Carbohydrate Research, 149 (1986) 85–99
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

BIOSYNTHESIS OF DISIALYLATED β-D-GALACTOPYRANOSYL-(1→3)-2-ACETAMIDO-2-DEOXY-β-D-GLUCOPYRANOSYL OLIGOSACCHARIDE CHAINS. IDENTIFICATION OF A β-D-GALACTOSIDE α-(2→3)- AND A 2-ACETAMIDO-2-DEOXY-β-D-GLUCOSIDE α-(2→6)-SIALYLTRANSFERASE IN REGENERATING RAT LIVER AND OTHER TISSUES*

HANS T. DE HEIJ, PIET L. KOPPEN, AND DIRK H. VAN DEN EIJNDEN

Department of Medical Chemistry, Vrije Universiteit, Van der Boechorststraat 7, NL-1007 MC Amsterdam (The Netherlands)

(Received March 14th, 1985; accepted for publication, May 2nd, 1985)

ABSTRACT

Regenerating rat liver microsomes contain a β-D-galactoside α-(2→3)- and a 2-acetamido-2-deoxy-β-D-glucoside α-(2→6)-sialyltransferase that are involved in the synthesis of the terminal α-NeuAc-(2→3)-β-D-Gal*p*-(1→3)-α-[NeuAc-(2→6)]-β-D-Glc*p*NAc-(1→R) group occurring in human milk oligosaccharides and the glycan chains of several *N*-glycoproteins. Analysis by liquid chromatography and methylation of the products of sialylation obtained when lacto-*N*-tetraose [β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc] was used as a substrate in the incubations *in vitro* indicated that the disialylated sequence is formed for >95% through the tetrasaccharide α-NeuAc-(2→3)-β-D-Gal-(1→3)-β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-D-Glc as one of two possible intermediates. This indicates that in the synthesis of the disialylated sequence the α-(2→3)- and the α-(2→6)-sialyltransferase act in a highly preferred order in which the α-(2→3) enzyme acts first. This order is imposed by the specificity of the α-(2→6)-sialyltransferase, which requires an α-NeuAc-(2→3)-β-D-Gal-(1→3)-β-D-GlcNAc-(1→R) sequence for optimal activity, and shows very low and no activity with β-D-Gal-(1→3)-β-D-GlcNAc-(1→R) and β-D-GlcNAc-(1→R) acceptor structures, respectively. Results obtained with normal rat, fetal calf, rabbit and human liver, and human placenta indicated that very similar or identical sialyltransferases occur in these tissues. It is suggested that these enzymes differ from the sialyltransferases that previously had been identified in fetal calf liver and human placenta.

INTRODUCTION

Structural investigations on *N*- and *O*-linked carbohydrate chains of glycoproteins have shown the existence of a wide variety of sialic acid linkages. These

*A preliminary account of this work has appeared[1].

0008-6215/86/$ 03.50

include α-NeuAc(Gc)-(2→3)-Gal*, α-NeuAc-(2→4)-Gal, α-NeuAc-(2→6)-Gal, α-NeuAc(Gc)-(2→3)-GalNAc, α-NeuAc(Gc)-(2→6)-GalNAc, α-NeuAc-(2→4)-GlcNAc, α-NeuAc-(2→6)-GlcNAc, and α-NeuAc(Gc)-(2→8)-NeuAc(Gc) linkages[2-7]. Some of these linkages are also found in gangliosides[8-10], and in oligosaccharides from human milk[11,12] and urine[2,13]. Based on the "one enzyme–one linkage" hypothesis[14,15], these sialic acid linkages imply the existence of at least eight different sialyltransferases involved in their synthesis. As sialyltransferases do not only specifically recognize the acceptor sugar, but also the penultimate sugar and the linkage between these two residues[16-20], as well as the aglycon portion of the acceptor structure[21], probably many more of these enzymes exist. Up till now, six sialyltransferases from various tissues have been purified and enzymically characterized[17-20,22-25]. In addition, several other sialyltransferase activities have been identified[26-31].

An interesting feature of the structure of *N*-linked glycans of several bovine-blood coagulation factors is the occurrence[32-34] of the terminal α-NeuAc-(2→3)-β-Gal-(1→3)-[α-NeuAc-(2→6)]-β-GlcNAc-(1→ group. This structure has been found first for the disialyllacto-*N*-tetraose from human milk[35] and, in addition to the coagulation factors, is present also in rat α_1-acid glycoprotein[36] and bovine fetuin[37]. This tetrasaccharide group shows similarity with the α-NeuAc-(2→3)-Galβ-(1→3)-[α-NeuAc-(2→6)]-α-GalNAc-(1→ group that occurs *O*-linked on many secretory and membrane-bound glycoproteins[2]. It has been shown that, in the synthesis of the latter structure, two different sialyltransferases were involved, which act in a highly preferred[38] or obligatory[29] order depending on the tissue source of the enzyme linking the sialyl group to the 2-acetamido-2-deoxy-D-galacto-pyranosyl residue.

In the present study, we investigated the biosynthesis of the α-NeuAc-(2→3)-β-Gal-(1→3)-[α-NeuAc-(2→6)]-β-GlcNAc group. Two sialyltransferases that are involved in this synthesis were identified in several tissues. The enzymes appeared to act in a preferred order in which the α-(2→3)-linked sialyl group was attached first to the β-Gal-(1→3)-β-GlcNAc acceptor structure. Special attention was paid to the question of whether the α-(2→6)-sialyltransferase attaching the sialyl group to the 2-acetamido-2-deoxy-β-D-glucopyranosyl residue is related to the previously described [α-NeuAc-(2→3)-β-Gal-(1→3)]-α-GalNAc-(1→R) α-(2→6)-sialyl-transferase of fetal calf liver[29].

EXPERIMENTAL

Materials. — Lacto-*N*-tetraose (**1**), LS-tetrasaccharide-a (**2**), -b (**3**), and -c (**4**), and disialyllacto-*N*-tetraose (**5**) were kindly donated by Dr. V. Ginsburg, National Institutes of Health, Bethesda, Maryland. GlcNAcβ→3Galβ→4Glc was kindly provided by Dr. A. H. L. Koenderman from our department. Newcastle-

*In abbreviated structures, all sugars are of the D-configuration and in the pyranose form.

disease virus neuraminidase was a generous gift of Mr. H. Diabaté, Justus-Liebig Universität, Giessen, F.R.G. Human placenta and human liver were obtained from the Department of Obstetrics and Gyneacology and the Department of Pathology, Academisch Ziekenhuis of the Vrije Universiteit, respectively. The other tissues were obtained from a local slaughterhouse or were dissected from laboratory animals. Unlabeled CMP-NeuAc[39], NeuAcα→3Galβ→3GalNAcα→*p*-nitrophenyl[21], NeuAcα→3Galol, NeuAcα→3Galβ→3GalNAcol, and NeuAcα→3Galβ→3(NeuAcα→6)GalNAcol[40] were prepared as described previously. CMP-[^{3}H]NeuAc (700 GBq/mol) and CMP-[^{14}C]NeuAc (60 GBq/mol) were purchased from New England Nuclear Corp. The protease inhibitors were purchased from Sigma Chemical Co. All other materials were obtained commercially.

β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc

1

α-NeuAc-(2→3)-β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc

2

β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc
6
↑
2
α-NeuAc

3

α-NeuAc-(2→6)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc

4

α-NeuAc-(2→3)-β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc
6
↑
2
α-NeuAc

5

Preparation of microsomal fractions. The tissue (~10 g) was homogenized in 0.25M sucrose containing mM EDTA and mM dithiothreitol (50 mL) by use of a Potter–Elvehjem system with a Teflon pestle rotating at 1400 r.p.m. for 2 min at 0°. The homogenate was centrifuged at 3000 *g* for 20 min. The sediment was discarded

and the supernatant was centrifuged at 100 000 *g* for 1 h. The pellet of this centrifugation step was resuspended in a small volume of the sucrose solution to yield a microsomal preparation at a protein concentration of 30–40 mg/mL.

Partial hepatectomy. Adult male rats of ~200 g were subjected to partial hepatectomy by removing the left lateral and medial lobes of the liver according to standard procedures[41]. Four days after operation, the rats were sacrificed and the regenerating livers directly homogenized in sucrose solution as described earlier.

Sialyltransferase assays. — The reaction mixture for the assay of CMP-NeuAc:Galβ→3GlcNAcβ→R α-(2→3)-sialyltransferase activity contained, in a volume of 30 μL, mM lacto-*N*-tetraose, 2.5mM CMP-[^{14}C]NeuAc (30 GBq/mol), 0.1M Tris maleate buffer (pH 6.7), 0.1mM protease inhibitors (mixture of phenylmethylsulfonyl fluoride, *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone and *N*-*p*-tosyl-L-lysine chloromethyl ketone), 0.8% (v/v) Triton X-100, and microsomal enzyme preparation (1.0–2.0 mg of protein). The reaction mixture was incubated for 30 min at 37°. Incorporation was estimated according to Paulson *et al.*[17] (assay 3) employing small columns of anion exchanger, except for the use of Dowex 1-X8 (100–200 mesh) in the AcO^- rather than the PO_4^{3-} form, and the elution with 0.05M sodium acetate (pH 8.3) instead of phosphate buffer.

CMP-NeuAc:(NeuAcα→3Galβ→3)GlcNAcβ→R α-(2→6)-sialyltransferase activity was assayed by use of a reaction mixture of 30 μL containing mM LS-tetrasaccharide-a (**2**), labeled in the sialic acid group either with ^{14}C (30 GBq/mol) or ^{3}H (74 GBq/mol), 2.5mM CMP-NeuAc, 0.1M Tris maleate buffer (pH 6.7), 0.1mM protease inhibitors as described earlier, 0.8% (v/v) Triton X-100, and microsomal enzyme preparation (1.0–2.0 mg of protein).

The reaction mixtures were incubated for 30 min at 37° whereafter the reaction was quenched by the addition of 0.05M ammonium acetate (0.5 mL, pH 5.2). The mixture was then analyzed on a column (1.6 × 200 cm) of Bio-Gel P-4 (200–400 mesh) at 37° equilibrated and eluted with 0.05M ammonium acetate (pH 5.2) at a flow of 15 mL/h. Fractions (4 mL) were collected and the radioactivity was determined by liquid-scintillation counting. In this system, a clear separation between LS-tetrasaccharide-a and the product disialyllacto-*N*-tetraose was obtained.

Product identification by liquid chromatography. — For routine product identification, samples were analyzed with a Spectra–Physics SP 8700 liquid chromatograph, equipped with a Rheodyne 7105 injection valve and a Hewlett–Packard HP 1040A diode-array detector operating at 195 nm. The chromatograms were recorded with a Hewlett–Packard HP 3390A integrator. The chromatography was performed in a column (4 × 250 mm) of Lichrosorb-NH_2 (particle size 5 μm, Merck) at an ambient temperature of 20° and a pressure of 11 MPa, essentially as described previously[40]. The mobile phase consisted of a mixture of acetonitrile (Lichrosorv grade, Merck) and de-ionized, distilled water containing 15mM KH_2PO_4 (pH 5.2). Starting at a ratio of 4:1, the acetonitrile content was decreased at a rate of 0.2%/min, and after 75 min of 0.5%/min. The flow was maintained at

2 mL/min and, in the case of radioactive samples, fractions (2 mL) were collected and the radioactivity was determined.

Large-scale synthesis of LS-tetrasaccharide-a and -b, and disialyllacto-N-tetraose. — For the synthesis of LS-tetrasaccharide-a and disialyllacto-*N*-tetraose, the incubation mixture with lacto-*N*-tetraose as a substrate and microsomes of regenerating rat liver as an enzyme source, as described under "sialyltransferase assay", was scaled up to 1 mL. After incubation for 16 h at 37°, the reaction was quenched by the addition of 0.05M ammonium acetate (2 mL, pH 5.2) and subsequently centrifuged at 100 000 *g* for 1 h to sediment the microsomal membranes. The resulting supernatant was applied to a column (1.6 × 200 cm) of Bio-Gel P-4 (200–400 mesh), as described earlier, in order to separate the substrates and the mono- and di-sialylated products. Fractions containing radioactive peaks were pooled and lyophilized. Further purification of the materials was accomplished by liquid chromatography using the system described earlier. Appropriate fractions were pooled, the solvents evaporated, and the salts removed by gel filtration on a column (1.5 × 50 cm) of Bio-Gel P-4.

To prepare LS-tetrasaccharide-b (**3**), di[^{14}C]sialyllacto-*N*-tetraose (**5**) (0.5 μmol) was incubated in 0.1M Tris maleate (pH 6.7) with bovine serum albumin (4 mg) and Newcastle-disease virus neuraminidase (5.4 mU; 1 U is the amount of enzyme releasing 1 μmol of NeuAc/min from fetuin in this incubation system) in 400 μL for 16 h at 37°. The reaction products, monosialyllacto-*N*-tetraose and *N*-acetylneuraminic acid, were separated by gel filtration on a column (1.6 × 200 cm) of Bio-Gel P-4 as described earlier.

Methylation analysis. — Purified-oligosaccharide material (0.2–0.3 μmol) was dissolved in 0.05M Tris acetate (500 μL, pH 8.3) containing a 30-fold molar excess of $NaBH_4$. After 1 h at 20°, the excess of $NaBH_4$ was eliminated and the reduced oligosaccharide was isolated and purified as described previously[29]. The reduced oligosaccharides were methylated with methylsulfinyl carbanion as base[42] as has been described in detail previously[28]. After isolation of the permethylated reduced oligosaccharide[28], the material was methanolyzed and acetylated according to Fournet *et al.*[43]. The resulting mixture of *O*-acetyl-*O*-methyl methyl glycosides was analyzed with a Hewlett–Packard 5993 gas chromatograph–quadrupole mass spectrometer–computer combination equipped with an ultra performance capillary column (12.5 × 0.2 mm i.d.) containing cross-linked methylsilicone. Helium was the carrier gas at a pressure of 50 kPa. The column temperature started at 55° and was increased 20°/min to 95°, then 2°/min to 145°, and finally 5°/min to 230°. Mass spectra were recorded at 70 eV at an ion-source temperature of 200° and pressure of 1.3 mPa.

RESULTS

Sialyltransferase activities in various tissues. — A specific acceptor substrate was available for each of the sialyltransferases studied. Although, in the case of

TABLE I

SIALYLTRANSFERASE ACTIVITIES IN SEVERAL TISSUES[a]

Tissue	*Activity (nmol · h⁻¹ · mg⁻¹ of protein) of*	
	α-(2→3)-SATase	*α-(2→6)SATase*
Regenerating rat liver	7.9	2.7
Rat liver	5.1	1.3
Fetal calf liver	6.8	1.7
Bovine liver	1.0	0.5
Rabbit liver	0.1	0.6
Porcine liver	0.2	0.4
Human liver	0.2	1.0
Human placenta	1.1	0.4
Bovine mammary gland	0.1	0.1
Human milk	<0.05	<0.05
Human mammary tumor	<0.05	<0.05

[a]Activities of CMP-NeuAc:Galβ→3GlcNAcβ→R α-(2→3)-sialyltransferase [α-(2→3)-SATase] and CMP-NeuAc:(NeuAcα→3Galβ→3)GlcNAcβ→R α-(2→6)-sialyltransferase [α-(2→6)-SATase] were assayed as described in the Experimental section. All enzyme preparations were microsomal fractions, except for human placenta of which a homogenate was used, and for human milk, which was defatted by centrifugation prior to use.

lacto-*N*-tetraose (Galβ→3GlcNAcβ→3Galβ→4Glc), the substrate used to assay the α-(2→3)-sialyltransferase, a sialic acid group could potentially be added to O-3 of the galactose and O-6 of the *N*-acetylglucosamine residues, it appears that under the assay conditions used >95% of sialic acid was transferred to the galactose residue. It has been demonstrated that at least two different α-(2→3)-sialyltransferases (the Galβ→3GalNAcα→R and Galβ→3(4)GlcNAcβ→R sialyltransferase) can act on lacto-*N*-tetraose (**1**) *in vitro*[18-20]. However, in view of the observation that the Galβ→3GalNAcα→R α-(2→3)-sialyltransferase acts on this substrate with a very low efficiency[18,19], the values of α-(2→3)-sialyltransferase activity assayed for various tissues, as given in Table I, represent fair estimates of the Galβ→3GlcNAcβ→R α-(2→3)-sialyltransferase activity.

As microsomal enzyme preparations catalyzed the transfer of a sialic acid group mainly to the terminal galactose group of lacto-*N*-tetraose (**1**), this tetrasaccharide could not be used to assay the activity of the 2-acetamido-2-deoxy-β-D-glucopyranosyl α-(2→6)-sialyltransferase. For this purpose GlcNAcβ→3Galβ→4-Glc could not be used either as no incorporation of sialic acid into this tetrasaccharide was observed, even with very active enzyme preparations. LS-tetrasaccharide-a (**2**), however, appeared to be an ideal substrate for assaying the α-(2→6)-sialyltransferase.

Of the tissues tested, liver of rat and fetal calf showed the highest activities of both the α-(2→3)- and α-(2→6)-sialyltransferase (Table I). Particularly high activities were found in regenerating rat liver. Adult bovine liver, however, showed

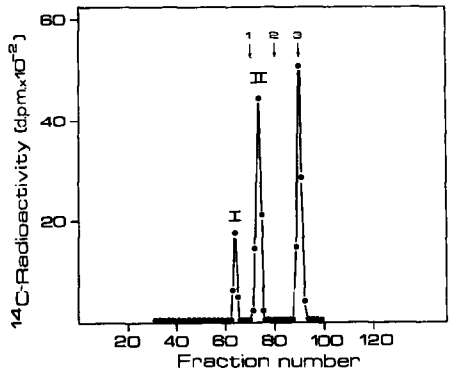


Fig. 1. Fractionation of the products of sialylation of lacto-*N*-tetraose (**1**) by gel filtration. Sialylated oligosaccharides obtained from an incubation with regenerating rat liver microsomes as an enzyme source were fractionated on a column (1.6 × 200 cm) of Bio-Gel P-4 (200–400 mesh), equilibrated and eluted with 50mM ammonium acetate (pH 5.2) at a flow rate of 15 mL/h. Fractions (4 mL) were collected and the ^{14}C radioactivity was determined. Appropriate fractions were pooled and lyopholized for further analysis. The elution position of the following reference compounds is indicated: (1) NeuAcα→3Galβ→3(NeuAcα→6)GalNAcol; (2) NeuAcα→3Galβ→3GalNAcol; and (3) NeuAc.

much lower activities than the fetal tissue. Very low activities were found in bovine mammary gland, human milk, and a human mammary tumor.

Product identification. — In order to identify the products formed by the various enzyme preparations, the incubations with microsomes from regenerating rat liver, rat liver, fetal calf liver, rabbit liver, human liver, and human placenta were conducted for a prolonged period of time (6 h). The radioactive products were fractionated on a calibrated column of Bio-Gel P-4 which separates di- and mono-sialyloligosaccharides, and the acceptor and sugar-donor substrates. Fig. 1 shows a typical example of such an experiment in which regenerating rat liver microsomes had been used as an enzyme source. The di- and mono-sialylated products were further identified by l.c. (Fig. 2). More than 95% of the mono-sialylated product (Fig. 1, peak II) was eluted from the Lichrosorb column identically with authentic LS-tetrasaccharide-a (**2**) (Fig. 2B). Only a very small part was coeluted with LS-tetrasaccharide-b (**3**). The occurrence of an α-(2→3)-linked sialyl group in the major component was confirmed by digesting the material with Newcastle-disease virus neuraminidase; this enzyme is known to specifically release the α-(2→3)-linked sialyl groups[44]. A quantitative conversion into lacto-*N*-tetraose (**1**) and sialic acid was obtained (data not shown). The disialylated compound (Fig. 1, peak I) had the same retention time as disialyllacto-*N*-tetraose (**5**) on l.c. (Fig. 2D). Upon digestion of this material with Newcastle-disease virus neuraminidase, a monosialylated material (III) was formed that could be isolated by gel filtration on Bio-Gel P-4 and was indistinguishable from LS-tetrasaccharide-b (**3**) by l.c. (Fig. 2C). Identical results were obtained with the other enzyme sources.

Methylation analysis. — In order to prove the structure of the di- and mono-sialylated products formed in the incubation with regenerating rat liver micro-

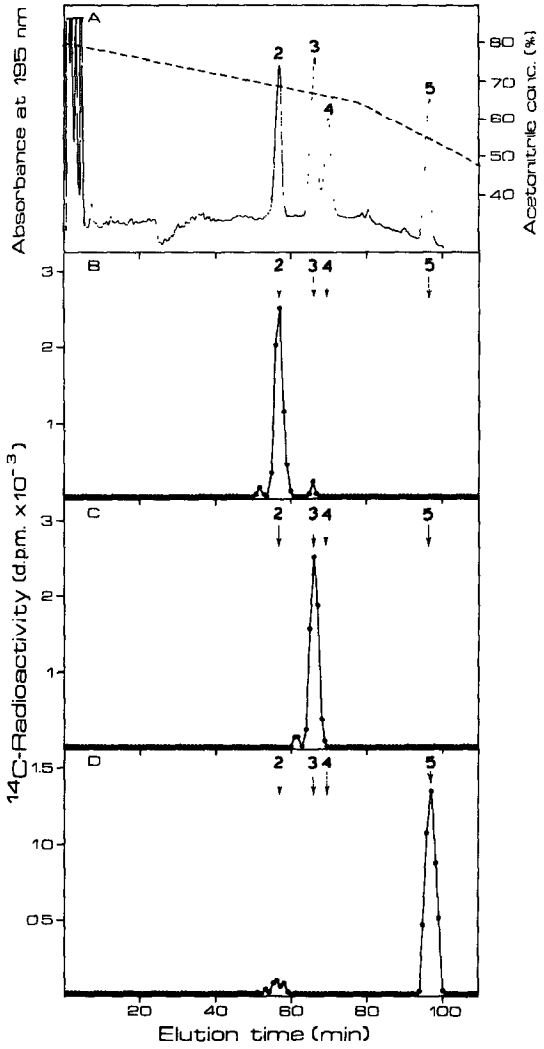


Fig. 2. Identification of sialylated oligosaccharides by l.c. A system capable of separating LS-tetrasaccharide-a (**2**), -b (**3**), and -c (**4**), and disialyllacto-*N*-tetraose (**5**) (panel A), described in the Experimental section, was used. The monosialylated (Fig. 1, peak II) and disialylated (Fig. 1, peak I) oligosaccharide products obtained from an incubation with regenerating rat liver microsomes (panels B and D, respectively), as well as the monosialylated oligosaccharide (III) (panel C), derived from the disialylated compound (I) by digestion with Newcastle-disease virus neuraminidase, were analyzed.

somes, and of the monosialylated product formed from the bisialylated compound I by digestion with Newcastle-disease virus neuraminidase, purified samples of the reduced compounds were subjected to methylation analysis (Table II). In the g.l. chromatogram of reduced material II (Fig. 1), methyl 3-*O*-acetyl-2,4,6-tri-*O*-methylgalactoside was present but no trace of the tetra-*O*-methylgalactoside. In

TABLE II

METHYLATION ANALYSIS OF SIALYLATED OLIGOSACCHARIDES[a]

Per-O-acetyl derivative of	*Sialylated oligosaccharides*		
	I	*II*	*III*
Methyl 2,3,4,6-tetra-*O*-methyl-D-galactoside	−	−	+
Methyl 2,4,6-tri-*O*-methyl-D-galactoside	+	+	+
Methyl 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-glucoside	−	+	−
Methyl 2-deoxy-4-*O*-methyl-2-(*N*-methylacetamido)-D-glucoside	+	−	+
1,2,3,5,6-Penta-*O*-methyl-D-glucitol	+	+	+
N-Acetyl, *N*-methyl-4,7,8,9-tetra-*O*-methylneuraminic acid methyl ester methyl glycoside	+	+	+

[a]Materials I, II and III, obtained by sialylation *in vitro* with regenerating rat liver microsomes and digestion with Newcastle-disease virus neuraminidase, were subjected to methylation analysis after reduction. The presence (+) or absence (−) of the per-*O*-acetyl derivatives of the methyl glycosides is indicated.

addition, a single *O*-methyl 2-acetamido-2-deoxyglucoside could be detected, which was identified as methyl 3-*O*-acetyl-2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-glucopyranoside by the abundance in its mass spectrum (not shown) of mass ions, *m/z* 45, 142, 169, 214, and 246, and the absence of the *m/z* 217 fragment according to the criteria of Fournet *et al.*[43]. Reduced material I (Fig. 1) showed the same substitution pattern for its galactosyl residues as material II (Table II, Fig. 3A). It yielded, however, the two anomeric forms of a single methyl 2-acetamido-2-deoxyglucoside, to which the structure of a 4-*O*-methyl derivative was attributed on the basis of the relative abundances of the characteristic mass ions, *m/z* 182, 215, 242, and 274 (Fig. 3B). The same 2-acetamido-2-deoxyglucose derivative was detected in the g.l. chromatogram of reduced material III (Table II). Furthermore, the two anomeric forms of methyl 2,3,4,6-tetra-*O*-methyl-D-galactopyranoside were observed in addition to methyl 2,4,6-tri-*O*-methyl-D-galactopyranoside. These results showed unequivocally that materials I, II, and III were disialyllacto-*N*-tetraose (**5**),LS-tetrasaccharide-a (**2**) and LS-tetrasaccharide-b (**3**), respectively.

Kinetics of sialic acid incorporation. — The substrate–intermediate–product relationship of lacto-*N*-tetraose (**1**), LS-tetrasaccharide-a (**2**), and disialyllacto-*N*-tetraose (**5**) was further investigated by studying the kinetics of sialic acid incorporation into lacto-*N*-tetraose (**1**). For this purpose, an incubation with regenerating rat liver microsomes was conducted in which the lacto-*N*-tetraose (**1**) concentration was 0.22mM. At various times, samples were withdrawn from the incubation mixture and the amount of products formed was estimated by analysis on a calibrated Bio-Gel P-4 column. The results (Fig. 4) showed that lacto-*N*-tetraose (**1**) was rapidly converted into LS-tetrasaccharide-a (**2**), and that the formation of disialyllacto-*N*-tetraose (**5**) started already at a low concentration of the monosialylated product. An almost complete conversion was obtained within 24 h.

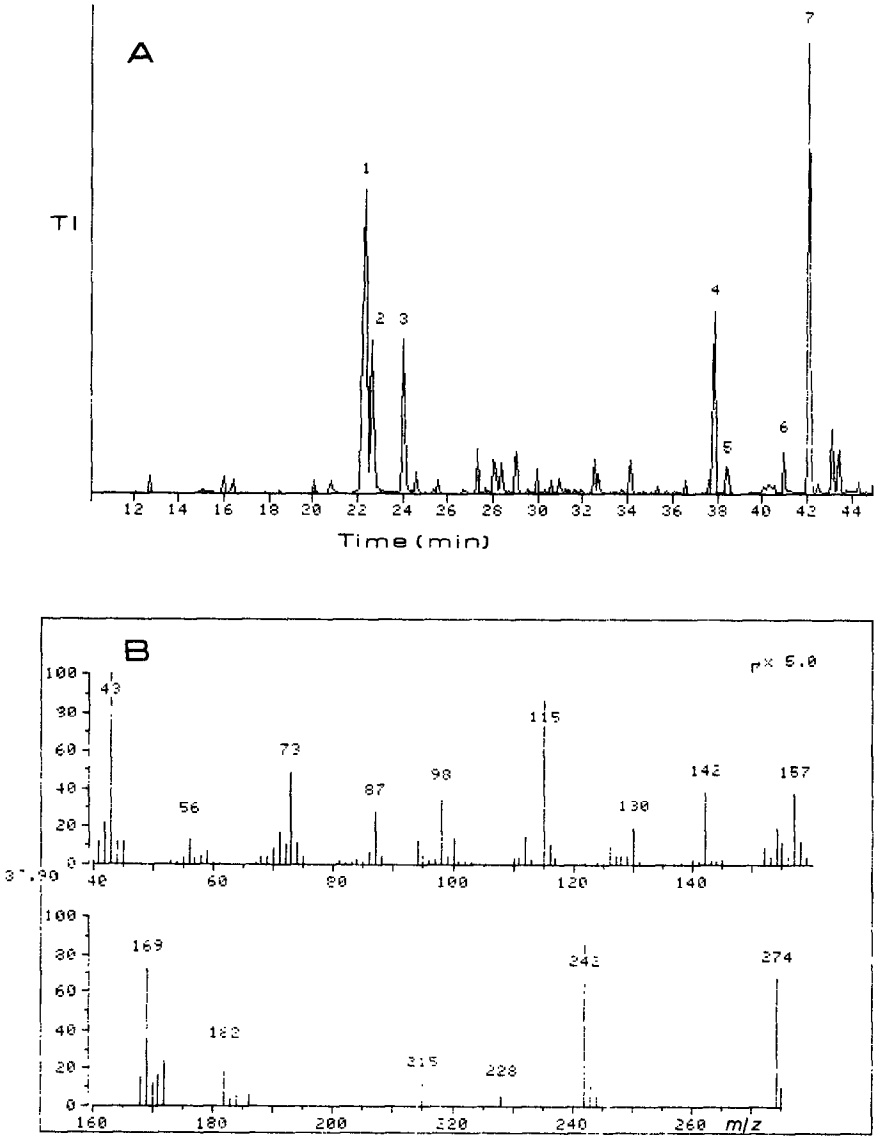


Fig. 3. Identification of methylated sugar derivatives. The disialylated oligosaccharide product (Fig. 1, peak I) was subjected to methylation analysis as described in the Experimental section: (A) In the gas-liquid chromatogram the following derivatives were identified: the per-*O*-acetyl derivatives of both anomeric forms of methyl 2,4,6-tri-*O*-methyl-D-galactoside (1 and 3); 1,2,3,5,6-penta-*O*-methyl-D-glucitol (2); both anomeric forms of methyl 2-deoxy-4-*O*-methyl-2-(*N*-methylacetamido)-D-glucoside (4 and 5); and both anomeric forms of the methyl ester methyl glycoside of *N*-acetyl-*N*-methyl-4,7,8,9-tetra-*O*-methylneuraminic acid (6 and 7). (B) Mass spectrum of the material eluted at 37.9 min (4) in (A). TI: total ion current.

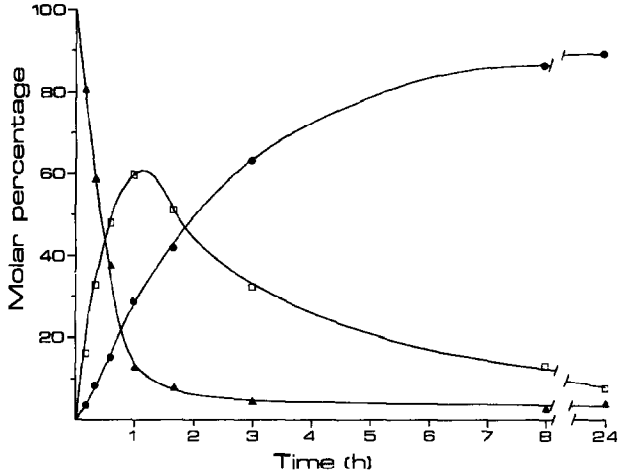


Fig. 4. Kinetics of sialic acid incorporation. The time course of incorporation of sialic acid into lacto-*N*-tetraose (**1**) (▲) by microsomes of regenerating rat liver to give LS-tetrasaccharide-a (**2**) (□) and disialyllacto-*N*-tetraose (**5**) (●) was determined by withdrawing samples from the reaction mixture at the times indicated and by analysis by gel filtration as described in the legend to Fig. 1.

Competition studies. — In order to establish the relationship between the sialyltransferase catalyzing the conversion of LS-tetrasaccharide-a (**2**) into disialyllacto-*N*-tetraose (**5**) and the previously described CMP-NeuAc:(NeuAcα→3Galβ→3)-GalNAcα→R α-(2→6)-sialyltransferase[29], which can specifically be assayed with NeuAcα→3Galβ→3GalNAcα→*p*-nitrophenyl[21], competition studies were carried out using microsomes from regenerating rat liver and fetal calf liver as enzyme sources, and LS-tetrasaccharide-a (**2**) and the *p*-nitrophenyl glycoside as acceptor substrates. Incorporation of sialic acid into each of these substrates separately could be established by analyzing the reaction mixtures on a calibrated column of Bio-Gel P-4 (Fig. 5). With both enzyme preparations, a competition between the two acceptor substrates was found (Table III).

DISCUSSION

The α-(2→3)- and α-(2→6)-sialyltransferase activities of regenerating rat liver and other tissues described herein clearly are involved in the biosynthesis of the disialyloligosaccharide structure, NeuAcα→3Galβ→3(NeuAcα→6)GlcNAcβ→R, which occurs in human milk oligosaccharides[11,12,35] and as the terminal group of the glycan chains of several *N*-glycoproteins[32–34,36,37]. Analysis of the sialylated products formed *in vitro* indicated that the disialyllated structure is formed mainly through one of two possible intermediate sequences, namely, NeuAcα→3Galβ→3-GlcNAcβ→R. This indicates that, in the preferred pathway, the action of the α-(2→3)-sialyltransferase preceeds that of the α-(2→6)-sialyltransferase. The α-(2→3) enzyme, which has already previously been purified from rat liver[25], can be

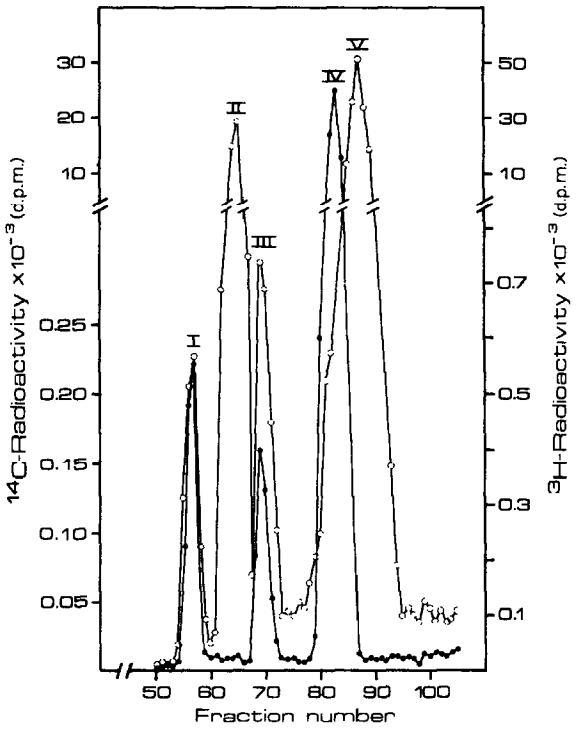


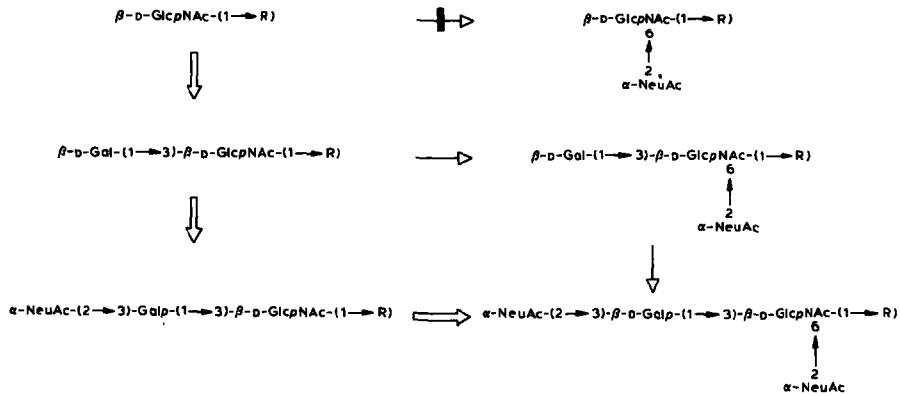
Fig. 5. Separation of sialylated oligosaccharides by gel filtration. The products of sialylation obtained from an incubation with fetal calf liver microsomes and ^{3}H-labeled LS-tetrasaccharide-a (**2**), *p*-$NO_2C_6H_4$ NeuAcα→3Galβ→3GalNAcα, and CMP-[^{14}C]NeuAc were analyzed on a calibrated column of Bio-Gel P-4 as described in the legend to Fig. 1. The various peak materials were identified as: (I) disialyllacto-*N*-tetraose (**5**); (II) LS-tetrasaccharide-a (**2**); (III) NeuAcα→3Galβ→3(NeuAcα→6)GalNAcα→*O*-*p*-$NO_2C_6H_4$; (IV) NeuAc; and (V) NeuAcα→3Galβ→3GalNAcα→*O*-*p*-$NO_2C_6H_4$ as described in this study and in reference[21]. ^{14}C- (●) and ^{3}H-radioactivity (○) were determined by liquid-scintillation counting.

TABLE III

COMPETITION STUDIES[a]

Acceptor concentration (mM)		*Incorporation of sialic acid (nmol · h^{-1}) by microsomes of*	
LS-tetrasaccharide-a (**2**)	*NeuAcα→3Galβ→GalNAcα→*O-p-$NO_2C_6H_4$	*Fetal calf liver*	*Regenerating rat liver*
2	0	2.6	5.9
0	2	2.5	5.8
2	2	1.4 + 1.0	3.9 + 1.9

[a]Sialyltransferase activities were assayed by use of the incubation mixture described in the Experimental section, except for the use of ^{3}H-labeled LS-tetrasaccharide-a (**2**) (labeled in the NeuAc group, 74 GBq/mol) and the *p*-nitrophenyl α-glycoside of [^{3}H]NeuAcα→3Galβ→3GalNAc (130 GBq/mol) as acceptor substrates at the concentrations indicated. Incorporation into each of the acceptors was established by gel filtration as described in Fig. 5.



Scheme 1. Proposed pathway for the biosynthesis of disialylated Galβ→3GlcNAcβ→ groups in milk oligosaccharides [R = 3)-β-D-Gal*p*-(1→4)-D-Glc] and glycans (R = oligosaccharide–protein) of *N*-glycoproteins: ⟹, major route: ⟶, minor route; and ▬, reaction cannot proceed.

classified as a CMP-NeuAc:Galβ→3(4)GlcNAc-R α-(2→3)-sialyltransferase[19]. It shows almost equal activity with lacto-*N*-tetraose (**1**) and LS-tetrasaccharide-b (**3**). Our data show that the α-(2→6)-sialyltransferase, on the other hand, acts with a high preference on LS-tetrasaccharide-a (**2**), and shows little activity with lacto-*N*-tetraose (**1**), and no activity at all with GlcNAcβ→3Galβ→4Glc. The specificity of this transferase, which can be designated as a CMP-NeuAc:(NeuAcα→3Galβ→3)-GlcNAc-β→R α-(2→6)-sialyltransferase, thus imposes the preferred pathway mentioned earlier and shown in Scheme 1. Our results essentially confirm recent findings on this pathway by Paulson *et al.*[30].

Although the two sialyltransferases have been identified by the analysis of products formed when milk oligosaccharides were used as substrates, indications were obtained that the pathway applies to the biosynthesis of the disialyl sequence of *N*-glycoproteins as well. When bovine asialoprothrombine, which has Galβ→4GlcNAcβ as well as Galβ→3GlcNAcβ disaccharide terminal groups[32], was sequentially sialylated with Galβ→4GlcNAc→R α-(2→6)-sialyltransferase from bovine colostrum[22] and a partially purified preparation of Galβ→3(4)GlcNAc→R α-(2→3)-sialyltransferase from rat liver[25], it was possible to obtain an additional incorporation of sialic acid by incubation with regenerating rat liver microsomes. The latter sialyl groups appeared to be largely resistant to digestion by Newcastle-disease virus neuraminidase, thus indicating that they were α-(2→6)-linked[45].

With all tissues investigated, LS-tetrasaccharide-a (**2**) was preferentially formed from lacto-*N*-tetraose (**1**) *in vitro*. The concentration of this sialooligosaccharide in human milk, however, is much lower[12] than that of LS-tetrasaccharide-b (**3**). This cannot be due to a relatively low activity of the α-(2→3)-sialyltransferase in human mammary gland, as human milk contains considerable amounts of disialyllacto-*N*-tetraose (**5**). More likely, the low concentration of LS-tetrasaccharide-a (**2**) is the result of its effective conversion by the α-(2→6)-sialyl-

transferase to the disialylated oligosaccharide **5**. On the other hand, the higher concentration of LS-tetrasaccharide-b (**3**) may be explained by assuming that this oligosaccharide, once formed, is α-(2→3)-sialylated only at a very low rate because this reaction is competitively inhibited by lacto-*N*-tetraose (**1**), which is abundantly present. Our earlier suggestion[1] that LS-tetrasaccharide-b (**3**) is possibly formed from disialyllacto-*N*-tetraose (**5**) by enzymic or chemical hydrolysis during production in the mammary gland, or subsequent transport and storage of the milk can be discounted, since radioactive disialyllacto-*N*-tetraose (**5**), when incubated in human milk at 37° up to several days, appeared to be completely stable[45].

Previously, a Galβ→4GlcNAc→R α-(2→3)-sialyltransferase has been identified in fetal calf liver by use of asialo-α_1-acid [^{3}H]glycoprotein as an acceptor[28]. The same sialyltransferase was shown to be abundantly present in human placenta, but could not be detected in rat liver[28]. Yet, an α-(2→3)-sialyltransferase acting on Galβ→4GlcNAc→R acceptor groups has been purified from rat liver[25]. This enzyme, however, preferably acts on Galβ→3GlcNAc→R groups and, therefore, has been classified as a Galβ→3(4)GlcNAc→R α-(2→3)-sialyltransferase[19]. In the present study, we have found that rat liver and fetal calf liver are relatively rich in this sialyltransferase activity, but that human placenta contains much less of it. Because of the difference in tissue and species distribution of the Galβ→4GlcNAc→R α-(2→3)- and the Galβ→3(4)GlcNAc→R α-(2→3)-sialyltransferase, we believe that the two activities have to be attributed to two different enzyme species.

The (NeuAcα→3Galβ→3)GlcNAcβ→R α-(2→6)-sialyltransferase identified in this study shows a substrate specificity that is analogous to that of a recently described (NeuAcα→3Galβ→3)GalNAcα→R α-(2→6)sialyltransferase in fetal calf liver[29]. Both sialyltransferases bind a sialyl group with an α-(2→6)-linkage to a 2-acetamido-2-deoxy-D-glucose residue, but require that this residue be substituted at O-3 with a NeuAcα→3Galβ group. Because of the resemblance in acceptor specificity of the two α-(2→6)-sialyltransferases, competition experiments were carried out. LS-tetrasaccharide-a (**2**) and the *p*-nitrophenyl α-glycoside of NeuAcα→3Galβ→3GalNAc, which are specific acceptor substrates for these two enzymes, respectively (see refs. 21 and 30, and this study), were used to establish whether these two enzymes are related. Since a competition was found with both the microsomes of regenerating rat liver and fetal calf liver (used as a sialyltransferase preparation), it may appear that both substrates compete for a common site on the sialyltransferases. This result does not necessarily indicate that only one sialyltransferase exists that acts on both substrates. Such an enzyme would be incapable to differentiate between a 2-acetamido-2-deoxy-β-D-glucopyranosyl and a 2-acetamido-2-deoxy-α-D-galactopyranosyl residue. It rather seems to us, that the two enzymes have a domain in common, which specifically recognizes the NeuAcα→3Galβ→3 group of the acceptor molecules. To clarify this point, however, the purification of one or both of the α-(2→6)-sialyltransferases is required.

REFERENCES

1 H. T. DE HEIJ AND D. H. VAN DEN EIJNDEN, *Abstr. Int. Carbohydr. Symp., XIIth,* (1984) 225.
2 J. MONTREUIL, *Adv. Carbohydr. Chem. Biochem.,* 37 (1980) 157–223.
3 E. G. BERGER, E. BUDDECKE, J. P. KAMERLING, A. KOBATA, J. C. PAULSON, AND J. F. G. VLIEGENTHART, *Experientia,* 38 (1982) 1129–1258.
4 A. SLOMIANY AND B. L. SLOMIANY, *J. Biol. Chem.,* 253 (1978) 7301–7306.
5 J. FINNE, *J. Biol. Chem.,* 257 (1982) 11 966–11 970.
6 S. INOUE AND G. MATSUMURA, *Carbohydr. Res.,* 74 (1979) 361–368.
7 M. IWASAKI, S. INOUE, U. KITAJIMA, H. NOMOTO, AND Y. INOUE, *Biochemistry,* 23 (1984) 305–310.
8 R. W. LEDEEN AND R. K. YU, in N. MARKS AND R. RODNIGHT (Eds.), *Research Methods in Neurochemistry,* Vol. 4, Plenum Press, New York. 1978, pp. 371–410.
9 B. C. MACHER AND C. C. SWEELEY, *Methods Enzymol.,* 50 (1978) 236–251.
10 K. WATANABE AND S. HAKOMORI, *Biochemistry,* 18 (1979) 5502–5504.
11 A. KOBATA, *Methods Enzymol.,* 28 (1972) 262–271.
12 D. F. SMITH, D. A. ZOPF, AND V. GINSBURG, *Anal. Biochem.,* 85 (1978) 602–608.
13 J. PARKKINEN AND J. FINNE, *Eur. J. Biochem.,* 136 (1983) 355–361.
14 A. HAGOPIAN AND E. H. EYLAR, *Arch. Biochem. Biophys.,* 128 (1968) 422–433.
15 S. ROSEMAN, *Chem. Phys. Lipids,* 5 (1970) 270–297.
16 H. SCHACHTER AND S. ROSEMAN, in W. J. LENNARZ (Ed.), *The Biochemistry of Glycoproteins and Proteoglycans,* Plenum Press, New York, 1980, pp. 85–160.
17 J. C. PAULSON, J. I. REARICK, AND R. L. HILL, *J. Biol. Chem.,* 252 (1977) 2363–2371.
18 J. I. REARICK, J. E. SADLER, J. C. PAULSON, AND R. L. HILL, *J. Biol. Chem.,* 254 (1979) 4444–4451.
19 J. WEINSTEIN, U. DE SOUZA-E-SILVA, AND J. C. PAULSON, *J. Biol. Chem.,* 257 (1982) 13 845–13 853.
20 D. H. JOZIASSE, M. L. E. BERGH, H. TER HART, P. L. KOPPEN, G. J. M. HOOGHWINKEL, AND D. H. VAN DEN EIJNDEN, *J. Biol. Chem.,* 260 (1985) 4941–4951.
21 M. L. E. BERGH AND D. H. VAN DEN EIJNDEN, *Eur. J. Biochem.,* 136 (1983) 113–118.
22 J. C. PAULSON, W. E. BERANEK, AND R. L. HILL, *J. Biol. Chem.,* 252 (1977) 2356–2362.
23 J. E. SADLER, J. I. REARICK, J. C. PAULSON, AND R. L. HILL, *J. Biol. Chem.,* 254 (1979) 4434–4443.
24 J. E. SADLER, J. I. REARICK, AND R. L. HILL, *J. Biol. Chem.,* 254 (1979) 5934–5941.
25 J. WEINSTEIN, U. DE SOUZA-E-SILVA, AND J. C. PAULSON, *J. Biol. Chem.,* 257 (1982) 13 835–13 844.
26 B. KAUFMAN, S. BASU, AND S. ROSEMAN, in S. M. ARONSON AND B. W. VOLK (Eds.), *Inborn Disorders of Sphingolipid Metabolism,* Pergamon, New York, 1967, pp. 193–213.
27 B. KAUFMAN, S. BASU, AND S. ROSEMAN, *J. Biol. Chem.,* 243 (1968) 5804-5807.
28 D. H. VAN DEN EIJNDEN AND W. E. C. M. SCHIPHORST, *J. Biol. Chem.,* 256 (1981) 3159–3162.
29 M. L. E. BERGH, G. J. M. HOOGHWINKEL, AND D. H. VAN DEN EIJNDEN, *J. Biol. Chem.,* 258 (1983) 7430–7436.
30 J. C. PAULSON, J. WEINSTEIN, AND U. DE SOUZA-E-SILVA, *Eur. J. Biochem.,* 140 (1984) 523–530.
31 E. R. VIMR, R. D. MCCOY, H. F. VOLLGER, N. C. WILKINSON, AND F. A. TROY, *Proc. Natl. Acad. Sci. U.S.A.,* 81 (1984) 1971–1975.
32 T. MIZUOCHI, K. YAMASHITA, K. FUJIKAWA, W. KISIEL, AND A. KOBATA, *J. Biol. Chem.,* 254 (1979) 6416–6425.
33 T. MIZUOCHI, K. YAMASHITA, K. FUJIKAWA, K. TITANI, AND A. KOBATA, *J. Biol. Chem.,* 255 (1980) 3526–3531.
34 T. MIZUOCHI, T. TANIGUCHI, K. FUJIKAWA, K. TITANI, AND A. KOBATA, *J. Biol. Chem.,* 258 (1983) 6020–6024.
35 L. GRIMMONPREZ AND J. MONTREUIL, *Bull. Soc. Chim. Biol.,* 18 (1968) 843–849.
36 H. YOSHIMA, A. MATSUMOTO, T. MIZUOCHI, T. KAWASAKI, AND A. KOBATA, *J. Biol. Chem.,* 256 (1981) 8476–8484.
37 E. BERMAN, *Abstr. Int. Carbohydr. Symp., XIIth,* (1984) 421.
38 T. A. BEIJER, 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.
39 D. H. VAN DEN EIJNDEN AND W. VAN DIJK, *Hoppe-Seyler's Z. Physiol. Chem.,* 353 (1972) 1817–1820.
40 M. L. E. BERGH, P. L. KOPPEN, AND D. H. VAN DEN EIJNDEN, *Carbohydr. Res.,* 94 (1981) 225–229.
41 G. M. HIGGINS AND R. M. ANDERSON, *Arch. Pathol.,* 12 (1931) 186–195.
42 S. HAKOMORI, *J. Biochem. (Tokyo),* 55 (1964) 205–208.
43 B. FOURNET, G. STRECKER, Y. LEROY, AND J. MONTREUIL, *Anal. Biochem.,* 116 (1981) 489–502.
44 R. DRZENIEK, *Histochem. J.,* 5 (1973) 271–290.
45 H. T. DE HEIJ, W. E. C. M. SCHIPHORST, AND D. H. VAN DEN EIJNDEN, unpublished results.