Carbohydrate Research, 149 (1986) 209–223
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

A BRAIN SIALYLTRANSFERASE HAVING A NARROW SPECIFICITY FOR *O*-GLYCOSYL-LINKED OLIGOSACCHARIDE CHAINS*

HÉLÈNE BAUBICHON-CORTAY, MIREILLE SERRES-GUILLAUMOND†, PIERRE LOUISOT‡, AND PIERRE BROQUET§

Laboratoire de Biochimie Générale et Médicale, Unité 189 de l'Institut National de la Santé et de la Recherche Médicale Alliée au Centre National de la Recherche Scientifique, Faculté de Médecine Lyon-Sud, BP 12, F-69921 Oullins (France)

(Received March 8th, 1985; accepted for publication in revised form, July 10th, 1985)

ABSTRACT

The existence of a brain sialyltransferase catalyzing the specific transfer of NeuAc on native fetuin was demonstrated. This enzyme was not able to sialylate either asialofetuin or desialylated and nondesialylated orosomucoid, transferrin, and bovine submaxillary mucin. It required the presence of Mn^{2+} for optimal activity. Morever, in fetuin, this activity was closely related to the proportion of NeuAc residues, but in liver tissue sialylation occurred only onto asialofetuin. In native fetuin, sialylation took place on *O*-glycan chains to give an *O*-disialyltetrasaccharidic structure. The Gal→GalNAc→protein was not an acceptor, but α-NeuAc-(2→3)-Gal→GalNAc→protein was, suggesting a specific transfer α-(2→6) to the GalNAc residue.

INTRODUCTION

Of any tissue, the brain possesses the highest concentration of sialoglycoconjugates[1]. The sialic acid content of the gangliosides accounts for 65% of the total sialic acid in the rat brain, 32% is bound to glycoproteins, and the rest is free sialic acid[2]. Some studies on brain sialyltransferases in the biosynthesis of glycoproteins have been reported for the rat brain by Van den Eijnden and Van Dijk[3], and Ng and Dain[4], and for the embryonic chicken brain by Den *et al.*[5], using either asialoglycoproteins or lactose as acceptors (for a review, see ref. 6). However, in the brain, the demonstration of one single sialyltransferase, or evidence for two or more distinct sialyltransferases, was not always provided. This was in particular the case for studies using as acceptor asialofetuin which is known to contain both *N*-

*This work was supported by the Institut National de la Santé et de la Recherche Médicale (INSERM U.189), the Centre National de la Recherche Scientifique (UA 514), and the University of Lyon, Lyon-Sud Medical School.

†Chargé de Recherche at the Institut National de la Santé et de la Recherche Médicale.

‡To whom all correspondence should be addressed.

§Chargé de Recherche at the Centre National de la Recherche Scientifique.

0008-6215/86/$ 03.50

and *O*-glycan chains, each of them possessing two types of sialyl linkage[7].

Other tissues have been extensively studied. Bauvois *et al.*[8] were able to distinguish, in human platelets, between an α-(2→3)- and an α-(2→6)-sialyltransferase having an *N*-glycan chain linkage specificity. Hill and assoc.[9,10] and Rearick *et al.*[11] have purified to homogeneity two sialyltransferases solubilized from the porcine submaxillary gland, specific for *O*-glycan chains in α-(2→3) and α-(2→6) linkages. Van den Eijnden *et al.* studied the enzymic transfer of sialic acid to bi- and tri-antennary glycopeptides of α_1-glycoprotein[12] and first detected an α-(2→3)-sialyltransferase to the β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl group in fetal calf liver[13]. In addition, Van den Eijnden and assoc. extensively studied the specificity of sialyltransferases from porcine liver by use of mucin-type acceptors[14,15], and from ovine[16] and porcine[17] submaxillary glands. Recently, they demonstrated the processing for sequential sialylation of *O*-glycans of fetuin in the fetal calf liver[18].

In the present work, we provide evidence for the existence of a brain sialyltransferase possessing a narrow specificity for *O*-glycan chains of native fetuin.

EXPERIMENTAL

Materials. — All reagents were of analytical grade. Fetuin (grade III), bovine submaxillary mucin, human serotransferrin, orosomucoid (α_1-acid glycoprotein), *N*-acetylneuraminic acid, sialyllactose, CMP-NeuAc, and neuraminidase–agarose (type X, from *Clostridium perfringens*) were obtained from Sigma (St Louis, USA); CMP-[^{14}C]NeuAc (specific activity 247 Ci/mol), CMP-[^{3}H]NeuAc (specific activity 18.9 Ci/mmol), GDP-L-[^{14}C]fucose (specific activity 170 Ci/mol), and UDP-D-[^{14}C]Gal (specific activity 337 Ci/mol) from New England Nuclear (Boston, U.S.A.), and NaB($^{3}H_4$) (5–20 Ci/mmol) was obtained from Commissariat à l'Énergie Atomique (CEA).

Rats were from OFA strain (issued from Sprague–Dawley strain) and mice from OF1 strain (IFFA-Credo, Les Oncins, France). Mongolian gerbils (*Meriones unguiculatus*) were bred in the laboratory. Pig submaxillary glands and sheep brains were obtained from local slaughterhouses.

Preparation of glycosyltransferase acceptors. — Desialylation of fetuin, bovine submaxillary mucin, orosomucoid, and serotransferrin was performed by mild acid hydrolysis (50mM H_2SO_4, 80°, 60 min) and monitored by the resorcinol–HCl method[19]. As a control for progressive desialylation, fetuin was digested with neuraminidase–agarose. Fetuin (1 g) was passed in a continuous flow (20 mL/h) through a small column (0.5 × 3 cm) containing neuraminidase–agarose (1 unit) in 0.1M acetate buffer (pH 5.0) and an aliquot (100 mg) was taken out every 30 min. Each aliquot was dialyzed against 0.1M NaCl and distilled water, and lyophilized.

Preparation of sialyltransferase (EC 2.4.99.1) from rat brain. — The rat brains were fractionated as described elsewhere[20] in 50mM Tris–HCl buffer (pH 7.1) and 330mM sucrose, and the microsomal pellets were frozen. Immediately before use,

pellets were thawed and suspended in 50mM Tris [2-amino-2-(hydroxymethyl)-1,3-propanediol] buffer (pH 7.1) (referred to later as "Tris buffer").

Preparation of glycoprotein β-D-galactosyltransferase (EC 2.4.1.38) from porcine submaxillary glands. — Porcine submaxillary gland microsomes were prepared according to Van den Eijnden *et al.*[21]. The final membrane suspension was in 0.2M MES [2-(*N*-morpholino)ethanesulfonic acid] buffer (pH 6.0), containing 50mM $MnCl_2$ and 5mM mercaptoethanol (referred to later as "MES buffer").

Sialyltransferase assay. — To Tris buffer (200 μL) containing brain microsomal proteins (400 μg) were added fetuin (400 μg), 0.5% Triton X-100, 5mM $MnCl_2$, 40mM MES (pH 6.0), and CMP-[^{14}C]NeuAc (50 nCi). The incubation was performed at 28° for 120 min, and the reaction stopped with a mixture of trichloroacetic acid (10% w/v) and phosphotungstic acid (5% w/v) (2 mL). The precipitate was filtered on GF-B Whatman filters as described previously[22].

L-*Fucosyltransferase assay.* — To Tris buffer (200 μL) containing brain microsomal proteins (400 μg) were added asialofetuin (400 μg), 0.5% (w/v) Triton X-100, and GDP-L-[^{14}C]fucose (20 nCi). The incubation was carried out at 25° and stopped as described for sialyltransferase.

D-*Galactosyltransferase assay.* — A suspension of porcine submaxillary gland microsomes was diluted five times with 50mM MES (pH 6.0) to lower the incorporation onto endogenous acceptors. To this diluted suspension (200 μL), corresponding to 500 μg of proteins, were added asialomucin (400 μg), 0.75% Triton X-100 (w/v), and UDP-D-[^{14}C]Gal (50 nCi). The incubation was performed at 30° for 30 min and stopped as described earlier.

Preparation of reduced oligosaccharides by NaOH–$NaBH_4$ treatment of native fetuin. — Alkaline $NaBH_4$ degradation of fetuin and separation of the reduced oligosaccharides on Sephadex G-25 chromatography were performed as described by Spiro and Bhoyroo[23]. The same procedure was used to prepare standard ^{3}H-labeled oligosaccharides for l.c. analysis. Native fetuin was submitted to alkaline treatment under reductive conditions in NaOH containing a mixture of labeled (5 mCi) and unlabeled $NaBH_4$. The tritiated oligosaccharides were chromatographed on paper in 4:1:1 butanol–ethanol–water for 48 h to remove radioactive contaminants. Oligosaccharides were recovered by water elution of the paper from −2 to +5 cm from the origin[24].

Preparation of reduced oligosaccharides from [^{14}C]*sialylated fetuin.* — To Tris buffer (2 mL) containing brain microsomal proteins (4 mg) were added fetuin (4 mg), 0.5% (w/v) Triton X-100, 5mM $MnCl_2$, 40mM MES (pH 6.0), and CMP-[^{14}C]NeuAc (800 nCi). The incubation was performed at 28° for 24 h, whereafter it was diluted to 100 mL with cold water and centrifuged at 100 000*g* for 60 min to remove the microsomes. The supernatant solution containing the [^{14}C]sialylated fetuin was lyophilized. The labeled products were desalted by gel filtration on a column (100 × 1 cm) of Bio-Gel P-4 (100–200 mesh) in 0.1M pyridine acetate (pH 5.0). After lyophilization, the labeled fetuin was submitted to alkaline

treatment under reductive conditions in 0.1M NaOH (4 mL) containing 0.8M $NaBH_4$ for 68 h at 37°, and purified as described by Spiro and Bhoyroo[23]. Labeled, reduced oligosaccharides were detected by scintillation counting in Instagel (Packard Instrument).

Glycosylation of acceptors. — Galactosylation of bovine submaxillary gland asialomucin ("GalNAc-protein") by porcine submaxillary gland microsomes. This was performed according to Van den Eijnden *et al.*[21]. To "MES buffer" (4 mL) containing submaxillary porcine membrane proteins (2.5 mg) used as a source of UDPgalactose:2-acetamido-2-deoxy-D-galactose 3-β-D-galactosyltransferase were added bovine asialomucin (8 mg), 0.75% (w/v) Triton X-100, and UDP-[^{14}C]Gal (1 μCi). The incubation was performed at 30° for 24 h. After dilution and centrifugation, labeled products were desalted by gel filtration on a Bio-Gel P-4 column and the reduced oligosaccharides (Gal→GalNAcol) were prepared as described earlier.

Sialylation of [^{14}C]Gal-asialomucin ("[^{14}C]Gal→GalNAc→protein") by submaxillary glands microsomes. To "MES buffer" (2 mL) containing membrane proteins (2.5 mg) were added [^{14}C]Gal→GalNAc→protein (4 mg), 0.75% (w/v) Triton X-100, and CMP-NeuAc (640 nmol). The incubation was performed at 30° for 24 h. The next steps for the isolation of NeuAc→[^{14}C]Gal→GalNAc→protein thus formed and corresponding reduced oligosaccharides (NeuAc→Gal→GalNAcol) were the same as described earlier. "NeuAc→[^{14}C]Gal→GalNAc→protein" was used as an acceptor for rat brain sialyltransferase (see later). The same synthesis was performed with CMP-[^{3}H]NeuAc, and the doubly labeled [^{3}H]NeuAc→[^{14}C]Gal→GalNAcol obtained was used as a standard for l.c. analysis.

The two products Gal→GalNAcol and NeuAc→Gal→GalNAcol were identified by l.c. on a $SiNH_2$ column according to the procedure of Bergh *et al.*[25,26] and Joziasse *et al.*[27] as described later.

Sialylation of NeuAc→[^{14}C]Gal→asialomucin ("NeuAc→[^{14}C]Gal→GalNAc→protein") by brain microsomes. To membrane proteins (2 mL) containing 4 mg of proteins were added NeuAc→[^{14}C]Gal→GalNAc→protein (4 mg), 0.5% (w/v) Triton X-100, 5mM $MnCl_2$, 40mM MES (pH 6.0), and CMP-[^{3}H]NeuAc (100 μCi). The incubation was performed at 28° for 24 h. After incubation, the glycoprotein, doubly-labeled with ^{3}H and ^{14}C, was isolated and the reduced oligosaccharides prepared as described earlier.

Hydrazinolysis of [^{14}C]sialylated fetuin. — The hydrazinolysis of fetuin was carried out according to a procedure adapted from Takasaki *et al.*[28]. [^{14}C]Sialylated fetuin (4 mg) was solubilized in anhydrous hydrazine (0.5 mL) and heated in a sealed tube for 9 h at 100°. The mixture was evaporated to dryness under N_2, and the residue freed from hydrazine by repeated addition and evaporation of toluene and dissolved in saturated $NaHCO_3$ solution (0.2 mL). Acetic anhydride (4 × 10 μL) was added at 10-min intervals at room temperature. The mixture was passed through a column of Dowex 50-X8 (100–200 mesh, H^+) which was washed with distilled water (5 bed vol.). The eluent and washings were pooled and

lyophilized. The product was chromatographed on a Bio-Gel P-4 column (200–400 mesh; 120 × 1 cm) in 0.1M pyridine acetate buffer (pH 5.0). The products prepared by hydrazynolysis of native fetuin, as described by Bendiak and Cook[29], were used as controls.

Identification of sialo-oligosaccharides by liquid chromatography. — The oligosaccharides were identified with a Waters apparatus consisting of two M6000 pumps, a M720 gradient generator, a M480 UV detector, and a M730 integrator. The oligosaccharide peaks were detected at 205 nm and 1-mL fractions were collected for the measurement of radioactivity by liquid scintillation. This method was adapted from that of Baenziger and Natowicz[30] by use of a Radial-Pak SAX column (Waters) which was run isocratically at a constant flow rate of 1 mL/min with 25mM KH_2PO_4 (pH 4.2) for 15 min, after which a linear gradient was started to increase the phosphate concentration to 500mM within 30 min. Alternatively, a method using a Radial $SiNH_2$ column (Waters) was adapted from that of Bergh *et al.*[25,26]. The column was run isocratically with a mixture of 4:1 acetonitrile–15mM KH_2PO_4 (pH 5.2) for 15 min, and then a linear gradient was started to decrease the acetonitrile content by 0.66%/min. The flow rate was 2 mL/min.

RESULTS AND DISCUSSION

Specificity of rat brain sialyltransferase activities towards various glycoprotein acceptors. — The activity of microsomal rat brain sialyltransferases was examined for various exogenous acceptors (fetuin, orosomucoid , transferrin, and bovine submaxillary mucin), either desialylated or not (Table I). Native fetuin was the best acceptor, and this unexpected result led us to determine whether the transfer of

TABLE I

SPECIFICITY OF RAT BRAIN MICROSOMAL SIALYLTRANSFERASES TOWARDS VARIOUS SIALYLATED AND DESIALYLATED ACCEPTORS

Acceptor[a]	*Specific activity*[b] *(pmol · mg^{-1} of proteins · 2h^{-1})*
None	4.6 + 0.7
Fetuin	36.0 + 4.0
Asialofetuin	6.3 + 1.3
Orosomucoid	3.9
Asialo-orosomucoid	3.5
Bovine mucin	3.6
Asialomucin	4.2
Transferrin	5.2
Asialotransferrin	5.1

[a]400 μg/assay. [b]The sialyltransferase activity was measured as the rate of transfer of [^{14}C]NeuAc to each acceptor. The incubations were performed for 2 h at 28° as described in the Experimental section. The values of specific activities onto exogenous acceptors included that of endogenous acceptor (which is mentioned in this Table).

sialic acid on native fetuin was performed by an original sialyltransferase. Rat brain fetuin sialyltransferase required the presence of cations for a good activity, the optimal concentration being 5mM (data not shown). The best specific activity was obtained with Mn^{2+} and to a lesser extend with Mg^{2+}, Ca^{2+}, and Co^{2+} in a decreasing order. To elucidate whether the same sialyltransferase was active either on native fetuin or on asialofetuin, the optimal conditions for transfer to the two substrates were determined. The optimal pH was nearly the same (6.0), but an important discrepancy in optimal temperatures was observed, namely 28° for native fetuin and 22° for asialofetuin (see Fig. 1). Thus, all incubations with fetuin as acceptor were performed at 28°.

Kinetic studies on rat brain fetuin sialyltransferase activity. — The relation between NeuAc transfer velocities and CMP-NeuAc concentrations for different concentrations of native fetuin is recorded in a Lineweaver–Burk plot (Fig. 2). A similar plot for the relation between NeuAc transfer velocities and native fetuin concentrations for different concentrations of CMP-NeuAc was also established (data not shown). Secondary plots (Insert in Fig. 2) are linear, indicating a single enzyme mechanism. From these secondary plots, the kinetic parameters were mathematically calculated and are given according to Cleland's nomenclature as

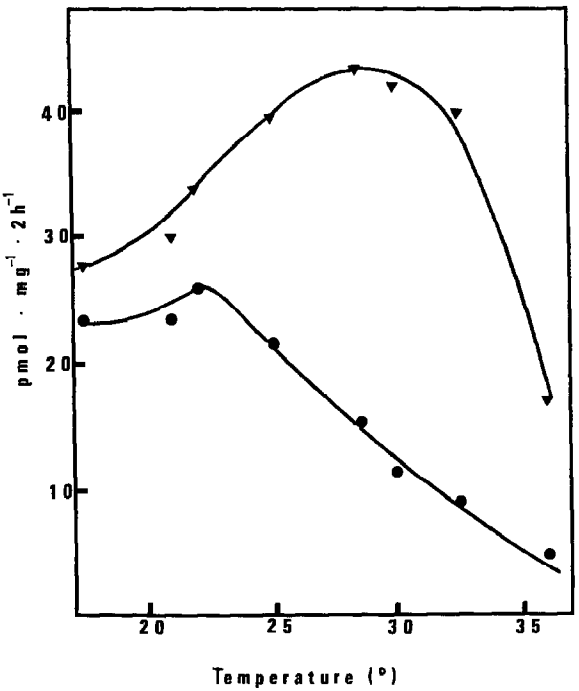

Fig. 1. Temperature dependence of the brain microsomal sialyltransferase activity with native fetuin and asialofetuin as acceptors. Incubations were performed for 2 h as described in the Experimental section. The sialyltransferase activity was determined for native fetuin (▼—▼) and for asialofetuin (●—●) as acceptor.

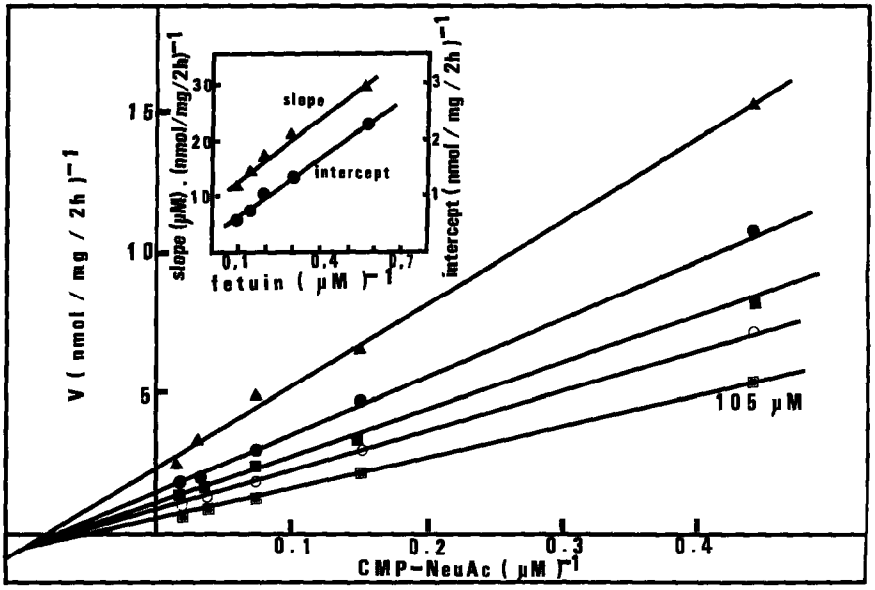

Fig. 2. Lineweaver–Burk plots. Effect of fetuin concentrations on sialyltransferase activity for various CMP-NeuAc concentrations. Sialyltransferase activity was determined as described in the Experimental section. The parameters were determined according to a linear regression procedure: (▲) 17.5, (●) 35, (■) 52.5, (○) 70, and (■) 105 µM. Inset: Secondary plots for slope and intercept *vs.* concentration of the other substrate. The intercept and slope were determined mathematically from the data.

described by Fromm[31] (Table II). These results indicate a BiBi sequential mechanism very similar to that of the rat brain asialofetuin fucosyltransferase[32] which was assumed to be a random BiBi mechanism.

Influence of sialic acid content of fetuin on sialyltransferase activity. — As native fetuin was a more effective acceptor than asialofetuin, we studied the influence of NeuAc residues in fetuin upon the sialyltransferase activity by progressively desialylating fetuin with neuraminidase–agarose from *Clostridium perfringens*. Since the range of specificity of this sialidase is wide, we did not expect any indication about the specificity of the sialyltransferase, but as shown in Fig. 3, the sialyltransferase activity was directly related to the *N*-acetylneuraminic acid

TABLE II

VALUE OF PARAMETERS OF SECONDARY LINEWEAVER–BURK PLOTS[a] FOR RAT BRAIN SIALYLTRANSFERASE

Secondary plot of	K_a[b]	K_b	K_{ia}	K_{ib}	V_{max}[c]
$1/V = f(1/A)$	34.5	135	11.2	43.4	3.88
$1/V = f(1/B)$	35.2	142.8	11.3	44.5	4.05

[a]The values of constants and V_{max} were given according to Fromm[22] and mathematically calculated from results obtained with CMP-NeuAc as substrate A (Fig. 2) and native fetuin as substrate B (not shown). [b]Constant expressed in µM. [c]Value of V_{max} expressed as nmol · mg^{-1} of proteins · $2\ h^{-1}$.

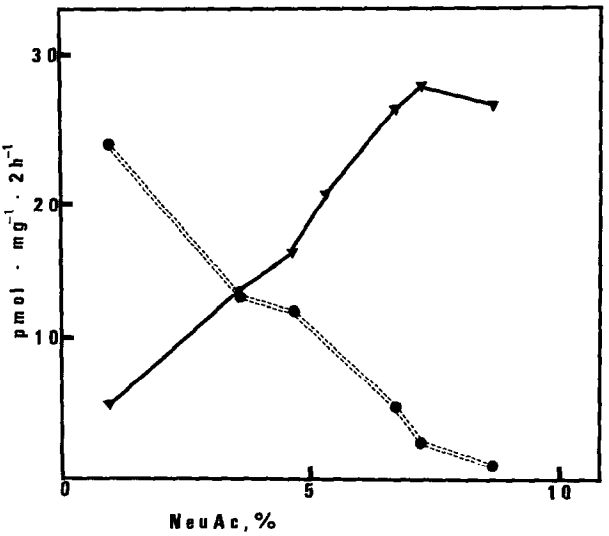

Fig. 3. Effect of NeuAc content on the rat brain sialyltransferase activity. The desialylation of native fetuin was carried out with neuraminidase–agarose for various times as described in the Experimental section. Incubations were performed for 2 hours for sialyltransferase (▼—▼) and for 10 min for L-fucosyltransferase (●==●).

content of fetuin. As a control, brain fucosyltransferase[33,34] activity was monitored in the same fractions and gave the expected results, *i.e.*, an increase in activity with a decrease of the NeuAc content of fetuin. Therefore, the presence of NeuAc residues seems to be essential for optimal sialyltransferase activity.

Comparative studies of fetuin sialyltransferase activity for various organs and animals. — A rat brain sialyltransferase acting on native fetuin has not been previously described. Thus, sialylation by brain and liver tissue of different animals was studied to establish whether this enzyme was related to one species (rat) or more widely distributed (Table III). Sialylation by the rat, mouse, and gerbil liver enzymes was only effective on asialo fetuin, as expected. Sialyl transfer by the sheep and gerbil brain enzymes took place at the same rate for native and asialo fetuin, whereas native fetuin was the best acceptor for the rat and mouse brain

TABLE III

MICROSOMAL SIALYLTRANSFERASE ACTIVITY FOR VARIOUS ORGANS AND ANIMALS[a]

Acceptor	*Brain*				*Liver*		
	Rat	*Mouse*	*Gerbil*	*Sheep*	*Rat*	*Mouse*	*Gerbil*
Fetuin	35.0	17.0	9.3	12.8	2.2	5.7	2.0
Asialofetuin	7.6	11.1	12.6	21.0	14.1	43.6	40.4

[a]Sialyltransferase activity was determined at 28° as described in the legend to Table I and expressed as pmol · mg^{-1} of proteins · 2 h^{-1}.

enzymes. These results suggest that, in the brain, the sialylation of native fetuin involves an unexpected sialyltransferase activity.

Identification of the nature of the glycan acceptor. — Native fetuin contains *N*-glycans and two types of *O*-glycans (mono- and di-sialoglycans). In order to identify the nature of the glycan acceptor, β-elimination was performed on native fetuin and on fetuin sialylated by rat brain microsomal sialyltransferase using CMP-[^{14}C]NeuAc as NeuAc donor. Reduced oligosaccharides were chromatographed on a Sephadex G-25 column. The chromatographic pattern for native fetuin gave the expected results (Fig. 4A), with a major peak (A) containing the asparagine-linked glycans and two peaks (B and C) corresponding to reduced *O*-glycan chains.

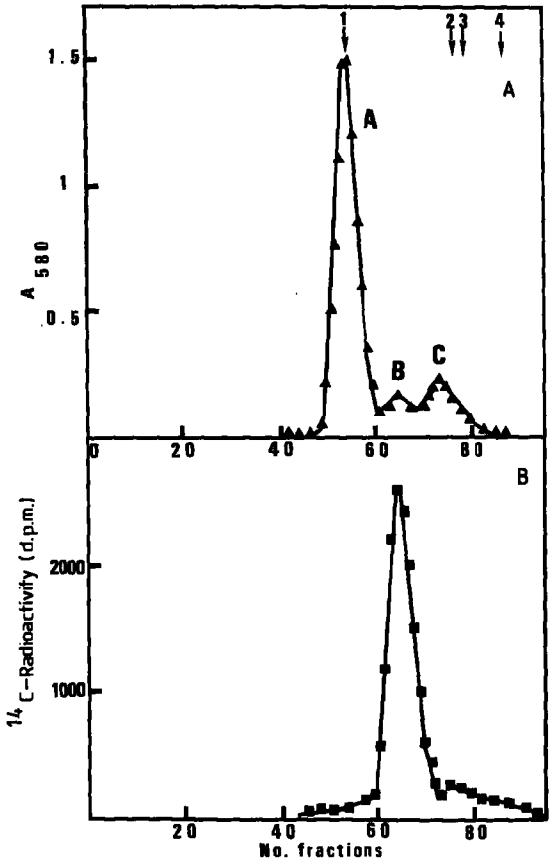

Fig. 4. Gel filtration on Sephadex G-25 of alkaline borohydride-treated native fetuin and [^{14}C]sialylated fetuin. The alkaline borohydride-treated samples were placed on a column (120 × 2 cm), equilibrated with 0.1M pyridine–acetate buffer (pH 5.0), and eluted with this buffer in 5-mL fractions collected every 20 min: (A) Sialic acid of the reduced oligosaccharides from native fetuin (▲—▲) determined by the resorcinol procedure[14], and (B) radioactivity of the reduced oligosaccharides from [^{14}C]sialylated fetuin (■—■). The arrows indicate elution volume of Dextran Blue (1), stachyose (2), sialyllactose (3), and NeuAc (4).

According to Spiro and Bhoyroo[23], peak B is the reduced disialyltetrasaccharide α-NeuAc-(2→3)-β-D-Gal*p*-(1→3)-[α-NeuAc-(2→6)]-D-GalNAcol and peak C is the monosialosyltrisaccharide α-NeuAc-(2→3)-β-D-Gal*p*-(1→3)-D-GalNAcol. Reduced, [^{14}C]sialylated glycans showed one single radioactive peak (Fig. 4B) corresponding to a product having a molecular weight lower than that of an *N*-glycan chain and compatible with a tetrasaccharide *O*-glycan structure.

As additional control, labeled, sialylated fetuin was hydrazinolyzed and *N*-reacetylated, as described by Bendiak and Cook[29], and the product chromatographed through a Bio-Gel P-4 column. The high-molecular-weight *N*-glycan fraction was not labeled, and the radioactivity was only recovered in the *O*-linked oligosaccharides and free *N*-acetylneuraminic acid (data not shown). More evidence for the labeling of *O*-glycan chains may be deduced from the result presented in Table I. Serotransferrin and orosomucoid, which are devoid of *O*-glycosyl-linked chains, were not acceptors. However, if the requirement for an *O*-glycan structure is necessary, some additional requirements are needed as bovine submaxillary mucin (GalNAcSer) and asialofetuin were very poor acceptors.

NeuAc content of the product. — Reduced, sialylated oligosaccharides were chromatographed on a SAX anion-exchange l.c. column as described by Baenziger and Natowicz[30] in order to determine the content of NeuAc groups. The pattern obtained from labeled, native fetuin showed two peaks (Fig. 5), the major being eluted with 100mM KH_2PO_4 buffer (*T* 27 min), which corresponds to a reduced disialo-oligosaccharide when compared with labeled, standard α-NeuAc-(2→3)-β-D-Gal*p*-(1→3)-[α-NeuAc-(2→6)]-D-GalNAcol obtained from native fetuin and with the results of Baenziger and Natowicz[30]. The minor peak, eluted first at a retention time of *T* 11 min with 25mM KH_2PO_4 buffer, may be due to the transfer of an NeuAc group by the residual enzymic acitivity at 28° of "asialofetuin:sialyltransferase" (as shown in Fig. 1). This peak corresponds to a monosialyl structure when compared with labeled, standard α-NeuAc-(2→3)-β-D-Gal*p*-(1→3)-D-GalNAcol from native fetuin.

Acceptors not commercially available were synthesized as follows. β-D-[^{14}C]Gal*p*-(1→3)-D-GalNAc was obtained by galactosylation of bovine asialomucin ("GalNAc→protein") with UDP-Gal:*N*-acetyl-D-galactosaminylprotein (1→3)-β-D-galactosyltransferase isolated from porcine submaxillary gland[21]. After subsequent β-elimination under reductive conditions, the reduced oligosaccharide was eluted at a retention time of *T* 5 min from an SAX column.

Sialylation of "[^{14}C]Gal→GalNAc→protein" by sialyltransferase from submaxillary gland and subsequent β-elimination led to a compound giving an l.c. pattern corresponding to a reduced monosialo-oligosaccharide (Fig. 5), which migrated at the same rate as the minor peak obtained from [^{14}C]sialylated native fetuin and a mono-[^{3}H]sialyl-oligosaccharide standard. However, if the SAX column was able to separate well reduced asialo-, mono-, di-, or trisialo-oligosaccharides, it was not able to distinguish between oligosaccharides having α-(2→3)- and -(2→6)-linked NeuAc groups. Sialylation of sialylated "[^{14}C]Gal→Gal-

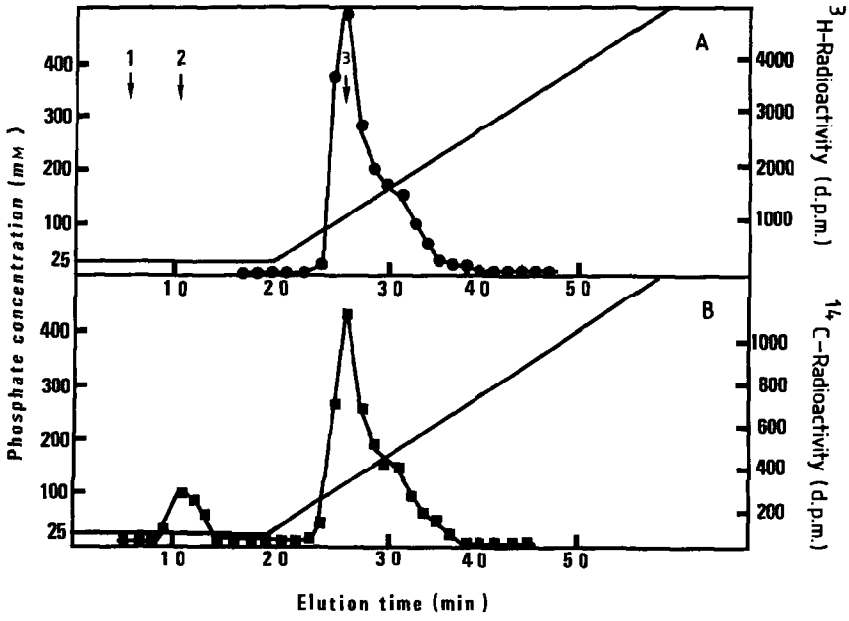

Fig. 5. Anionic liquid chromatography under elevated pressure of reduced sialo-oligosaccharides obtained by β-elimination. The reduced oligosaccharides were chromatographed on a Radial-Pak SAX Waters column which was eluted for 15 min with 25mM KH_2PO_4 (pH 4.2), and then with a linear gradient up to 500mM KH_2PO_4 (pH 4.2). The radioactivity was determined by liquid-scintillation counting on 1-mL fractions: (A, ●—●) reduced oligosaccharides from [^{3}H]sialylated NeuAc→Gal→GalNAc→protein, and (B, ■—■) reduced oligosaccharides from [^{14}C]sialylated fetuin. Reference oligosaccharides: (1) β-D-Gal*p*-(1→3)-D-GalNAcol, (2) α-NeuAc-(2→3)-β-D-Gal*p*-(1→3)-D-GalNAcol biosynthesized or obtained from native fetuin under reductive conditions, and (3) ^{3}H-standard α-NeuAc-(2→3)-β-D-Gal*p*-(1→3)-[α-NeuAc-(2→6)]-D-GalNAcol.

NAc→protein" with sialyltransferase from rat brain led, after β-elimination, to a reduced disialo-oligosaccharide giving the same l.c. pattern as the reduced disialo-oligosaccharide obtained from [^{14}C]sialylated, native fetuin and a [^{3}H]disialo-oligosaccharide standard (Fig. 5). Sialylation of unsialylated "[^{14}C]Gal→GalNAc→protein" with rat brain sialyltransferase gave, as only reduced oligosaccharide after β-elimination, [^{14}C]Gal→GalNAcol, and no sialylated oligosaccharide was detected (data not shown). These results clearly indicate that a monosialyl compound is required as a substrate for the rat brain fetuin:sialyltransferase to give a disialyl compound.

Identification of the sialyl linkage. — The structure[7] of the two *O*-glycan chains of fetuin are α-NeuAc-(2→3)-β-D-Gal*p*-(1→3)-α-D-Gal*p*NAc-Ser (or Thr) (glycan A) and α-NeuAc-(2→3)-β-D-Gal*p*-(1→3)-[α-NeuAc-(2→6)]-α-D-Gal*p*-NAc-Ser (or Thr) (glycan B). As discussed earlier, the observation that the product obtained with rat brain fetuin:sialyltransferase is a disialoglycan, strongly suggests an α-(2→6) sialylation of glycan A by this enzyme to give glycan B. An α-(2→8)-linked NeuAc group to a NeuAc residue seems excluded for the following reasons:

No incorporation was detected into gangliosides extracted according to a modified procedure from Svennerholm and Fredman[35]. The incorporation of NeuAc groups on colominic acid [a putative acceptor for an α-(2→8)-NeuAc linkage[36]] was not effective under the present sialylation conditions (data not shown). Finally, an α-NeuAc-(2→8)-NeuAc group was only reported for *N*- and not for *O*-glycan chains[37].

As a control, the various biosynthesized compounds were analyzed by l.c. with an $SiNH_2$ column, according to a method adapted from Bergh *et al.*[16] and Sherblom and Bourassa[38]. The biosynthesized, reduced [^{3}H]NeuAc→[^{14}C]Gal→GalNAcol and standard mono-[^{3}H]sialyloligosaccharide obtained from native fetuin were eluted at the same retention time (*T* 21 min) (Fig. 6). The structure of

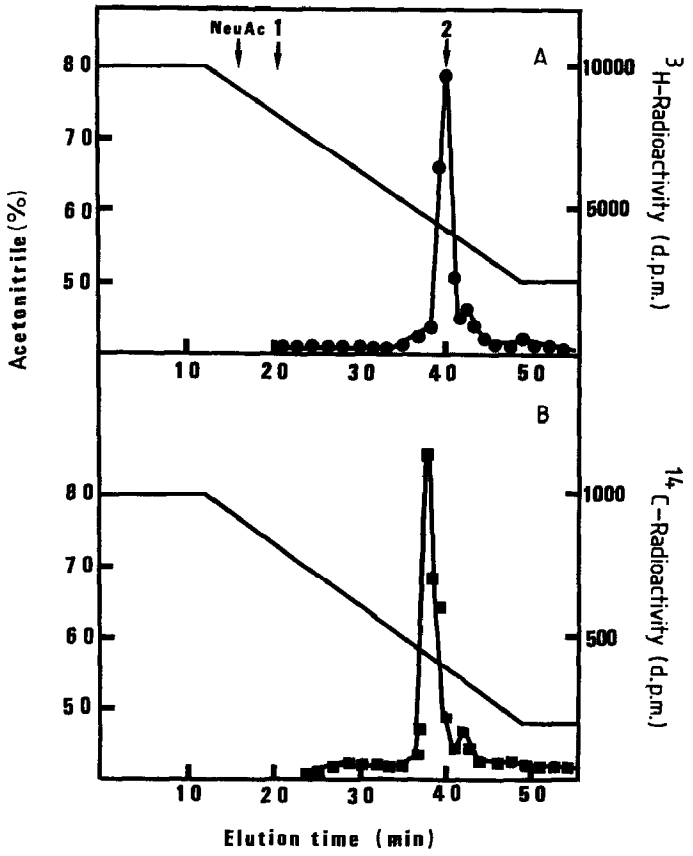

Fig. 6. Liquid chromatography under elevated pressure on $SiNH_2$ of sialo-oligosaccharides obtained by β-elimination. The reduced oligosaccharides were chromatographed on a Radial-Pak $SiNH_2$ Waters column which was eluted for 15 min with 4:1 acetonitrile–15mM KH_2PO_4 (pH 5.2), and then by a linear gradient with a decrease of 0.66%/min in the acetonitrile content: (A, ●—●) reduced oligosaccharides from [^{3}H]sialylated NeuAc→Gal→GalNAc→protein, and (B, ■—■) reduced oligosaccharides from [^{14}C]sialylated fetuin. Reference oligosaccharides: (1) α-NeuAc-(2→3)-β-D-Gal*p*-(1→3)-GalNAcol biosynthesized or obtained from native fetuin, and (2) ^{3}H-standard α-NeuAc-(2→3)-β-D-Gal*p*-(1→3)-[α-NeuAc-(2→6)]-D-GalNAcol prepared from native fetuin.

this biosynthesized, reduced oligosaccharide was also confirmed by comparison with the results of Bergh *et al.*[16] who found a retention time of 35 min (corresponding to ~76% of acetonitrile) for α-NeuAc-(2→3)-β-D-Gal*p*-(1→3)-GalNAcol and 56 min (~63% of acetonitrile) for β-D-Gal*p*-(1→3)-[α-NeuAc-(2→6)]-GalNAcol. Under our own chromatographic conditions, a retention time of 21 min corresponded to a concentration of ~76% of acetonitrile in the eluent. Thus, the chain obtained by sialylation of "Gal→GalNAc→protein" with sialyltransferase of porcine submaxillary is, under the present incubation conditions, essentially α-NeuAc-(2→3)-β-D-Gal*p*-(1→3)-GalNAc, even if the α-(2→3)- and α-(2→6)-sialyltransferases are both present in porcine submaxillary gland[9]. Furthermore, the standard monosialyloligosaccharide from native fetuin, eluted at the same retention time, has the structure: α-NeuAc-(2→3)-β-D-Gal*p*-(1→3)-GalNAcol. A β-D-Gal-(1→3)-[α-NeuAc-(2→6)]-GalNAc structure has never been observed in fetuin. The results described herein are in discrepancy with those obtained by Sherblom and Bourassa[38] who found that 85% of the NeuAc groups transfer were in α-(2→6) linkage to GalNAc residues. However, the acceptor used was asialofetuin instead of galactosylated asialomucin, and it has been previously reported[17] that the acceptor residue may determine the specificity of a sialyltransferase. Our results are in better agreement with those of Sadler *et al.*[39] who reported for the α-(2→6)-sialyltransferase from porcine submaxillary gland a K_m value ~100 times that of the α-(2→3)-sialyltransferase of the same gland.

The reduced product obtained from [^{14}C]sialylated native fetuin was eluted at a retention time of 38 min. The same retention time was found for the standard di[^{3}H]sialyloligosaccharide and the reduced disialo-oligosaccharide obtained by sialylation of "NeuAc→[^{14}C]Gal→GalNAc→protein" with the rat brain sialyltransferase. When native fetuin was used as an acceptor for rat brain sialyltransferase, all our results provided evidence that the glycan A of fetuin is the acceptor and that the structure of the product obtained corresponded to that of glycan B.

The substrate properties of the rat brain sialyltransferase and its specific requirement for an α-(2→3)-linked NeuAc group to a Gal residue for NeuAc transfer onto GalNAc are very close to those of the sialyltransferase described by Bergh *et al.*[18] for the synthesis of *O*-glycosyl oligosaccharide chains of fetuin in fetal calf liver[18]. These properties differ from those of ovine[16] and porcine[10] submaxillary α-(2→6)-sialyltransferases which sialylate GalNAc→protein, β-D-Gal*p*-(1→3)-α-D-Gal*p*NAc→protein and α-NeuAc-(2→3)-β-D-Gal*p*-(1→3)-α-D-Gal*p*NAc→protein[16,40]. In adult porcine liver, Bergh *et al.*[15] demonstrated that only the α-(2→3)-sialyltransferase was present. These results are corroborated by our study of adult rat, mouse, and gerbil livers (Table III). In fetal calf liver, Bergh *et al.*[18] demonstrated the existence of both α-(2→3)- and α-(2→6)-sialyltransferase, the latter acting only when a NeuAc group α-(2→3)-linked to a Gal residue was present. These results suggest that the pathway proposed by Bergh *et al.*[18] for the synthesis of the *O*-glycosyl-linked chains of fetuin, in fetal calf liver tissue but not

in adult calf liver, may also exist in some nonfetal tissues, such as mature rat brain tissue.

ACKNOWLEDGMENTS

The authors thank Marie Jo Peschard for skillful technical assistance, Pascal George for assistance in liquid chromatography analysis, and Y. Bonhomme from LIPHA Co. for a kind gift of the initial strain of mongolian gerbils.

REFERENCES

1 L. SVENNERHOLM, *Handb. Neurochem.*, 3 (1970) 425–452.
2 E. G. BRUNNGRABER, L. A. WITTING, C. HABERLAND, AND B. BROWN, *Brain Res.*, 38 (1972) 151–161.
3 D. H. VAN DEN EIJNDEN AND W. VAN DIJK, *Biochim. Biophys. Acta*, 62 (1974) 136–149.
4 S. S. NG AND J. A. DAIN, *J. Neurochem.*, 29 (1977) 1075–1083.
5 H. DEN, B. KAUFMAN, E. J. MCGUIRE, AND S. ROSEMAN, *J. Biol. Chem.*, 250 (1975) 739–746.
6 R. SCHAUER, *Cell Biol. Monogr.*, 10 (1982).
7 B. NILSSON, N. E. NORDEN, AND S. SVENSSON, *J. Biol. Chem.*, 254 (1979) 4545–4553.
8 B. BAUVOIS, R. CACAN, B. FOURNET, J. CAEN, J. MONTREUIL, AND A. VERBERT, *Eur. J. Biochem.*, 121 (1982) 567–572.
9 J. E. SADLER, J. I. REARICK, J. C. PAULSON, AND L. H. HILL, *J. Biol. Chem.*, 254 (1979) 4434–4443.
10 J. E. SADLER, J. I. REARICK, AND R. L. HILL, *J. Biol. Chem.*, 254 (1979) 5934–5941.
11 J. I. REARICK, J. E. SADLER, J. C. PAULSON, AND R. L. HILL, *J. Biol. Chem.*, 254 (1979) 4444–4451.
12 D. H. VAN DEN EIJNDEN, D. H. JOZIASSE, H. DORLAND, H. VAN HALBEEK, J. F. G. VLIEGENTHART, AND K. SCHMID, *Biochem. Biophys. Res. Commun.*, 92 (1980) 839–845.
13 D. H. VAN DEN EIJNDEN AND W. E. C. M. SCHIPHORST, *J. Biol. Chem.*, 256 (1981) 3159–3162.
14 D. H. VAN DEN EIJNDEN, M. L. E. BERGH, B. DIELEMAN, AND W. E. C. M. SCHIPHORST, *Hoppe-Seyler's Z. Physiol. Chem.*, 362 (1981) 113–124.
15 M. L. E. BERGH, G. J. M. HOOGHWINKEL, AND D. H. VAN DEN EIJNDEN, *Biochim. Biophys. Acta*, 660 (1981) 161–169.
16 M. L. E. BERGH, P. L. KOPPEN, AND D. H. VAN DEN EIJNDEN, *Biochem. J.*, 201 (1982) 411–415.
17 M. L. E. BERGH AND D. H. VAN DEN EIJNDEN, *Eur. J. Biochem.*, 136 (1983) 113–118.
18 M. L. E. BERGH, G. J. M. HOOGHWINKEL, AND D. H. VAN DEN EIJNDEN, *J. Biol. Chem.*, 258 (1983) 7430–7436.
19 L. SVENNERHOLM, *Biochim. Biophys. Acta*, 24 (1957) 604–611.
20 P. BROQUET, M. N. PEREZ-GONZALEZ, AND P. LOUISOT, *Biochimie*, 60 (1978) 1261–1271.
21 D. H. VAN DEN EIJNDEN, R. A. BARNEVELD, AND V. E. C. M. SCHIPHORST, *Eur. J. Biochem.*, 95 (1979) 623–637.
22 P. BROQUET, R. MORELIS, AND P. LOUISOT, *J. Neurochem.*, 24 (1975) 989–995.
23 R. G. SPIRO AND V. D. BHOYROO, *J. Biol. Chem.*, 249 (1974) 5704–5717.
24 C. J. LIANG, K. YAMASHITA, C. G. MUELLENBERG, H. SHICHI, AND A. KOBATA, *J. Biol. Chem.*, 254 (1979) 6417–6423.
25 M. L. E. BERGH, P. L. KOPPEN, AND D. H. VAN DEN EIJNDEN, *Carbohydr. Res.*, 94 (1981) 225–229.
26 M. L. E. BERGH, P. L. KOPPEN, AND D. H. VAN DEN EIJNDEN, *Carbohydr. Res.*, 117 (1983) 275–278.
27 D. H. JOZIASSE, W. M. BLANKEN, P. L. KOPPEN, AND D. H. VAN DEN EIJNDEN, *Carbohydr. Res.*, 119 (1983) 303–309.
28 S. TAKASAKI, T. MIZUOCHI, AND A. KOBATA, *Methods Enzymol.*, 83 (1982) 263–268.
29 B. BENDIAK AND G. M. W. COOK, *Biochem. J.*, 213 (1983) 253–260.
30 J. V. BAENZIGER AND M. NATOWICZ, *Anal. Biochem.*, 112 (1981) 357–361.
31 H. J. FROMM, *Mol. Biol. Biochem. Biophys.*, 22 (1975).
32 P. BROQUET, M. N. PEREZ-GONZALEZ, AND P. LOUISOT, *J. Neurochem.*, 32 (1979) 599–606.
33 P. BROQUET, M. LÉON, AND P. LOUISOT, *Eur. J. Biochem.*, 123 (1982) 9–13.
34 P. BROQUET, M. SERRES-GUILLAUMOND, H. BAUBICHON-CORTAY, M. J. PESCHARD, AND P. LOUISOT, *FEBS Lett.*, 174 (1984) 43–46.

35 L. SVENNERHOLM AND P. FREDMAN, *Biochim. Biophys. Acta*, 617 (1980) 97–109.
36 F. A. TROY, I. K. VIJAY, AND N. TESCHE, *J. Biol. Chem.*, 250 (1975) 156–163.
37 J. FINNE, *J. Biol. Chem.*, 257 (1982) 11 966–11 970.
38 A. P. SHERBLOM AND C. R. BOURASSA, *Biochim. Biophys. Acta*, 761 (1983) 94–102.
39 J. E. SADLER, J. I. REARICK, J. C. PAULSON, AND R. L. HILL, *Proc. Int. Symp. Glycoconjugates, IVth*, (1979) 763–766
40 T. A. BEYER, J. I. REARICK, J. C. PAULSON, J. P. PRIEELS, J. E. SADLER, AND R. L. HILL, *J. Biol. Chem.*, 254 (1979) 12 531–12 541.