) The

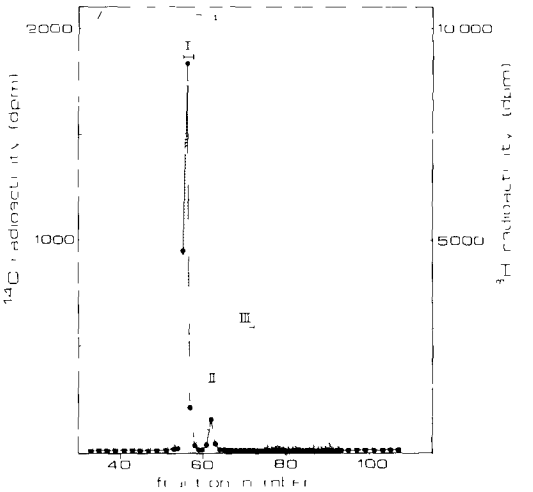

Fig 1 Fractionation of sialylated oligosaccharides by gel filtration Reduced acidic oligosaccharides obtained from [^{14}C]Gal-GalNAc-protein sialylated by porcine liver cell-free preparation in vitro were applied to a column of Bio-Gel P-4, equilibrated in 0 05 M pyridine acetate, pH 5 0, and eluted with the same bufter (flow rate 10 ml/h) Fractions of 4 ml were collected and assayed for ^{14}C (●——●) and ^{3}H radioactivity (○ ○) The column was calibrated with (1) NeuAcα(2 → 3)Galβ(1 → 3)[NeuAcα(2 → 6)]GalNAcOH, (2) NeuAcα(2 → 3)Galβ(1 → 3)GalNAcOH, (3) NeuAcα(2 → 6)-GalNAcOH, (4) NeuAcα(2 → 3)GalOH and (5) galactose The bars indicate the fractions that were pooled for further analysis

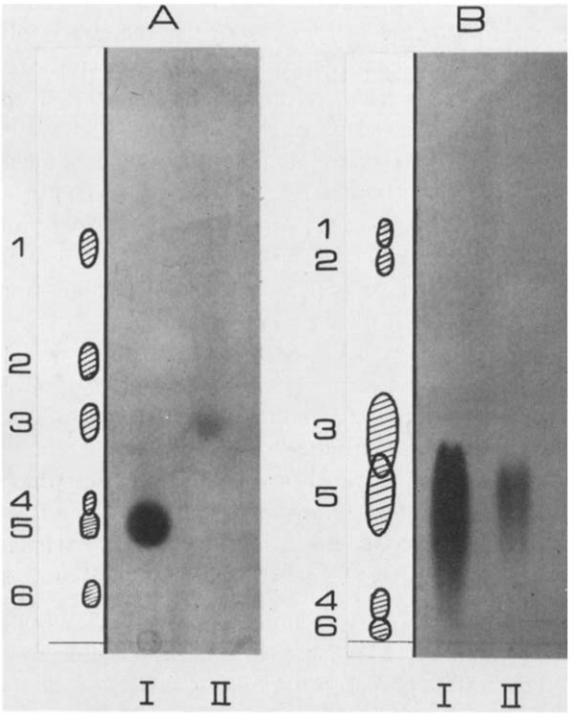

Fig 2 TLC of sialylated oligosaccharides Sialylated oligosaccharide fractions I and II were run on (A) a silica gel 60 plate (Merck) in solvent system A and (B) a cellulose plate (Merck) in system B Reference compounds are (1) NeuAcOH, (2) NeuAc, (3) NeuAcα(2 → 6)GalNAcOH, (4) NeuAcα(2 → 3)Galβ(1 → 4)Glc, (5) NeuAcα(2 → 3)Galβ(1 → 3)GalNAcOH and (6) NeuAcα(2 → 3)Galβ(1 → 3)-[NeuAcα(2 → 6)]GalNAcOH Radioactive compounds were detected by autoradiography and references by resorcinol-hydrochloric acid (silica gel) or periodate-resorcinol (cellulose)

major peak (I) contained 97% of the ^{14}C radioactivity applied to the column and its elution volume corresponded to that of a sialic acid-containing trisaccharide Upon TLC in systems A and B, the oligosaccharide material of peak I migrated as a single spot with the same mobility as authentic NeuAcα(2 → 3)-Galβ(1 → 3)GalNAcOH (Fig 2) The sialic acid content of oligosaccharide I was assayed by the thiobarbituric acid method [19] after neuraminidase digestion The amounts of [^{14}C]galactose and GalNAcO^{3}H were estimated from the specific radioactivity of the UDP-[^{14}C]Gal used for the galactosylation and that of GalNAcO^{3}H formed in the reductive β-elimination step from unglycosylated GalNAc residues, and isolated from the neutral oligosaccharide fraction [4] A molar ratio of NeuAc and GalNacOH to Gal of 1 02 and 1 09 to 1 00, respectively, was found, indicating that oligosaccharide I (Fig 1) was a sialic acid-containing trisaccharide The neutral disaccharide resulting after the neuraminidase digestion of this trisaccharide migrated like authentic Galβ(1 → 3)GalNAcO^{3}H upon TLC in systems C and D Also in gas chromatographic analysis it had the same relative retention time with reference to the internal standard lactose as Galβ(1 → 3)GalNAcO^{3}H The second peak (II) comprised 3% of the total ^{14}C radioactivity applied to the Bio-Gel column TLC in systems A and B revealed that oligosaccharide II consisted of one single component, which definitely migrated different from NeuAcα(2 → 6)GalNAcOH (Fig 2) After digestion of oligosaccharide II with neuraminidase, the ^{14}C as well as ^{3}H radioactivity in the digest moved together over the same distance as GalOH and unlike GalNAcOH upon high-voltage electrophoresis Hence, it is likely that oligosaccharide II is identical to NeuAc-GalOH, which probably was formed in the β-elimination step as a result of peeling from the parent NeuAc-Gal-GalNAc trisaccharide

The third peak from the Bio-Gel P-4 column (peak III, Fig 1), containing only ^{3}H radioactivity, was not identical to reduced NeuAc or any NeuAc-containing disaccharide Because of the limited amount of material, this peak was not investigated further

The reduced neutral oligosaccharide fraction obtained from the β-elimination of the sialylated [^{14}C]Gal-GalNAc-protein, was resolved on Bio-Gel P-2 into two peaks, which could be identified as the neutral precursoe chain [^{14}C]Galβ(1 → 3)GalNAcO^{3}H and GalNAcO^{3}H, respectively, in a way identical to that described before [4]

Methylation studies

To establish the exact site of sialic acid attachment in [^{14}C]Gal-GalNAc-protein, the trisaccharide product obtained by Bio-Gel P-4 fractionation (peak I, Fig 1) was subjected to methylation analysis As controls, the neuraminidase-treated product and the neutral precursor chain [^{14}C]Gal-GalNAcO^{3}H were treated similarly Essentially all ^{14}C radioactivity in the trisaccharide product was recovered in the

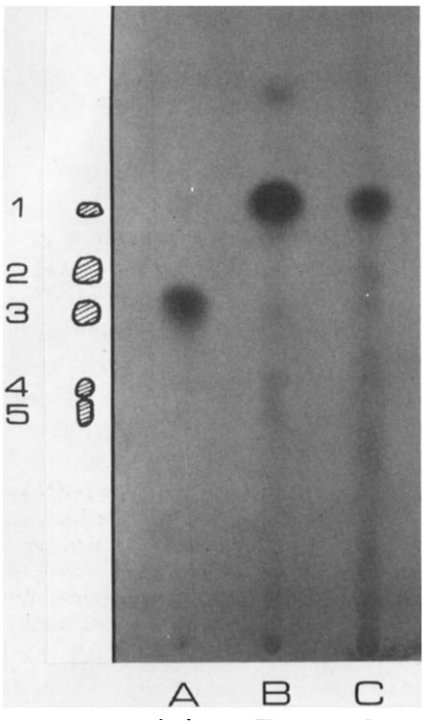

Fig 3 TLC of methyl ethers of [^{14}C]galactose derived from (A) trisaccharide I, obtained from sialylated [^{14}C]Gal-GalNAc-protein, (B) the disaccharide [^{14}C]Galβ(1 → 3)-GalNAcOH obtained by β-elimination of the acceptor [^{14}C]-Gal-GalNAc-protein and (C) neuraminidase-treated trisaccharide I Radioactivity was detected by autoradiography and reference compounds by aniline phthalate References are (1) tetramethylgalactose, (2) 2,3 6-trimethylgalactose, (2) 2,4,6-trimethylgalactose, (4) 2 3,4-trimethylgalactose and (5) 3,4,6-trimethylgalactose

methylated sugar fraction and migrated like 2,4,6-trimethylgalactose, whereas the precursor chain and the neuraminidase-treated product yielded tetramethylgalactose only (Fig 3) Hence, the transfer of sialic acid had taken place to position C-3 of galactose in Galβ(1 → 3)GalNAc chain No minor transfer of sialic acid to position C-6 of galactose or to position C-6 of GalNAc in the trisaccharide had taken place, since at the sites in lane A (Fig 3) where 2,3,4-trimethylgalactose and tetramethylgalactose would have been expected, no radioactivity higher than background levels could be detected by liquid scintillation counting of these areas scraped off from the silica gel plate

TABLE I

KINETIC DATA OF PORCINE LIVER Galβ(1 → 3)GalNAc-R α(2 → 3) SIALYLTRANSFERASE

Acceptor	K_m (mM)	V (nmol/h per mg protein)
Porcine submaxillary		
Asialo/afuco-mucin	1 7	17 3
Ganglioside G_{M1} *	7 6	50
Ganglioside G_{M1} **	2 9	20 2

* Concentration = 0–3 mM
** Concentration >3 mM

Sialylation of ganglioside G_{M1}

In addition to [^{14}C]Gal-GalNAc-protein and porcine submaxillary asialo/afuco-mucin, ganglioside G_{M1} could serve as acceptor for the porcine liver sialyltransferase Sialylation of ganglioside G_{M1} under the conditions described did not obey Michaelis-Menten kinetics, and two sets of kinetic parameters were obtained one for a high acceptor concentration range, and another one for the lower range (Table I) To identify the sialylated ganglioside product, the standard incubation system was scaled up 10-fold and incubated for 17 h The radioactive product, isolated according to Fishman et al [20], moved identically to ganglioside G_{D1a} upon TLC in a system capable of separating the two possible products, G_{D1a} and G_{D1b} [21] Most of the ^{14}C label (more than 90%) could be removed from the glycolipid product by neuraminidase treatment [22]

Competition experiments and heat inactivation studies

Since [^{14}C]Gal-GalNAc-protein, porcine submaxillary asialo/afuco-mucin and ganglioside G_{M1} have the same terminal oligosaccharide sequence Galβ(1 → 3)-GalNAc-R, it was of importance to examine whether the sialic acid transfers to glycoprotein and glycolipid acceptors were accomplished by two different, highly substrate-specific enzymes or by one single enzyme acting on both substrates Therefore, competition experiments were carried out, in which porcine submaxillary asialo/afuco-mucin and ganglioside G_{M1}

were used as the glycoprotein and glycolipid acceptor, respectively Employing the standard sialyltransferase assay, the apparent K_m and the V values were determined for the sialylation of ganglioside G_{M1} and of porcine submaxillary asialo/afuco-mucin separately (Table I) The kinetic constants were obtained from double-reciprocal plots Using the data from Table I, it was possible to calculate to what extent incorporation into ganglioside G_{M1} and porcine submaxillary asialo/afuco-mucin could be expected when both acceptors are sialylated simultaneously, either in the case where one enzyme was acting, or in the case of two independent transferases [23] Since for sialylation of the glycolipid two sets of kinetic constants were found (Table I), both sets were used for the calculations, each set in its appropriate concentration range The experimental data of sialic acid incorporation into both acceptors are very similar to the theoretical values calculated for one single sialyltransferase acting on both substrates (Fig 4)

Additional support was obtained by heat inactivation experiments Both acceptors (ganglioside G_{M1} and porcine submaxillary asialo/afuco-mucin) were

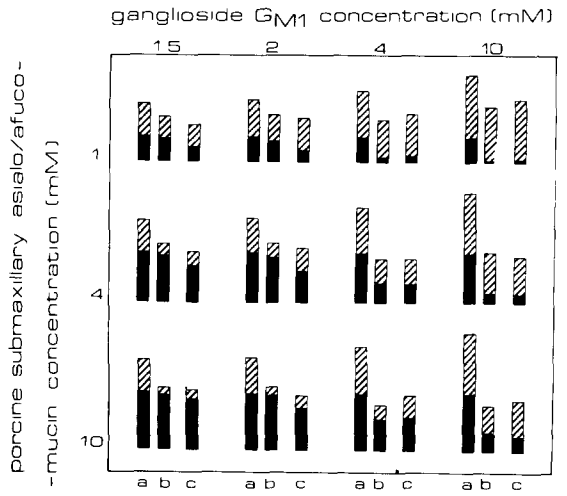

Fig 4 Rate of sialic acid incorporation into porcine submaxillary asialo/afuco-mucin and ganglioside G_{M1} Both acceptors were incubated simultaneously with a porcine liver sialyltransferase preparation, at various concentrations as indicated The theoretical rates of incorporation into the glyco lipid (▨) and into mucin (▬) were calculated from the data in Table I for two independent sialyltransferases (a) and for one single sialyltransferase acting on both substrates (b), and compared with the experimental values (c)

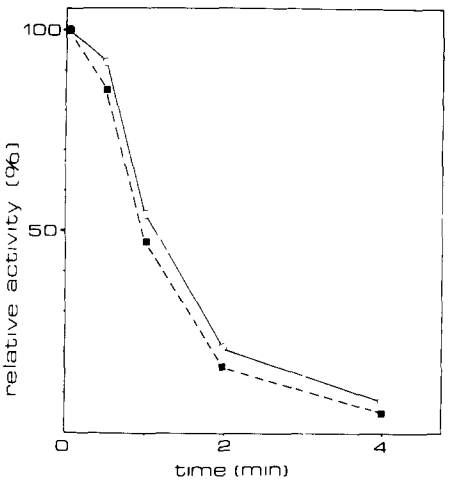

Fig 5 Effect of heat treatment on porcine liver sialyltransferase Porcine liver cell-free preparations were heated at 56°C for 0 5, 1, 2 and 4 min Sialyltransferase activities of these partially inactivated preparations were measured with ganglioside G_{M1} (■ - - - - - - ■) and porcine submaxillary asialo/afuco-mucin (o——o) as acceptors in the standard sialyltransferase assay

incubated with porcine liver sialyltransferase preparations which were partially inactivated by heat treatment The decay in transferase activity of these preparations as a function of heating time was very similar with both acceptors (Fig 5) Together these results strongly suggest that only one sialyltransferase is responsible for the sialylation of the asialo/afuco-mucin as well as ganglioside G_{M1}

Discussion

Porcine liver microsomes appear to be capable of introducing sialic acid from CMP-NeuAc into porcine submaxillary asialo/afuco-mucin, [^{14}C]galactosylated ovine submaxillary asialo-mucin as well as ganglioside G_{M1} Since these acceptor molecules all possess terminal Galβ(1 → 3)GalNAc disaccharide units, the possibility existed that one single sialyltransferase would be responsible for these incorporations Studies on the glycosylation of glycolipids are often hampered by the appearance of micelle formation of the acceptor glycolipid, which might depend on the concentration of the acceptor molecule, the type and the concentration of the detergent used in addition to effects of ions Indeed, two sets of kinetic param-

eters were obtained for the sialylation of ganglioside G_{M1} (Table I) Therefore, the competition experiments with the ganglioside substrate and porcine submaxillary asialo/afuco-mucin were carried out in a broad range of substrate concentrations At all concentrations the results of these experiments indicated that the in vivo sialylation of the glycoprotein and the glycolipid acceptor was due to the action of one single sialyltransferase (Fig 4) This conclusion was further supported by the heat inactivation experiment, which showed a very similar decay in sialyltransferase activity towards both acceptors (Fig 5) It should be noted that this conclusion is in contrast with the early hypothesis that glycosyltransferases involved in the biosynthesis of glycoproteins belong to a set of enzymes different from those involved in glycolipid biosynthesis [24] In favor of this view was the observation that asialofetuin and ganglioside G_{M1} were sialylated by two different transferases from rat brain [25] Other studies, however, revealed that bovine submaxillary asialomucin and ganglioside G_{M1} were sialylated by a single enzyme from normal and transformed mouse and hamster cells [18] Recently, a sialyltransferase purified from porcine submaxillary glands [26] was shown to be capable of sialylating both antifreeze glycoprotein and ganglioside G_{M1} [27], which is in agreement with our results using porcine liver sialyltransferase

The type of linkage which is accomplished by the porcine liver sialyltransferase can be inferred directly from the results of the methylation experiments Since in the methylation analysis of the product chain obtained from sialylated [^{14}C]Gal-GalNAc protein only 2,4,6-trimethyl[^{14}C]galactose has been formed, whereas the precursor chain and the neuraminidase-treated control yield tetramethyl[^{14}C]galactose (Fig 3), the transfer of sialic acid exclusively has taken place to position C-3 of the galactose residue on the acceptor glycoprotein The same type of sialic acid attachment most probably was achieved with ganglioside G_{M1} as a substrate, which was converted to a product migrating like ganglioside G_{D1a} Apparently, the enzyme only recognizes Galβ(1 → 3)-GalNAc sequences, but has no specificity with regard to the anomeric configuration of the GalNAc residue, which is α in mucins [28] and β in gangliosides [29] Therefore, the enzyme can be designated as a Galβ(1 → 3)GalNAc-R α(2 → 3) sialyltransferase

Towards low molecular weight acceptors the enzyme seems to be less specific Porcine liver microsomes are capable of sialylating lactose, to produce both 3′-sialyllactose and 6′-sialyllactose [30] The latter product seems to be formed by an enzyme involved in the sialylation of complex-type serum glycoproteins, which has been reported to occur in porcine liver [31], and shows the same specificity as the rat liver [16] and bovine colostrum [32] β-galactoside α(2 → 6) sialyltransferase The formation of 3′-sialyllactose by porcine liver microsomes on the other hand, cannot be the result of the action of an analogue Galβ(1 → 4)GlcNAc-R α(2 → 3) sialyltransferase, since such an enzyme activity, which recently has been shown to occur in fetal calf liver, is absent from porcine liver [33] Therefore, it is feasible that this formation has to be attributed to the Galβ(1 → 3)GalNAc-R α(2 → 3) sialyltransferase described in this study

The same specificity towards mucin-type acceptors, ganglioside G_{M1} and lactose has been attributed to the afore-mentioned β-galactoside α(2 → 3) sialyltransferase from porcine submaxillary gland [27] In addition, indications have been obtained for the presence of a Galβ(1 → 3)GalNAc-R sialyltransferase in bovine colostrum [34], rat brain [35], TA3-Ha cancer cells [36], rat liver and rat mammary adenocarcinoma [37] as well as 3T3 cells [38] This sialyltransferase thus seems to have a widespread occurrence

It should be noted that the methodology described here bypasses the need of extensive enzyme purification procedures By using an acceptor molecule exclusively bearing a radioactive label in the terminal glycose residue, micromethylation analysis not only allows the unequivocal determination of the specificity of a glycosyltransferase present in crude tissue preparations or body fluids, but also eliminates the risk of missing an unknown enzyme activity towards the acceptor used Our result, that no tetramethyl-[^{14}C]galactose could be detected upon methylation of the sialylated oligosaccharides, therefore clearly demonstrates that the sialyltransferase capable of linking sialic acid to position C-6 of GalNAc on mucins [39,40] is not present in porcine liver In addition, also the fact that no disaccharide NeuAcα(2 → 6)GalNAcO^{3}H was formed fully

confirms the conclusion of our previous study [3] that porcine liver sialyltransferase is incapable of transferring sialic acid to GalNAc monosaccharide units on mucins

This result, however, does not necessarily imply that such a sialyltransferase is absent from liver tissue in general For example, fetuin contains a tetrasaccharide side chain of the structure NeuAcα(2 → 3)-Galβ(1 → 3)[NeuAcα(2 → 6)]GalNAc [11], suggesting that fetal calf liver, in contrast to porcine liver, might contain a GalNAc α(2 → 6) sialyltransferase If so, the question can be put forward whether the occurrence of this enzyme in liver tissue is specific to certain species, or can be attributed to a fetal stage of mammalian development

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