Biochem. J. (1983) **213**, 253–260
Printed in Great Britain

Comparative rates of transfer of *N*-acetylneuraminic acid to acceptors bearing one or more Gal(β1-4)GlcNAc terminus by the Gal(β1-4)GlcNAc(NeuAc-Gal) (α2-6)-sialyltransferase from embryonic chicken liver

Utilization of oligosaccharides as acceptors in sialyltransferase assays

Brad BENDIAK and Geoffrey M. W. COOK
Department of Pharmacology, University of Cambridge, Hills Road, Cambridge CB2 2QD, U.K.

(*Received 25 January 1983/Accepted 22 March 1983*)

Using a number of branched and unbranched oligosaccharides, glycoproteins and artificial glycoproteins bearing Gal(β1-4)GlcNAc-R termini as acceptors (where R represents H, oligosaccharide, oligosaccharide–protein or fatty acid–protein), the comparative rates of transfer of NeuAc by the Gal(β1-4)GlcNAc(NeuAc-Gal) (α2-6)-sialyltransferase of embryonic chicken liver were determined. Acceptor substrates were utilized at levels approximating physiological, near the K_m value of the best acceptor, desialylated α_1 acid glycoprotein. The sialyltransferase has a marked preference for multi-branched acceptors. From the specificity data, it is concluded that the enzyme binds at least two Gal(β1-4)GlcNAc termini of an acceptor molecule, and that the relative orientation of the branches is an important factor determining the rate of catalysis by the enzyme. The use of oligosaccharides as acceptors to study sialyltransferase catalyses is emphasized. Results are discussed in the context of the mode of assembly of sialoside termini of known glycoprotein structures *in vivo*.

Sialic acids are common constituents of glycoproteins, and are present on oligosaccharide chains that are either *N*-glycosidically linked to asparagine or *O*-glycosidically linked to serine or threonine residues of glycoproteins (Montreuil, 1980; Kornfeld & Kornfeld, 1980). The principal subcellular site of sialylation of glycoproteins is the Golgi apparatus (Schachter *et al.*, 1970; Schachter & Roseman, 1980) and sialic acid transfer may be localized to, or at least concentrated in, *trans* Golgi cisternae (Bennett & O'Shaughnessy, 1981). CMP-NeuAc, the sugar donor for sialylation, apparently enters Golgi cisternae via a transport molecule located in Golgi membranes (Sommers & Hirschberg, 1982). NeuAc is subsequently transferred to acceptor glycoproteins, the precise positioning of which depends upon the catalytic specificities of the sialyltransferases present in the Golgi apparatus.

Structural evidence to date indicates that sialic acids are found in eight different linkages in the glycoproteins of vertebrates. These include NeuAc-(α2-3, -4 or -6)Gal (Mizuochi *et al.*, 1980*a*; Takasaki *et al.*, 1979), NeuAc(Gc)(α2-3 or -6)-GalNAc (Carlson, 1968; Spiro & Bhoyroo, 1974; Inoue *et al.*, 1981), NeuAc(α2-4 or -6)GlcNAc (Slomiany & Slomiany, 1978; Takasaki *et al.*, 1979) and NeuAc(α2-8)NeuAc (Finne, 1982). This implies the existence of at least eight different sialyltransferases, based on the one-enzyme-one-linkage hypothesis (Hagopian & Eylar, 1968). Additional evidence, based on specificities of sialyltransferases, indicates that the NeuAc(α2-6)Gal(β1-4)GlcNAc and NeuGc(α2-6)Gal(β1-3)GalNAc linkages (Paulson *et al.*, 1977; Aminoff *et al.*, 1979) as well as the NeuAc(α2-3)Gal[β1-3(4)]GlcNAc and NeuAc(α2-3)Gal(β1-3)GalNAc linkages (Rearick *et al.*, 1979; Weinstein *et al.*, 1982) require separate enzymes for their synthesis, indicating the probability of the existence of at least ten sialyltransferases. It is important that the specificities of sialyltransferases and their patterns of expression in different cell types be understood, in order to clarify the mode of assembly of sialoside termini of glycoproteins by the various cells of an organism.

With regard to the synthesis of complex-type

Abbreviations used: NeuGc, *N*-glycollylneuraminic acid; BSA, bovine serum albumin.

asparagine-linked oligosaccharides of glycoproteins *in vivo*, there is now ample structural evidence that indicates that NeuAc is not randomly transferred to Gal(β1-4)GlcNAc branches. The structures of galactoprotein (Fukuda & Hakomori, 1979), fetuin (Nilsson *et al.*, 1979), caeruloplasmin (Yamashita *et al.*, 1981), human α_1 proteinase inhibitor (Mega *et al.*, 1980), human lactotransferrin (Spik *et al.*, 1982), human proline-rich glycoprotein from parotid saliva (Reddy *et al.*, 1982) and human immunoglobulin A_1 (Baenziger & Kornfeld, 1974) indicate preferential transfer of NeuAc by sialyltransferases to particular Gal(β1-4)GlcNAc termini of bi- and tri-antennary oligosaccharides.

Previous studies of sialyltransferase activity in smooth membranes of embryonic chicken liver indicated the presence of two sialyltransferases, one utilizing the acceptor structure Gal(β1-4)GlcNAcβ-R (where R represents H, oligosaccharide–protein or fatty acid–protein), a second using the structure Gal(β1-3)GalNAcα-R as an acceptor (Bendiak & Zalik, 1981). The enzyme that transferred NeuAc to Gal(β1-4)GlcNAc structures utilized the disaccharide Gal(β1-4)GlcNAc to a lesser extent than glycoproteins bearing Gal(β1-4)GlcNAc termini as part of a larger oligosaccharide structure. It was deemed that structures underlying the terminal disaccharide might be necessary for catalysis to proceed at a maximal rate.

Recently, we documented the purification of the sialyltransferase that transfers NeuAc to Gal(β1-4)GlcNAc structures, free of activity recognizing Gal(β1-3)GalNAc termini. The product of its catalysis and kinetic mechanism were described (Bendiak & Cook, 1982). In the present paper, we report the comparative rates of transfer of NeuAc by the enzyme to various galactoside-terminated structures at approximate physiological acceptor concentrations, near the K_m value for the best acceptor, desialylated α_1 acid glycoprotein. The general utility of oligosaccharides as acceptors for assaying sialyltransferases is pointed out.

Experimental

Preparation of substrates

CMP-[^{14}C]NeuAc (sp. radioactivity 1.68 Ci/mol) was obtained from New England Nuclear. Fetuin (Spiro method) was purchased from Gibco. Human α_1 acid glycoprotein and antithrombin III were generously supplied by Dr. Y. L. Hao, National Fractionation Center, Bethesda, MD, U.S.A. The structures of the carbohydrate moieties of these glycoproteins have been reported and verified (Fournet *et al.*, 1978; Nilsson *et al.*, 1979; Franzén *et al.*, 1980; Mizuochi *et al.*, 1980*b*; Krusius & Finne, 1981; Yoshima *et al.*, 1981). These glycoproteins were desialylated (Bendiak & Zalik, 1981) and desialylated agalactofetuin was prepared from desialylated fetuin by a single round of the Smith degradation, as previously described (Bendiak & Zalik, 1981). Antifreeze glycoprotein from the antarctic fish *Trematomus borchgrevinkii* was supplied by Dr. A. L. DeVries, Department of Physiology, University of Illinois, Urbana-Champaign, IL, U.S.A. Several compounds (Table 1, structures 9–12 and 14) were obtained from Dr. R. U. Lemieux, Chembiomed, W5-56, Chemistry Building, University of Alberta, Edmonton, Alberta, Canada. The synthesis of similar compounds has been described previously by Lemieux *et al.* (1982). Dr. J. Lönngren, Department of Organic Chemistry, Arrhenius Laboratory, University of Stockholm, Stockholm, Sweden, provided several oligosaccharides (Table 1, structures 15 and 17–20), the synthesis of which has been reported (Arnarp & Lönngren, 1981). Dr. A. Veyrières, Laboratoire de Chimie Organique Multifonctionelle, Université de Paris-Sud, Orsay Cédex, France, supplied two trisaccharides (Table 1, structures 16 and 17; Alais & Veyrières, 1981).

Large-scale preparations of the *N*-glycosidically-linked oligosaccharide of native as well as desialylated fetuin were performed by using the hydrazinolysis procedure (Mizuochi *et al.*, 1980*a*). Anhydrous hydrazine was prepared by the method of Day & Whiting (1970), except that only two-thirds of the upper hydrazine layer was distilled. Fetuin (1 g) or desialylated fetuin (from 1 g of native fetuin) were treated for 24 h at 100°C in hydrazine (40 ml) in sealed glass tubes. Hydrazine was removed by freeze-drying, care being taken to collect ice (approx. 100 ml) in the trap of the freeze-dryer first, so that hydrazine hydrate was present upon cleaning the trap.

The de-*N*-acetylated oligosaccharide thus produced was either re-*N*-acetylated directly, or separated from some amino acid hydrazides by passage through a column (2 m × 2.1 cm) of Bio-Gel P4, and subsequently re-*N*-acetylated. *N*-Acetylation was carried out on oligosaccharide dissolved in saturated $NaHCO_3$ (50 ml at 0°C), followed by two 5 ml portions of acetic anhydride at 15 min intervals. The solution was then maintained at 4°C for 24 h, and subsequently stored frozen. Five separate batches were passed through a Bio-Gel P4 column (195 cm × 2.1 cm; 200–400 mesh) in 25 mM-sodium acetate/HCl, pH 5.5, at room temperature. Fractions (8.4 ml) were collected and portions (0.2 ml) tested for hexose by the phenol/H_2SO_4 reaction (Dubois *et al.*, 1956) and for NeuAc, after hydrolysis in 0.05 M-H_2SO_4 at 80°C for 1 h, by the method of Warren (1959). Fractions containing the high-molecular-weight oligosaccharide were pooled, concentrated by rotary evaporation (40°C) to about 40 ml and desalted by

passage, in distilled water, through a Sephadex G-15 column (2.6 cm × 43 cm; three batches). Hexose was determined as described above, and chloride was detected with silver nitrate solution. Sugar constituents of the *N*-linked oligosaccharide of desialylated fetuin were quantified by the procedures described by Spiro (1966), except that monosaccharides were separated by the more convenient one-step paper-chromatographic system described by Kim *et al.* (1971) with ethyl acetate/pyridine/water (12:5:4, by vol.) as the solvent system.

Sialyltransferase assay

β-D-Galactoside (α2-6)-sialyltransferase was purified on average 500-fold, by using procedures previously published (Bendiak & Cook, 1982). The standard assay mixture contained, in a total of 100 μl, 1.25–3.0 μg of protein of the purified enzyme preparation [506 ± 13.6 nmol · h^{-1} · (mg of protein)$^{-1}$], 210 μM-CMP-[^{14}C]NeuAc, 590 μM terminal galactoside or 'potential acceptor sites' of acceptor molecules (Table 1), 0.5% (v/v) Triton X-100 and 50 mM-cacodylate/HCl, pH 5.5. This solution was briefly vortex-mixed and incubated at 30°C for 20 min.

When glycoproteins were used as acceptors, product was quantified by precipitation with trichloroacetic acid/phosphotungstic acid as previously described (Bendiak & Zalik, 1981). Oligosaccharides were assayed by separation of sialylated product from CMP-NeuAc and any free NeuAc on Dowex AG2 columns (X8; 16.5 cm × 0.5 cm; Bendiak & Zalik, 1981). In the present paper, this procedure has been extended to include the utilization of all oligosaccharide acceptors ranging in size from two to 11 monosaccharides (see the Results section, Fig. 3). To achieve good separations, the following experimental details are important. First, for the column dimensions, we use 0.5 cm × 20 cm glass barrel Econo-columns (Bio-Rad). Secondly, the concentration of the competing chloride anion is critical. Owing to the marked variation in the pH of Tris buffers with temperature, the eluent should be prepared by adjusting a solution of 15 mM-Tris base to pH 7.0 with conc. HCl at 4°C. Thirdly, as we have experienced differences between batches of Dowex AG2 (X8; 200–400 mesh), the length of the resin bed may require alteration by 0.5–1.0 cm to achieve total product separation. This can be easily checked by determining the migration of NeuAc-lactose and NeuAc through columns.

All assays contained controls in which no exogenous acceptor was present; negligible endogenous activity was found in every case. Transfer to antifreeze glycoprotein 7, a neutral glycopeptide bearing 20 neutral amino acids and Gal(β1-3)-GalNAc structures (Shier *et al.*, 1975), was also quantified by the above column assay, as the product of its sialylation by a membranous preparation of embryonic chicken liver (purified 60-fold in sialyltransferase activity; Bendiak & Cook, 1982) migrated within the volume (5 ml) routinely collected.

Results

Acceptor preparation

After hydrazinolysis and re-*N*-acetylation, oligosaccharide was passed through a Bio-Gel P4 column (Fig. 1). The fractions containing high-molecular-weight oligosaccharide of native fetuin (Fig. 1*a*, fractions 36–43) were pooled and desalted by passage through Sephadex G-15; the yield was 176 mg (96% of theoretical yield, based on an M_r of 48000 for fetuin; Spiro, 1973). The lower-mol-

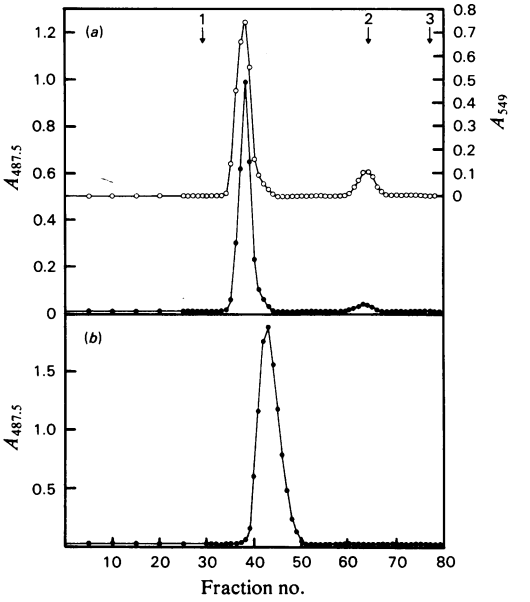
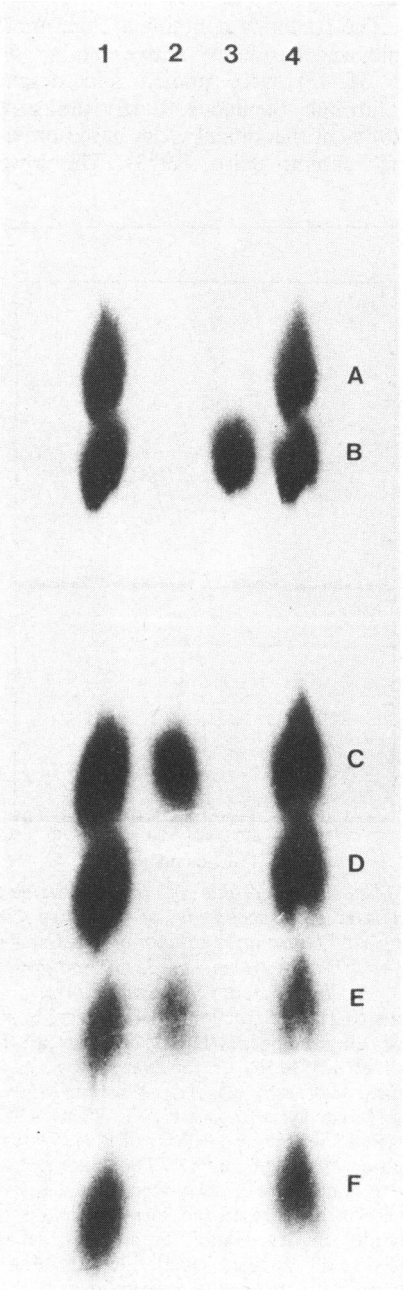


Fig. 1. *The elution profile of the hydrazine-treated, re-N-acetylated oligosaccharides of (a) native fetuin and (b) desialylated fetuin on a column of Bio-Gel P4 (200–400 mesh; 195 cm × 2.1 cm) in 25 mM-sodium acetate/HCl, pH 5.5, at room temperature*

Portions (0.2 ml) of fractions (8.4 ml) were analysed for hexose by the phenol/H_2SO_4 reaction (●; $A_{487.5}$; Dubois *et al.*, 1956) or for NeuAc by the thiobarbituric acid assay (○; A_{549}; Warren, 1959) after mild acid hydrolysis (0.05 M-H_2SO_4, 80°C, 1 h). The arrows indicate elution positions of Blue Dextran (1) stachyose (2) and glucose (3). The oligosaccharides of native fetuin were *N*-acetylated immediately after removal of hydrazine; the large *N*-linked oligosaccharide of desialylated fetuin was separated from the small *O*-linked saccharide after hydrazine treatment and subsequently *N*-acetylated.

ecular-weight oligosaccharide (fractions 60–67) was obtained in low yield (30–32% of theoretical yield, as judged by comparative peak integration of NeuAc and hexose) (Fig. 1*a*). Considerable degradation of the latter structure is presumed, Ogata & Lloyd (1982) and Takasaki *et al.* (1982) having reported similar degradation of *O*-linked oligosaccharides by the hydrazinolysis procedure.



The high-molecular-weight oligosaccharide of desialylated fetuin, after re-*N*-acetylation, was also passed through Bio-Gel P4 (Fig. 1*b*). Fractions 39–49 were pooled and desalted as above (yield 121mg; 96.5% of theoretical yield). A low-molecular-weight saccharide was not detected in this sample, as the hydrazine-released oligosaccharides were separated before *N*-acetylation on the same Bio-Gel P4 column (results not shown).

Visual detection of sugars present in the large desialylated oligosaccharide of fetuin is shown in Fig. 2. Compositional analysis of this material revealed mannose/galactose/glucosamine in the ratio of 3.0:3.0:4.7, a similar ratio having been observed by Nilsson *et al.* (1979) for this oligosaccharide. It is likely that the glucosamine residue at the reducing end of the oligosaccharide is modified during hydrazinolysis, as Saeed & Williams (1980) and Takasaki *et al.* (1982) have reported, but the precise chemical nature of these modifications has yet to be elucidated.

The sialyltransferase assay using oligosaccharides as acceptors

The migration of the large oligosaccharide of native fetuin (prepared by hydrazinolysis and re-*N*-acetylation) on the Dowex AG2(X8) columns used in the routine assay procedure is shown in Fig. 3. Of this oligosaccharide, which bears three sialic acid residues, 98% was eluted in the first 5ml. The product of sialyltransferase action on the desialylated large oligosaccharide of fetuin bears only one sialic acid residue (Bendiak & Cook, 1982). This structure migrates more rapidly through the columns than the trisialylated structure; 100% of the product was eluted in the collected 5ml (Fig. 3).

Comparative rates of transfer of NeuAc to galactoside acceptors by the β-galactoside (α2-6)-sialyltransferase

The rates of transfer of NeuAc to oligosaccharides and glycoprotein acceptors were deter-

Fig. 2. *Visual detection of monosaccharide constituents of the large oligosaccharide of desialylated fetuin released by hydrazine treatment*

Re-*N*-acetylated oligosaccharide (Fig. 1*b*) was hydrolysed and amino sugars were separated from neutral sugars by the method of Spiro (1966). Samples were spotted on Whatman 3MM paper and chromatographed for 15h in ethyl acetate/pyridine/water (12:5:4; Kim *et al.*, 1971). Lane 2, neutral sugar fraction; lane 3, amino sugar fraction; lanes 1 and 4, standards. The positions of galactosamine hydrochloride (A), glucosamine hydrochloride (B), galactose (C), glucose (D), mannose (E) and fucose (F) are indicated. The chromatogram was stained with the silver reagent (Trevelyan *et al.*, 1950). Sugars stain with different intensities; equimolar amounts (0.5 μmol) of the standards are shown.

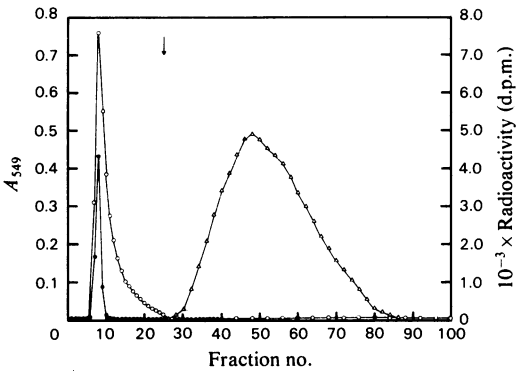


Fig. 3. *The migration of sialic acid-containing oligosaccharides on the columns of the sialyltransferase assay system*

Purified oligosaccharide was dissolved in 200 μl of 15 mM-Tris/HCl, pH 7.0 (at 4°C). The solution was placed on a Dowex AG2 column (0.5 × 16.5 cm; X8; Cl^- form; 200–400 mesh) in 15 mM-Tris/HCl, pH 7.0 (4°C). Material was run into the column, and the column was washed with the same buffer, collecting 0.2 ml fractions. (○), Migration of the trisialylated large oligosaccharide of fetuin (fractions 36–43; Fig. 1*a*, subsequently desalted); sialic acid was detected by the method of Warren (1959) after mild acid hydrolysis (0.05 M-H_2SO_4, 80°C, 1 h). ●, Migration of the [^{14}C]NeuAc-labelled, monosialylated large oligosaccharide of fetuin, prepared by the action of the Gal(β1-4)GlcNAc(α2-6)-sialyltransferase on the desialylated oligosaccharide (Fig. 1*b*), purified and desalted on Sephadex G-15. △, Migration of NeuAc, detected by the method of Warren (1959). The arrow indicates the volume (first 5 ml) that is routinely collected in the sialyltransferase assay, when oligosaccharides (two to 11 sugars) were used as acceptors.

mined at a constant concentration of CMP-NeuAc and terminal galactoside on acceptor molecules (Table 1). These concentrations were carefully chosen. The level of CMP-NeuAc used was five times the $K_m^{\text{CMP-NeuAc}}$ value for the enzyme (42 μM; Bendiak & Cook, 1982) to ensure that variation in reaction velocity was due to the structure of the acceptor molecule. Acceptors were used at a level 1.75 times the $K_m^{\text{terminal galactose}}$ value for the best acceptor, desialylated α_1 acid glycoprotein (336 μM; Bendiak & Cook, 1982). This value, near the K_m, was chosen so that comparisons could be made between acceptors at levels approximating physiological. Subsaturating acceptor concentrations were surmised to represent the situation *in vivo* more realistically than saturating concentrations. Typically, enzymes display a K_m near or above the physiological concentration of substrate, so that some measure of control can be exerted on the rate of product formation (Cleland, 1970). Clearly, if concentrations were used that were saturating for desialylated α_1 acid glycoprotein, yet unsaturating with respect to various other substrate analogues, then valid comparisons of reaction rates would not be possible. Second, a slightly higher level than the K_m (1.75 times) was chosen to keep incorporation of [^{14}C]NeuAc reasonably high for experimental reproducibility.

Discussion

The *N*-glycosidically-linked oligosaccharides of native and desialylated fetuin were prepared in good yield (over 95%) by the hydrazinolysis/re-*N*-acetylation procedure. On the basis of the compositional analyses and apparent molecular weight on Bio-Gel P4, the oligosaccharides appear intact after hydrazinolysis, although the reducing-end *N*-acetylglucosamine is likely to be modified by the procedure, and may exist as a mixture of compounds derived from *N*-acetylglucosamine. The *O*-glycosidically-linked oligosaccharides are largely degraded as low yields (approx. 30%) of these oligosaccharides were obtained.

An assay procedure using oligosaccharides as acceptors has been extended beyond the use of disaccharides (Bendiak & Zalik, 1981) to include all acceptors ranging from two to 11 monosaccharides in size. This procedure offers advantages over high-voltage electrophoresis (Roseman *et al.*, 1966) in being more rapid, and over the previous column procedure of Paulson *et al.* (1977) in that sialylated product is recovered quantitatively and correction factors need not be applied for every saccharide used as an acceptor. The more rapid migration rate of sialylated product, as compared with both CMP-NeuAc and NeuAc, is probably related to its lower charge/mass ratio. Separation of product from NeuAc is also essential, as it is often present in small quantities (approx. 1%) in commercial preparations of CMP-NeuAc. The *N*-linked oligosaccharide of fetuin, bearing three sialic acid residues, appears to trail on the columns of Dowex AG2(X8), which may be due to the co-operative effects of three charges on one molecule. Nevertheless, 98% of this oligosaccharide was eluted in the assay volume (5 ml).

The comparative rates of transfer of NeuAc to various acceptor substrates indicate that the disaccharide Gal(β1-4)GlcNAc is the essential portion of the acceptor molecule for transfer to occur. Structures bearing Gal(β1-3)GlcNAc or Gal(β1-3)GalNAc termini, which have a very different conformation (Lemieux *et al.*, 1980), were not utilized by the enzyme, although Gal(β1-4)Glc, which is similar in structure to Gal(β1-4)GlcNAc,

Table 1. *Acceptor specificity of the sialyltransferase*

Activity, measured as described in the Experimental section, is expressed as the percentage of radioactivity incorporated using desialylated α_1 acid glycoprotein as the reference value of 100%. All acceptors were present at a terminal galactoside concentration of 590 μM. In the case of native fetuin, α_1 acid glycoprotein, antithrombin III and desialylated agalactofetuin, this value refers to 'potential acceptor sites'. CMP-[^{14}C]NeuAc was utilized at a constant concentration of 210 μM. Six assays were performed with each acceptor. Residue X (compound 21, the large oligosaccharide of desialylated fetuin) represents an *N*-acetylglucosamine that is probably modified to some extent by hydrazine treatment (Saeed & Williams, 1980). Results are means ± S.E.M.

	Acceptor	Activity (%)
1.	α_1 acid glycoprotein	10.1 ± 0.41
2.	Desialylated α_1 acid glycoprotein	100
3.	Fetuin	8.1 ± 1.34
4.	Desialylated fetuin	67.2 ± 2.17
5.	Desialylated agalactofetuin	<1
6.	Antithrombin III	10.2 ± 0.54
7.	Desialylated antithrombin III	36.0 ± 2.08
8.	Antifreeze glycoprotein	<1
9.	{Gal(β1-4)GlcNAcβ1-O-[CH_2]$_8$C(=O)N}$_{16}$-BSA	42.8 ± 1.80
10.	{Gal(β1-3)GlcNAcβ1-O-[CH_2]$_8$C(=O)N}$_{26}$-BSA	<1
11.	{Gal(β1-3)GalNAcβ1-O-[CH_2]$_8$C(=O)N}$_{14}$-BSA	<1
12.	Gal(β1-4)GlcNAcβ1-O-[CH_2]$_8$C(=O)-OCH_3	<1
13.	Gal(β1-4)Glc	5.0 ± 0.60
14.	Gal(β1-4)GlcNAc	6.8 ± 0.60
15.	Gal(β1-4)GlcNAc(β1-2)Man	8.4 ± 0.82
16.	Gal(β1-4)GlcNAc(β1-3)Man	1.3 ± 0.36
17.	Gal(β1-4)GlcNAc(β1-6)Man	4.7 ± 0.34
18.	Gal(β1-4)GlcNAcβ1-2,4-Man-Gal(β1-4)GlcNAcβ1	13.9 ± 1.11
19.	Gal(β1-4)GlcNAcβ1-2,6-Man-Gal(β1-4)GlcNAcβ1	4.9 ± 0.71
20.	Gal(β1-4)GlcNAc(β1-2)Manα1-3,6-Man-Gal(β1-4)GlcNAc(β1-2)Manα1	5.9 ± 0.38
21.	Gal(β1-4)GlcNAcβ1-2,4-Manα1-Gal(β1-4)GlcNAcβ1; 3,6-Man(β1-4)GlcNAcβ1-X; Gal(β1-4)GlcNAc(β1-2)Manα1	48.9 ± 2.72

but bears a 2-hydroxy instead of a 2-acetamido function, was utilized to a small extent.

Of interest was our finding that molecules with just one Gal(β1-4)GlcNAc terminus were relatively poor acceptors for the sialyltransferase. The best of these was a trisaccharide, Gal(β1-4)GlcNAc(β1-2)Man, a structure found commonly in glycoproteins. Yet at the same galactoside concentration, the rate of transfer to desialylated α_1 acid glycoprotein was 12 times greater than to this trisaccharide. Originally, it was surmised (Bendiak & Zalik, 1981) that some oligosaccharide structure internal to the Gal(β1-4)GlcNAc termini of an acceptor molecule might be recognized by the

sialyltransferase. However, the result obtained with the artificial 'glycoprotein' (structure 9, Table 1) shows that this molecule, which does not remotely resemble natural glycoproteins in having a hydrocarbon 'core' structure, is utilized at an appreciable rate by the enzyme. If a specific hydroxy, acetamido or other partial sugar structure in the core region of natural oligosaccharides was a mandatory requirement for binding of the acceptor to the enzyme, then this molecule would not have been expected to act as an acceptor. The notable feature of this chemically-prepared 'glycoprotein' is the presence of a high number (16) of Gal(β1-4)GlcNAc termini. Indeed, it was found that all good acceptors (molecules where the rate of transfer of NeuAc was at least one-third or greater than that of desialylated α_1 acid glycoprotein) carried more than one Gal(β1-4)GlcNAc terminus per molecule.

From the specificity studies we make the following conclusions. First, that more than one Gal(β1-4)GlcNAcβ-R terminus of an acceptor molecule is bound by the sialyltransferase. Secondly, the internal structure of complex oligosaccharides is not actually recognized by the sialyltransferase but serves as a support for the display of Gal(β1-4)GlcNAc termini in an appropriate orientation to the enzyme. Thirdly, the ability of Gal(β1-4)GlcNAc termini to adopt a relative conformation that enables them to be bound by the sialyltransferase is probably an important factor in determining the rate of catalysis.

Presumably, not all Gal(β1-4)GlcNAc termini in natural oligosaccharides are capable of assuming an orientation, relative to other branches on the molecule and the active site of the enzyme, which would permit their sialylation. In tri-branched structures, for example, sialic acid has always been found, to date, in an (α2-3)-linkage to the Gal(β1-4)GlcNAc(β1-4)Man branch (Dorland *et al.*, 1978; Zinn *et al.*, 1978; Nilsson *et al.*, 1979; Mega *et al.*, 1980; Yamashita *et al.*, 1981). Other branches may contain NeuAc(α2-6)Gal or NeuAc(α2-3)Gal linkages, depending upon the individual glycoprotein, but the linkages are not random, sialic acid apparently being transferred specifically to certain galactose residues in individual glycoproteins.

Evidence relating to the specificity of other Gal(β1-4)GlcNAc (α2-6)-sialyltransferases has been reported. It has long been known that the disaccharide Gal(β1-4)GlcNAc is often a poor or ineffective acceptor for sialyltransferases that readily transfer NeuAc to *N*-glycosidically-linked oligosaccharides bearing this same disaccharide at branch termini (Spiro & Spiro, 1968; Paulson *et al.*, 1977; Bendiak & Zalik, 1981). The bovine colostrum β-galactoside (α2-6)-sialyltransferase has been found to transfer NeuAc to specific Gal(β1-4)GlcNAc termini of di- and tri-branched oligosaccharides (Van den Eijnden *et al.*, 1980). Rat liver β-galactoside (α2-6)-sialyltransferase has been found to utilize desialylated glycoproteins at a considerably higher rate than an octasaccharide bearing two Gal(β1-4)GlcNAc branches (Miyagi & Tsuiki, 1982) and has appreciably lower K_m values for the Gal(β1-4)GlcNAcβ-R sequence in desialylated glycoproteins than for Gal(β1-4)GlcNAc or Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc (Weinstein *et al.*, 1982). The results reported here indicate that the number and relative conformation of Gal(β1-4)GlcNAc branches of an acceptor molecule are probable factors in recognition by the embryonic chicken liver sialyltransferase.

Oberholtzer *et al.* (1981) have reported hydrogen-bonding within the structure of the complex oligosaccharide of native fetuin. Carver & Grey (1981) have compiled detailed proton-magnetic-resonance data showing that branching patterns of *N*-linked oligosaccharides result in specific 'microenvironments' for particular protons, and, accordingly, for individual sugar residues. As yet, however, information concerning the preferred three-dimensional conformation of these structures is limited. Further studies of sialyltransferases will be required, in order to elucidate the precise residue(s) and hydroxy group(s) to which NeuAc is transferred in natural oligosaccharides. In addition, large acceptors, bearing more than one Gal(β1-4)GlcNAc branch, will be necessary to determine branch preferences of sialyltransferases that act on these structures. Since not all sialic residues would be added simultaneously to an oligosaccharide it is also reasonable to assume that some oligosaccharides that contain NeuAc transferred to one branch may be preferable acceptors for certain sialyltransferases. As shown in the present study, the preparation and effective use of complex oligosaccharides as acceptors in studies of sialyltransferases can elucidate interesting aspects of the substrate specificities of these enzymes. The correlation of sialyltransferase branch preferences with oligosaccharide conformation remains an important task for the future.

We thank Dr. R. U. Lemieux, Dr. J. Lönngren, Dr. A. Veyrières, Dr. A. L. DeVries and Dr. Y. L. Hao for their gifts of glycoproteins and oligosaccharides. B. B. is a recipient of a Commonwealth Scholarship. G. M. W. C. is a member of the External Scientific Staff, Medical Research Council, London.

References

Alais, J. & Veyrières, A. (1981) *J. Chem. Soc. Perkin 1* 377–381

Aminoff, D., Gathmann, W. D. & Baig, M. M. (1979) *J. Biol. Chem.* **254**, 8909–8913

Arnarp, J. & Lönngren, J. (1981) *J. Chem. Soc. Perkin 1* 2070–2074

Baenziger, J. & Kornfeld, S. (1974) *J. Biol. Chem.* **249**, 7260–7269

Bendiak, B. & Cook, G. M. W. (1982) *Eur. J. Biochem.* **128**, 355–362
Bendiak, B. & Zalik, S. E. (1981) *Can. J. Biochem.* **59**, 171–180
Bennett, G. & O'Shaughnessy, D. (1981) *J. Cell Biol.* **88**, 1–15
Carlson, D. M. (1968) *J. Biol. Chem.* **243**, 616–626
Carver, J. P. & Grey, A. A. (1981) *Biochemistry* **20**, 6607–6616
Cleland, W. W. (1970) *Enzymes* **2**, 1–65
Day, A. C. & Whiting, M. C. (1970) *Org. Synth.* **50**, 3–6
Dorland, L., Haverkamp, J., Vliegenthart, J. F. G., Strecker, G., Michalski, J., Fournet, B., Spik, G. & Montreuil, J. (1978) *Eur. J. Biochem.* **87**, 323–329
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956) *Anal. Chem.* **28**, 350–356
Finne, J. (1982) *J. Biol. Chem.* **257**, 11966–11970
Fournet, B., Montreuil, J., Strecker, G., Dorland, L., Haverkamp, J., Vliegenthart, J. F. G., Binette, J. P. & Schmid, K. (1978) *Biochemistry* **17**, 5206–5214
Franzén, L., Svensson, S. & Larm, O. (1980) *J. Biol. Chem.* **255**, 5090–5093
Fukuda, M. & Hakomori, S. (1979) *J. Biol. Chem.* **254**, 5451–5457
Hagopian, A. & Eylar, E. H. (1968) *Arch. Biochem. Biophys.* **128**, 422–433
Inoue, S., Iwasaki, M. & Matsumura, G. (1981) *Biochem. Biophys. Res. Commun.* **102**, 1295–1301
Kim, Y. S., Perdomo, J. & Nordberg, J. (1971) *J. Biol. Chem.* **246**, 5466–5476
Kornfeld, R. & Kornfeld, S. (1980) in *The Biochemistry of Glycoproteins and Proteoglycans* (Lennarz, W. J., ed.), pp. 1–34, Plenum Press, New York
Krusius, T. & Finne, J. (1981) *Carbohydr. Res.* **90**, 203–214
Lemieux, R. U., Bock, K., Delbaere, L. T. J., Koto, S. & Rao, V. S. (1980) *Can. J. Chem.* **58**, 631–653
Lemieux, R. U., Abbas, S. Z., Burzynska, M. H. & Ratcliffe, R. M. (1982) *Can. J. Chem.* **60**, 63–67
Mega, T., Lujan, E. & Yoshida, A. (1980) *J. Biol. Chem.* **255**, 4057–4061
Miyagi, T. & Tsuiki, S. (1982) *Eur. J. Biochem.*, **126**, 253–261
Mizuochi, T., Yamashita, K., Fujikawa, K., Titani, K. & Kobata, A. (1980*a*) *J. Biol. Chem.* **255**, 3526–3531
Mizuochi, T., Fujii, J., Kurachi, K. & Kobata, A. (1980*b*) *Arch. Biochem. Biophys.* **203**, 458–465
Montreuil, J. (1980) *Adv. Carbohydr. Chem. Biochem.* **37**, 157–223
Nilsson, B., Nordén, N. E. & Svensson, S. (1979) *J. Biol. Chem.* **254**, 4545–4553
Oberholtzer, J. C., Englander, S. W. & Horwitz, A. F. (1981) *Biochemistry* **20**, 4785–4792
Ogata, S. & Lloyd, K. O. (1982) *Anal. Biochem.* **119**, 351–359
Paulson, J. C., Rearick, J. I. & Hill, R. L. (1977) *J. Biol. Chem.* **252**, 2363–2371
Rearick, J. I., Sadler, J. E., Paulson, J. C. & Hill, R. L. (1979) *J. Biol. Chem.* **254**, 4444–4451
Reddy, M. S., Levine, M. J. & Tabak, L. A. (1982) *Biochem. Biophys. Res. Commun.* **104**, 882–888
Roseman, S., Carlson, D. M., Jourdian, G. W., McGuire, E. J., Kaufman, B., Basu, S. & Bartholomew, B. (1966) *Methods Enzymol.* **8**, 354–372
Saeed, M. S. & Williams, J. M. (1980) *Carbohydr. Res.* **84**, 83–94
Schachter, H. & Roseman, S. (1980) in *The Biochemistry of Glycoproteins and Proteoglycans* (Lennarz, W. J., ed.), pp. 85–160, Plenum Press, New York
Schachter, H., Jabbal, I., Hudgin, R. L., Pinteric, L., McGuire, E. J. & Roseman, S. (1970) *J. Biol. Chem.* **245**, 1090–1100
Shier, W. T., Lin, Y. & DeVries, A. L. (1975) *FEBS Lett.* **54**, 135–138
Slomiany, A. & Slomiany, B. L. (1978) *J. Biol. Chem.* **253**, 7301–7306
Sommers, L. W. & Hirschberg, C. B. (1982) *J. Biol. Chem.* **257**, 10811–10817
Spik, G., Strecker, G., Fournet, B., Bouquelet, S., Montreuil, J., Dorland, L., Van Halbeek, H. & Vliegenthart, J. F. G. (1982) *Eur. J. Biochem.* **121**, 413–419
Spiro, M. J. & Spiro, R. G. (1968) *J. Biol. Chem.* **243**, 6520–6528
Spiro, R. G. (1966) *Methods Enzymol.* **8**, 3–26
Spiro, R. G. (1973) in *Advances in Protein Chemistry* (Anfinsen, C. B., Edsall, J. T. & Richards, F. M., eds.), pp. 349–467, Academic Press, London
Spiro, R. G. & Bhoyroo, V. D. (1974) *J. Biol. Chem.* **249**, 5704–5717
Takasaki, S., Yamashita, K., Suzuki, K., Iwanaga, S. & Kobata, A. (1979) *J. Biol. Chem.* **254**, 8548–8553
Takasaki, S., Mizuochi, T. & Kobata, A. (1982) *Methods Enzymol.* **83**, 263–268
Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950) *Nature (London)* **166**, 444–445
Van den Eijnden, D. H., Joziasse, D. H., Dorland, L., Van Halbeek, H., Vliegenthart, J. F. G. & Schmid, K. (1980) *Biochem. Biophys. Res. Commun.* **92**, 839–845
Warren, L. (1959) *J. Biol. Chem.* **234**, 1971–1975
Weinstein, J., de Souza-e-Silva, U. & Paulson, J. C. (1982) *J. Biol. Chem.* **257**, 13845–13853
Yamashita, K., Liang, C., Funakoshi, S. & Kobata, A. (1981) *J. Biol. Chem.* **256**, 1283–1289
Yoshima, H., Matsumoto, A., Mizuochi, T., Kawasaki, T. & Kobata, A. (1981) *J. Biol. Chem.* **256**, 8476–8484
Zinn, A. B., Marshall, J. S. & Carlson, D. M. (1978) *J. Biol. Chem.* **253**, 6768–6773