Biosynthesis of Mammalian Glycoproteins

GLYCOSYLATION PATHWAYS IN THE SYNTHESIS OF THE NONREDUCING TERMINAL SEQUENCES*

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Six purified glycosyltransferases (α-galactoside α2 → 6 sialyltransferase, a β-galactoside α2 → 3 sialyltransferase, an α-N-acetylgalactosaminide α2 → 6 sialyltransferase, a β-galactoside α1 → 2 fucosyltransferase, a β-N-acetylgalactosaminide α1 → 3 fucosyltransferase, and a (fucosyl α1 → 2) galactoside α1 → 3 N-acetylgalactosaminyltransferase) have been used to study the biosynthetic pathways for formation of the nonreducing terminal oligosaccharide sequences in mammalian glycoproteins. The two glycoproteins used as model acceptor substrates in this study were human asialotransferrin, which contains the nonreducing terminal oligosaccharide sequence Galβ1 → 4GlcNAcβ1 → 2Man, and antifreeze glycoprotein, which contains oligosaccharides with the structure, Galβ1 → 3GalNAcα1 → O-Thr. Sequential action of the six glycosyltransferases on these model substrates led to the formation of previously described oligosaccharide structures.

The studies reported here indicate that the substrate specificities of the individual enzymes dictate the structures that can be synthesized and the pathways by which they may be formed. The actions of a number of the transferases are mutually exclusive, thereby prohibiting the formation of theoretically possible oligosaccharide structures. Oligosaccharides with the terminal sequence NeuAca2 → 3(Fucα1 → 2)Galβ1 → 3GalNAc and NeuAca2 → 6Galβ1 → 4(Fucα1 → 3)GlcNAc cannot be formed because the prior incorporation of sialic acid by the sialyltransferases yields products that are not acceptor substrates for the fucosyltransferases, and vice versa. Synthesis of other products requires that the enzymes act sequentially in a specific order. The structures NeuAca2 → 6(Fucα1 → 2)Galβ1 → 4GlcNAc, Fucα1 → 2Galβ1 → 4(Fucα1 → 3)GlcNAc, GalNAcα1 → 3(Fucα1 → 2)Galβ1 → 4GlcNAc, and GalNAcα1 → 3(Fucα1 → 2)Galβ1 → 3GalNAc can only be synthesized if the fucosyl α1 → 2 galactose linkage is formed first. Synthesis of the pentasaccharide sequences GalNAcα1 → 3(Fucα1 → 2)Galβ1 → 3(NeuAca2 → 6)GalNAc and GalNAcα1 → 3(Fucα1 → 2)Galβ1 → 4(Fucα1 → 3)GlcNAc requires that the N-acetylgalactosaminyltransferase act last on the former structure and that the α1 → 3 fucosyltransferase act last on the latter. In those instances where a product can be formed by one of two possible pathways, the comparisons of reaction rates indicate that one pathway is usually preferred. Synthesis of the disialylated product, NeuAca2 → 3Galβ1 → 3(NeuAca2 → 6)GalNAc is more than thirty times faster if the NeuAca2 → 3Gal linkage is formed initially. Similarly, synthesis of the structure Fucα1 → 2Galβ1 → 3(NeuAca2 → 6)GalNAc is much more rapid if the sialic acid is transferred first.

These results suggest that oligosaccharide biosynthesis *in vitro* is regulated not only by the glycosyltransferases available but also by the order in which they act. Based on these findings, pathways are proposed for the biosynthesis of many of the structures found at the nonreducing termini of the oligosaccharide chains in mammalian glycoproteins.

Sialic acid, fucose, galactose, and N-acetylgalactosamine are often located at the nonreducing termini of the oligosaccharide prosthetic groups of mammalian glycoproteins, as indicated in four typical oligosaccharide structures shown in Fig. 1. The presence or absence of each of these sugars in an oligosaccharide, or variations in the nature of their attachment to an adjacent sugar residue, often contribute to the structural heterogeneity found in glycoproteins.

The biosynthesis of the structures shown in Fig. 1 requires the participation of several glycosyltransferases, each of which is bound to membranes of the endoplasmic reticulum or the Golgi apparatus, and transfers a sugar from an appropriate nucleotide sugar to glycoprotein acceptors. At present, little is known about mechanisms that allow these transferases to act in concert to determine the oligosaccharide structures of a given glycoprotein, but recently several of the glycosyltransferases that are responsible for incorporating one of the terminal sugars shown in Fig. 1 have been purified to homogeneity and their enzymic properties examined. This report describes the use of six of these transferases in elucidating possible biosynthetic pathways that lead to formation of some of the structures shown in Fig. 1.

At least five sialyltransferases are required in the biosynthesis of the major sialic acid linkages observed in mammalian glycoproteins, three of which are now available in homogeneous form and have been characterized enzymatically. One, from bovine colostrum, designated β-galactoside α2 → 6 sialyltransferase (3, 6), catalyzes Reaction 1.
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Fig. 1. Structures of the oligosaccharide prosthetic groups.

I, human lactoferrin (1); II, glycophorin (2); III, porcine submaxillary mucin (3); and IV, equine gastric mucin (4); these illustrate the presence of either sialic acid, fucose, galactose, or N-acetylgalactosamine at a terminal, nonreducing position in the oligosaccharide group. The terminal residues are underlined.

A second, from porcine submaxillary gland, designated \( \beta \)-galactoside \( \alpha_2 \rightarrow 3 \) sialyltransferase (7, 8), catalysts Reaction 5 and has been puriﬁed in homogeneous form from human plasma (14) and porcine submaxillary glands (15, 16). This enzyme, the (fucosyl \( \alpha_1 \rightarrow 2 \) galactoside \( \alpha_1 \rightarrow 3 \) N-acetylgalactosaminytransferase, catalyzes Reaction 6.

In addition to the enzymes that will be discussed in this report, two of the three galactosyltransferases required for the synthesis of structures shown in Fig. 1 have been puriﬁed or separated from other sialyltransferase activities. One catalyzes the synthesis of sialic acid in the sequence NeuAc\(_2 \rightarrow 3\) Gal\(_\beta_1 \rightarrow 4\) GlcNAc, the same as that in Structure I (Fig. 1), except that the sialic acid is in \( \alpha_2 \rightarrow 3 \) rather than \( \alpha_2 \rightarrow 6 \) linkage to galactose. The other utilizes the product of another sialyltransferase to produce the sequence NeuAc\(_2 \rightarrow 8\) NeuAc\(_2 \rightarrow X \) (10).

Three fucosyltransferases are required for the synthesis of the fucosidic linkages shown in Fig. 1, two of which have been extensively puriﬁed and are examined in this report. One puriﬁed to homogeneity from porcine submaxillary glands, designated \( \beta \)-galactoside \( \alpha_1 \rightarrow 2 \) fucosyltransferase (11), cat-

A third from the same source, designated \( \alpha \)-N-acetylgalactosaminide \( \alpha_1 \rightarrow 3 \) fucosyltransferase (Reaction 5), are mutually exclusive; that is, both enzymes can utilize the same acceptor substrate, but neither can use the product of the other transferase as an acceptor substrate (21). In the present report these studies were extended to examine the interactions of the six enzymes listed above in the glycosylation of two model glycoprotein acceptor substrates, human asialotransferrin and the anti-freeze glycoprotein of Antarctic ﬁsh, whose structures are shown in Fig. 2. These proteins have terminal oligosaccharide sequences, either Gal\(_\beta_1 \rightarrow 3\) GalNAc\(_2 \rightarrow O-Thr/瑟 (anti-freeze glycoprotein) (22) or Gal\(_\beta_1 \rightarrow 4\) GlcNAc\(_2 \rightarrow 2\) Man(isalotransferrin) (23), that contain an acceptor group for each of the highly puriﬁed glycosyltransferases that catalyze Reactions 1 to 6. It has been found that the six enzymes studied can act sequentially to form nonreducing terminal structures that are known to occur in mammalian glycoproteins. Further, it was found that the substrate speciﬁcities of the enzyme preparation also catalyzes the incorporation of fucose into acceptors with the terminal sequence Gal\(_\beta_1 \rightarrow 3\) GalNAc to give the product Gal\(_\beta_1 \rightarrow 3\) (Fuc\(_\alpha_1 \rightarrow 4\) GlcNAc). Considerable evidence suggests that a single enzyme may catalyze both reactions (12), although final, unambiguous proof that the two activities reside in one enzyme is lacking at present. The substrate speciﬁcity of the fucosyltransferase that forms the fucosyl \( \alpha_1 \rightarrow 6 \) N-acetylgalactosaminyaryl groups in Structure 1 (Fig. 1) has been examined (13), but the enzyme has not been extensively puriﬁed.

Finally, the N-acetylgalactosaminytransferase that synthesizes the A blood group antigenic structure has been obtained in homogeneous form from human plasma (14) and porcine submaxillary glands (15, 16). This enzyme, the (fucosyl \( \alpha_1 \rightarrow 2 \) galactoside \( \alpha_1 \rightarrow 3 \) N-acetylgalactosaminytransferase, catalyzes Reaction 7 and has been puriﬁed in homogeneous form from bovine and human milk (18, 19) and shown to catalyze the formation of the sequence Gal\(_\beta_1 \rightarrow 4\) GlcNAc, common to all complex asparagine-linked oligosaccharides. A second galactosyltransferase, purified from human plasma and designated (fucosyl \( \alpha_1 \rightarrow 2 \) galactoside \( \alpha_1 \rightarrow 3 \) N-acetylgalactosaminytransferase, catalyzes Reaction 8.

It was reported earlier that the actions of two of the foregoing glycosyltransferases, the \( \beta \)-galactoside \( \alpha_2 \rightarrow 6 \) sialyltransferase (Reaction 1) and the \( \beta \)-N-acetylgalactosaminide \( \alpha_1 \rightarrow 3 \) fucosyltransferase (Reaction 5), are mutually exclusive; that is, both enzymes can utilize the same acceptor substrate, but neither can use the product of the other transferase as an acceptor substrate (21). In the present report these studies were extended to examine the interactions of the six enzymes listed above in the glycosylation of two model glycoprotein acceptor substrates, human asialotransferrin and the anti-freeze glycoprotein of Antarctic fish, whose structures are shown in Fig. 2. These proteins have terminal oligosaccharide sequences, either Gal\(_\beta_1 \rightarrow 3\) GalNAc\(_2 \rightarrow O-Thr/瑟 (anti-freeze glycoprotein) (22) or Gal\(_\beta_1 \rightarrow 4\) GlcNAc\(_2 \rightarrow 2\) Man(isalotransferrin) (23), that contain an acceptor group for each of the highly purified glycosyltransferases that catalyze Reactions 1 to 6. It has been found that the six enzymes studied can act sequentially to form nonreducing terminal structures that are known to occur in mammalian glycoproteins. Further, it was found that the substrate speciﬁcities of...
the glycosyltransferases could account for the absence of many theoretically possible structures in naturally occurring oligosaccharides of glycoproteins. Preliminary reports of this work have been presented (24, 25).

**EXPERIMENTAL PROCEDURES**

**Materials**

CMP-[4,5,6,7,8,9-3H]NeuAc (235 mCi/mmol), CMP-[4-14C]NeuAc (1.86 mCi/mmol), GDP[U-14C]Fuc (222 mCi/mmol), and UDP-[1,4-3H]GalNAc (47.2 mCi/mmol) were obtained from New England Nuclear. Unlabeled GDP-Fuc was a gift from Dr. Robert Barker (Chemistry Department, Cornell University, Ithaca, New York). Unlabeled UDP-GalNAc was prepared as previously described (18). Antifreeze glycoprotein was purified as described earlier (26) from the serum of Dissostichus mawsoni, which was a gift from Dr. A. L. DeVries (Department of Physiology, University of Illinois, Urbana, Illinois). Human transferrin and insolubilized Clostridium perfringens neuraminidase (type IX-A) were purchased from Sigma Chemical Co. All other reagents were of the highest quality commercially available. The following enzymes were prepared as previously described: β-galactoside α2 → 3 sialyltransferase (7), α-N-acetylgalactosaminidase α2 → 6 sialytransferase (9), and (fucosyl α1 → 2) galactoside α1 → 3 N-acetylgalactosaminyltransferase (15) from porcine submaxillary glands; β-galactoside α2 → 6 sialytransferase from bovine colostrum (5); and β-N-acetylgalactosaminidase α1 → 3 fucosyltransferase from human milk (12).

**Preparation of Asialotransferrin**

Approximately 100 mg of human serum transferrin in 10 ml of 0.05 M sodium cacodylate, pH 5.5, were incubated with 0.055 unit of the immobilized neuraminidase (0.2 ml) at 37°C for 18 h. The insoluble enzyme was collected in a small column and washed with 5 ml of water. The fractions flowing through the column were pooled, dialyzed against water, and lyophilized. More than 98% of the sialic acid of human milk (12).

**Synthesis of Glycosylated Derivatives of Asialotransferrin and Antifreeze Glycoprotein**

All enzymic reactions were performed at 37°C. Except where otherwise noted, the glycosylated derivatives were separated from reaction mixtures by gel filtration on columns (0.8 x 10 cm) of Sephadex G-50 (fine) equilibrated in 0.2 M NaCl. The extent of glycosylation for each reaction was determined from the amount of radioactive sugar incorporated into the glycoprotein substrate. The concentration of potential acceptor sites was calculated from the galactose content of the acceptor substrate as determined enzymatically with galactose dehydrogenase (28). Each derivative is identified below by a trivial name used throughout this paper, followed by the structure of the nonreducing terminal portion of the glycoprotein oligosaccharide in parentheses. These structures are based on the substrate specificities of the enzymes described previously (6, 8, 9, 11, 16).

**Fucosyl α1 → 2 Transferferrin (Fucα1 → 2Galβ1 → 4GlcNAc−)**

This was prepared by incubating 3.4 mg of asialotransferrin (0.2 µmol of galactose) with 54 µM of the β-galactoside α1 → 2 fucosyltransferase, 15 µM of sodium cacodylate, pH 6.0, 6 µM of MnCl₂, and 0.4 µM of GDP-fucose (1600 cpm/µmol) in a volume of 0.3 ml. After 24 h, another 0.4 µM of GDP-fucose was added, and the reaction continued for 24 h. Incorporation of 0.93 mol of fucose/mol of galactose was obtained.

**Fucosyl α1 → 3 Transferferrin (Galβ1 → 4Fucα1 → 3GlcNAc−)**

This was prepared as described previously (29).

**Fucosyl α1 → 3 (Fucosyl α1 → 2) Transferferrin (Fucα1 → 2Galβ1 → 4Fucα1 → 3GlcNAc−)**

This was prepared by incubating 1.2 mg of fucosyl α1 → 2 transferferrin (70 µmol of galactose) with 15 µM of Mops/NaOH, pH 7.5, 1.5 µM of MnCl₂, 0.47 µM of GDP-fucose (1850 cpm/µmol), and 36 µM of the β-N-acetylgalactosaminidase α1 → 3 fucosyltransferase in a volume of 0.3 ml for 18 h, after which additional 0.47 µM of GDP-fucose was added and the reaction continued for 24 h. Incorporation of an additional 0.91 mol of fucose/mol of galactose was obtained.

**Staly α2 → 6 Transferferrin (NeuAca2 → 6Galβ1 → 4GlcNAc−)**

This was prepared as described previously (21), except that asialotransferrin was used in place of native transferrin. Incorporation of 0.93 mol of sialic acid/mol of galactose was obtained.

**Staly α2 → 6 (Fucosyl α2 → 2) Transferferrin (NeuAca2 → 6Fucα2 → 2Galβ1 → 4Fucα2 → 3GlcNAc−)**

This was synthesized by incubating 0.08 µg of fucosyl α2 → 2 transferferrin (50 nmol of galactose) with 25 µM of sodium cacodylate, pH 7.0, 0.95 µM of CMP-NeuAc (2550 cpm/µmol), and 30 µM of the β-galactoside α2 → 6 sialyltransferase in a volume of 0.25 ml for 24 h. An additional 0.48 µM of CMP-NeuAc was added and the reaction continued for 24 h. Incorporation of 0.6 mol of sialic acid/mol of galactose was obtained.

**Staly α2 → 6 3Fucosyl α2 + 2 Antifreeze Glycoprotein (Fucα1 + 2Galβ1 + 4Fucα2 + 3GlcNAc−)**

This was prepared by incubating 3.4 mg of asialotransferrin (0.2 pmol of galactose) with 2.5 µM of the (fucosyl α1 → 2) galactoside α1 → 3 N-acetylgalactosaminyltransferase, 30 µM of Mes/NaOH, pH 6.0, 6 µM of MnCl₂, 0.93 mg of Triton X-100, and 0.11 µM of UDP-GalNAc (1850 cpm/µmol) in a volume of 0.3 ml. After 18 h, an additional 0.11 µM of UDP-GalNAc and 2.5 µM of the enzyme were added and the reaction continued for 24 h. Incorporation of 0.82 mol of N-acetylgalactosamine/mol of galactose was achieved.

**Staly α2 → 3 Antifreeze Glycoprotein (NeuAca2 → 3Galβ1 → 4GlcNAc−)**

This was prepared by incubating 0.08 µg of antifreeze glycoprotein (0.99 µmol of galactose) with 30 µM of sodium cacodylate, pH 6.5, 0.3 µg of bovine serum albumin, 3 mg of Triton X-100, 3.5 µM of CMP-NeuAc (2600 cpm/µmol), and 10 µM of the β-galactoside α2 → 3 sialyltransferase in a volume of 0.3 ml. After 8 h, 2.3 µM of CMP-NeuAc and another 10 µl of the enzyme were added, and the reaction continued for 16 h. The sialylated product was isolated by gel filtration on a column (1.5 x 33 cm) of Sephadex G-25 in 0.1 M NaCl. Incorporation of 0.96 mol of sialic acid/mol of galactose was obtained.

**Staly α2 → 3 Antifreeze Glycoprotein (Galβ1 → 3NeuAca2 → 3Galβ1 → 3GlcNAc−)**

This was prepared as described above for sialyl α2 → 3 antifreeze glycoprotein except that 2 aliquots of 18 µM of the α-N-acetylgalactosaminidase α2 → 6 sialyltransferase were used. Incorporation of 1.06 mol of sialic acid/mol of galactose was obtained.

**Fucosyl α1 → 2 Antifreeze Glycoprotein (Fucα1 → 2Galβ1 → 4GlcNAc−)**

This was prepared by incubating 0.34 µg of antifreeze glycoprotein (0.6 µmol of galactose) with 27 µM of the β-galactoside α1 → 2 fucosyltransferase, 15 µM of sodium cacodylate, pH 6.0, 6 µM of MnCl₂, and 0.87 µM of GDP-fucose (1800 cpm/µmol) in a final volume 0.3 ml. After 24 h, another 0.87 µM of GDP-fucose was added, and the reaction was continued for another 16 h. Incorporation of 0.97 mol of fucose/mol of galactose was obtained.

**Fucosyl α1 → 2 (Staly α2 → 5) Antifreeze Glycoprotein (Fucα1 → 2Galβ1 → 5NeuAca2 → 5Galβ1 → 5GlcNAc−)**

This was prepared by incubating 0.08 µg of fucosyl α1 → 2 antifreeze glycoprotein (100 µmol of galactose) with 27 µM of the β-galactoside α1 → 2 fucosyltransferase, 10 µM of sodium cacodylate, pH 6.0, 4 µM of MnCl₂, and 0.47

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**Fig. 2. Structures of the oligosaccharide prosthetic groups of A, antifreeze glycoprotein (22) and B, human asialotransferrin (23).**

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**Note:** This text is a transcription of the first page of a scientific article discussing the synthesis and structure of glycosylated derivatives of glycoproteins, focusing on the processes of glycosylation and the enzymes involved in this process. The article includes detailed experimental procedures and structures of the oligosaccharide groups of antifreeze glycoprotein and human asialotransferrin. The text is rich in scientific terminology and biological data, providing a comprehensive overview of the glycosylation pathways in glycoprotein biosynthesis.
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μmol of GDP-fucose (1850 cpm/nmol) in a final volume of 0.2 ml. After 18 h, another 0.47 μmol of GDP-fucose and 27 μM of the fucosyltransferase were added, and the reaction continued for 24 h. Incorporation of 0.65 μmol of fucose/mol of galactose was achieved. N-Acetylglalactosaminyl a1 → 3 (Fucosyl a1 → 2) Antifreeze Glycoprotein (GalNAcα1 → 3[Fucal → 2]Galβ1 → 3GalNAcα1) → 5. This was performed as described above for the corresponding transferase except that 0.034 mg of fucosyl a1 → 2 antifreeze glycoprotein (50 nmol of galactose) was used as the acceptor. Incorporation of 0.98 μmol of N-acetylgalactosamine/mol of galactose was obtained.

Measurement of the Extent and Rate of the Glycosyltransferase Reactions

The maximum extent of incorporation for each enzyme with the various antifreeze glycoprotein and transferrin derivatives was determined by incubating the acceptors with a large excess of the transferase for 24 h at 37°C. Reaction conditions and substrate concentrations for each of the transferases are given under “Results.” Glycosylated products were isolated by gel filtration on columns (0.8 × 10 cm) of Sephadex G-50 (fine) equilibrated in 0.2 M NaCl. The entire reaction mixture was applied to the column, and the column was developed with 0.2 M NaCl. The first 1.5 ml of effluent was discarded, and the next 2.0 ml containing all of the glycosylated product was collected into a scintillation vial with 2.5 ml of Aquasol II (New England Nuclear) and counted.

The rate of formation of glycosylated products was followed by assaying the reactions after varying times of incubation. Each of the reactions contained 5 nmol of potential acceptor sites in a total volume of 50 μl. The composition of the reaction mixtures for the individual enzymes is given under “Results.” At different times a 5-μl aliquot was removed and the reaction terminated by diluting each aliquot into 100 μl of 10 mM GMP for the fucosyltransferases, 10 mM CTP for the sialyltransferases, or 10 mM UMP for the N-acetylgalactosaminyltransferase. The glycoprotein product of the reaction was then purified by gel filtration chromatography as described above for the substrate specificity studies.

Analytical Procedures

Sialic acid was determined by the periodate/resorcinol procedure (27), fucose by the Diehle Shettle procedure (30), and galactose by an enzymatic procedure using galactose dehydrogenase (28). Neuraminidase was assayed by following the release of 14C-sialic acid from enzymatically prepared [14C]NeuAc α2 → 6 sialic acid glycoprotein as described previously (31).

RESULTS

Preparation of Acceptor Substrates—The various acceptor substrates were prepared from either the antifreeze glycoprotein of Antarctic fish which contains the O-linked structure Galβ1 → 3GalNAcαThr (Fig. 2), or asialo human transferrin, which contains the biantennary oligosaccharide structure (Fig. 2) with the terminal sequence Galβ1 → 4GlcNAc.

Each of the possible mono- and diglycosylated derivatives of these two glycoproteins was synthesized with each of the glycosyltransferases as described under “Experimental Procedures.” The values reported in each case for the extent of glycosylation represent the maximum amount of a given sugar associated with these sialyltransferases which concomitantly synthesized CMP-NeuAc. Under the conditions used for glycosylation, the equilibrium heavily favored the formation of products. However, when the β-galactoside α2 → 3 sialyltransferase was incubated with CMP-NeuAc with a specific radioactivity of 12,400 cpm/nmol and sialyl α2 → 3 antifreeze glycoprotein with a specific radioactivity of 2,600 cpm/nmol of sialic acid under the conditions given in Table I, the exchange of radiolabeled sialic acid catalyzed by the reverse reaction of the transferase caused the apparent incorporation of another 0.58 mol of sialic acid/mol of galactose when, in fact, no net change in sialic acid content had occurred. Similar results were obtained with the α-N-acetylgalactosaminyl α2 → 6 sialyltransferase. To avoid this complication, the substrate specificity studies with the β-galactoside α2 → 3 sialyltransferase and the α-N-acetylgalactosaminyl α2 → 6 sialyltransferase were performed with CMP-NeuAc of the same specific radioactivity as that used to synthesize the monosialylated antifreeze glycoprotein derivatives. No reversibility of this type was observed with the other four glycosyltransferases used in this study as shown by the results in Tables I and II.

The results obtained with the antifreeze glycoprotein derivatives are given in Table I. The native glycoprotein (A) was an acceptor for the β-galactoside α2 → 3 sialyltransferase, the α-N-acetylgalactosaminyl α2 → 6 sialyltransferase, and the β-galactoside α1 → 2 fucosyltransferase. The presence of sialic acid in α2 → 6 linkage to the N-acetylgalactosamine (B) had little effect on the incorporation of either a second residue of sialic acid or a fucose residue. If the sialyl α2 → 3 galactose linkage was formed initially (U) then the subsequent action of the α2 → 6 sialyltransferase was unaffected, but the action of the fucosyltransferase was completely blocked. If the fucosyl α1 → 2 galactose linkage (D) was formed first, then incorporation of sialic acid by the α2 → 3 sialyltransferase was blocked, and incorporation of sialic acid by the α2 → 6 sialyltransferase was severely inhibited. In addition, incorporation of fucose in α1 → 2 linkage converted the antifreeze glycoprotein into a substrate for the α-N-acetylgalactosaminyltransferase. The fucosyl α1 → 2 (sialyl α2 → 6) antifreeze glycoprotein derivative (E) was also an acceptor for the α-N-acetylgalactosaminyltransferase, but in this case the presence of the sialic acid partially inhibited incorporation of α-N-acetyl-
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Reactions contained from 3 to 10 nmol of potential acceptor sites. The β-galactoside α2 → 3 sialyltransferase and the N-acetylgalactosaminide α2 → 6 sialyltransferase reaction mixtures (65 μl) contained 5 μmol of sodium cacodylate, pH 6.5, 50 μg of bovine serum albumin, 0.5 mg of Triton X-100, 54 nmol of CMP-NeuAc (2600 cpm/nmol), and 6.6 mU of either the α2 → 3 sialyltransferase or the α2 → 6 sialyltransferase. Reaction mixtures for the β-galactoside α2 → 3 sialyltransferase (100 μl) contained 5 μmol of sodium cacodylate, pH 6.0, 2 μmol of MnCl₂, 50 μg of bovine serum albumin, 60 nmol of GDP-fucose (16,000 cpm/nmol), and 13.5 mU of the fucosyltransferase. The (fucosyl α1 → 2) galactoside α1 → 3 N-acetylgalactosaminyltransferase reaction mixtures (100 μl) contained 10 μmol of Mes/NaOH, pH 6.0, 2 μmol of MnCl₂, 1 mg of Triton X-100, 45 nmol of UDP-GalNAc (19,300 cpm/nmol), and 2.1 mU of the transferase.

The α-N-acetylgalactosaminide α2 → 6 sialyltransferase was blocked. In contrast, initial attachment of sialic acid in α2 → 6 linkage to the N-acetylgalactosamine α1 → 2 fucosyltransferase. The presence of sialic acid in the α2 → 2 glicosyltransferase was blocked. However, this triglycosylated product could be formed if the α2 → 6 sialyltransferase than was the native antifreeze glycoprotein (Fig. 3A). In contrast, the sialyl α2 → 6 linkage was actually a better substrate for the α-N-acetylgalactosaminide α2 → 6 sialyltransferase than was the native antifreeze glycoprotein (Fig. 3A). In contrast, sialyl α2 → 3 antifreeze glycoprotein was actually a better substrate for the α-N-acetylgalactosaminide α2 → 6 sialyltransferase than was the native antifreeze glycoprotein (Fig. 3B). The rate of formation of the disialylated product was more than five times faster than formation of sialyl α2 → 6 antifreeze glycoprotein. Fig. 3B also shows that the rate of formation of the sialyl α2 → 6 N-acetylgalactosamine linkage in fucosyl α1 → 2 antifreeze glycoprotein was drastically decreased and that the extent of the reaction was limited. Unlike the other transferases, the β-galactoside α1 → 2 fucosyltransferase does not show absolute specificity for the linkage of the nonreducing terminal sugar residue in the acceptor substrate. Both the Galβ1 → 3GalNAc sequence in antifreeze glycoprotein and the Galβ1 → 4GlcNAc sequence

<table>
<thead>
<tr>
<th>Acceptor substrate</th>
<th>Glycosyltransferase reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Galβ1 → 3GalNAcThr</td>
<td>α2 → 6NeuAcT0α2 → 3GalNAcT0α1 → 2FucTα1 → 3GalNAcT0</td>
</tr>
<tr>
<td>B. Galβ1 → 3GalNAcThr</td>
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<tr>
<td>C. NeuAc2 → 3Galβ1 → 3GalNAcThr</td>
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<td>D. NeuAc2 → 3Galβ1 → 3GalNAcThr</td>
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</tr>
<tr>
<td>E. FucO1 → 2Galβ1 → 3GalNAcThr</td>
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</tr>
<tr>
<td>F. FucO1 → 2Galβ1 → 3GalNAcThr</td>
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<tr>
<td>G. Galβ1 → 3GalNAcThr</td>
<td>0</td>
</tr>
<tr>
<td>H. Galβ1 → 3GalNAcThr</td>
<td>0</td>
</tr>
</tbody>
</table>

α-N-Acetylgalactosaminide α2 → 6 sialyltransferase.
β-Galactoside α2 → 3 sialyltransferase.
β-Galactoside α1 → 2 fucosyltransferase.
(Fucosyl α1 → 2) galactoside α1 → 3 N-acetylgalactosaminyltransferase.
Not determined since earlier studies showed that this is not an acceptor for the transferases indicated.

Galactosamine. For the converse experiment in which the N-acetylgalactosaminyl α1 → 3 (fucosyl α1 → 2) antifreeze, derivative (F) was used as the acceptor, the transfer of sialic acid by the α2 → 6 sialyltransferase was blocked.

The reactions on the transferases on the transferrin derivatives are shown in Table II. Asialotransferrin (A) was an acceptor for the β-galactoside α2 → 6 sialyltransferase, the N-acetylgalactosaminide α1 → 3 fucosyltransferase, and the α-N-acetylgalactosaminyl (α1 + 3 fucose, but completely blocked the incorporation of sialic acid. Finally, the sialic acid in the fucosyl α1 → 2 (Sialyl α1 → 2) transferrin (G) prevented the incorporation of N-acetylgalactosamine linkage in fucosyl α1 → 2 antifreeze glycoprotein. Fig. 3B shows the results of such studies with each of the derivatives that was found to be an acceptor for the various transferases.

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<td>86</td>
</tr>
<tr>
<td>C. NeuAc2 → 3Galβ1 → 3GalNAcThr</td>
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<tr>
<td>D. NeuAc2 → 3Galβ1 → 3GalNAcThr</td>
<td>88</td>
</tr>
<tr>
<td>E. FucO1 → 2Galβ1 → 3GalNAcThr</td>
<td>19</td>
</tr>
<tr>
<td>F. FucO1 → 2Galβ1 → 3GalNAcThr</td>
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</tr>
<tr>
<td>G. Galβ1 → 3GalNAcThr</td>
<td>0</td>
</tr>
<tr>
<td>H. Galβ1 → 3GalNAcThr</td>
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</tr>
</tbody>
</table>

α-N-Acetylgalactosaminide α2 → 6 sialyltransferase.
β-Galactoside α2 → 3 sialyltransferase.
β-Galactoside α1 → 2 fucosyltransferase.
(Fucosyl α1 → 2) galactoside α1 → 3 N-acetylgalactosaminyltransferase.
Not determined since earlier studies showed that this is not an acceptor for the transferases indicated.

Relative Rate of Glycosyltransferase Reactions—Since both the rate and the extent of reaction may be important factors in determining the products formed by the transferases in vivo, it was of interest to measure the time course of glycosylation with these enzymes. Fig. 3 shows the results of such studies with each of the derivatives that was found to be an acceptor for the various transferases.

Substitution of antifreeze glycoprotein with sialic acid in α2 → 6 linkage to the N-acetylgalactosamine caused a 6-fold decrease in the rate of formation of the sialyl α2 → 3 galactose linkage relative to the rate observed with the native glycoprotein (Fig. 3A). In contrast, sialyl α2 → 3 antifreeze glycoprotein was actually a better substrate for the α-N-acetylgalactosaminide α2 → 6 sialyltransferase than was the native antifreeze glycoprotein (Fig. 3B). The rate of formation of the disialylated product was more than five times faster than formation of sialyl α2 → 6 antifreeze glycoprotein. Fig. 3B also shows that the rate of formation of the sialyl α2 → 6 N-acetylgalactosamine linkage in fucosyl α1 → 2 antifreeze glycoprotein was drastically decreased and that the extent of the reaction was limited.
in asialotransferrin were fucosylated. However, transfer to antifreeze glycoprotein which has the same disaccharide sequence as one of its natural substrates, porcine submaxillary mucin, was more than eight times faster than transfer to asialotransferrin (Fig. 3C). Substitution of antifreeze glycoprotein with sialic acid in \( \alpha_2 + 6 \) linkage to the \( \beta \)-N-acetylglucosaminide caused only a 50% decrease in the rate of fucosylation and a 20 to 30% decrease in the extent of saturation. This extent of fucosylation appears to be the practical limit for the terminal galactosamine that could not be sialylated by the \( \alpha_2 + 6 \) sialyltransferase. The transfer of fucose into 6% of the available sites (Table II) corresponds well to the 7% fucosylation of the penultimate N-acetylglucosamine residue for lactose and 2'-fucosyllactose of 80 mM and 4 mM, respectively (11). The approach to saturation was slower with the \( \beta \)-N-acetylglucosaminide \( \alpha_2 + 3 \) fucosyltransferase displayed a markedly biphasic saturation curve (Fig. 3E). Similar results have been reported for the sialylation of asialo-al-acid glycoprotein by this \( \beta \)-N-acetylglucosaminide \( \alpha_2 + 3 \) sialyltransferase was almost completely inhibited (Fig. 3D). Inhibition was not due to a slower reaction rate, since maximum incorporation was achieved after 3 h and could not be increased by prolonging the reaction or by adding more of the \( \beta \)-N-acetylglucosaminide \( \alpha_2 + 3 \) fucosyltransferase. The transfer of fucose into 6% of the available sites (Table II) corresponds well to the 7% galactose that could not be sialylated by the \( \beta \)-galactoside \( \alpha_2 + 6 \) sialyltransferase (see under “Experimental Procedures”), suggesting that fucose is incorporated solely into the non sia- lylated branch in this acceptor, as previously reported (21).

In contrast, fucosylation of sialyl \( \alpha_2 + 6 \) transferrin by the \( N \)-acetylglucosaminide \( \alpha_1 \rightarrow 3 \) fucosyltransferase was as described in Table I.
The (fucosyl α1 → 2) galactoside α1 → 3 N-acetylglactosaminyltransferase is distinct from the other enzymes studied, because it does not utilize either of the unsubstituted glycoprotein substrates as acceptors. Previous studies have shown an absolute requirement for the Fucα1 → 2Gal structure in acceptor substrates (16, 32, 33) and, as seen in Tables I and II, only those substrates that were previously fucosylated with the α1 → 2 fucosyltransferase could serve as acceptors. The rate of transfer to the antifreeze glycoprotein derivative, which closely resembles the native substrate, porcine submaxillary mucin, was only about twice as fast as transfer to the transferrin derivative (Fig. 3F). Substitution of fucosyl α1 → 2 antifreeze glycoprotein with sialic acid in α2 → 6 linkage to the N-acetylgalactosamine decreased the rate of incorporation to about 10% of that observed with the unsubstituted derivative.

DISCUSSION

The six glycosyltransferases used in this study are capable of acting sequentially to form many of the structures observed at the nonreducing termini of the oligosaccharide chains on mammalian glycoproteins. The results reported herein suggest that the structures produced in vivo are determined in large part by the substrate specificities of glycosyltransferases competing for a common substrate. Since in many instances the prior action of one transferase will block or inhibit the subsequent action of another transferase, oligosaccharide biosynthesis in vivo is probably regulated not only by the enzymes which are available to act on a particular substrate, but also by the order in which they are allowed to act.

The biosynthetic interactions of the glycosyltransferases involved in the formation of “mucin type” oligosaccharide structures are summarized in Fig. 4. Shown in the center is the substrate Gal[β1 → 3GalNAc (a), the core structure in many of the O-linked oligosaccharides in glycoproteins. Three of the enzymes studied here, the α-N-acetylgalactosaminidase α2 → 6 sialyltransferase (I), the β-galactoside α2 → 3 sialyltransferase (II), and the β-galactoside α1 → 2 fucosyltransferase (III) can utilize this substrate to form the trisaccharide structures (b), (c), and (d), respectively. These product can then serve as acceptor substrates in the formation of larger oligosaccharides.

Synthesis of the disialylated structure (e), an O-linked carbohydrate chain found on glycophrin (2) and fetuin (34), can proceed by the initial attachment of either of the sialic acid residues. However, since structure (b) is a poor substrate for the β-galactoside α2 → 3 sialyltransferase (II) and structure (c) is the best of the known acceptors for the α-N-acetylgalactosaminidase α2 → 6 sialyltransferase (I), the preferred pathway appears to be the initial transfer of sialic acid to the galactose followed by transfer of the second sialic acid to the N-acetylgalactosaminide. Similarly, the Fucα1 → 2Gal[β1 → 3(NeuAcα2 → 6GalNAc) tetrasaccharide structure (g), which is found in porcine submaxillary mucin (3), can be synthesized by the initial transfer of either sugar residue. It has been proposed that in vivo, the transfer of the fucose proceeds that of the sialic acid (35, 36). However, in light of the finding that the fucosylated structure (d) is such a poor substrate for the α-N-acetylgalactosaminidase α2 → 6 sialyltransferase, it is likely that the terminal structure preceding (d) is such a poor substrate that the reaction proceeds very slowly.

"Fig. 3. Time course of incorporation of different monosaccharides by the six different glycosyltransferases into derivatives of either anti-freeze glycoprotein or transferrin. A, β-galactoside α2 → 3 sialyltransferase. Reaction mixture (Table I) contained 139 nmol of CMP-NeuAc (10,000 cpm/nmol) and 3.3 mU of transferase. Acceptors were antifreeze glycoprotein (O) and NeuAc α2 → 6 antifreeze glycoprotein (W). B, α-N-acetylgalactosaminidase α2 → 6 sialyltransferase. Reaction mixtures (Table I) contained 30 nmol of GDP-fucose (11,700 cpm/nmol) and 3.3 mU of fucosyltransferase. Acceptors were asialotransferrin (O), Fucα1 → 2 antifreeze glycoprotein (V), and NeuAcα2 → 6 antifreeze glycoprotein (W). C, β-galactoside α2 → 3 sialyltransferase. Reaction mixtures (Table I) contained 20 nmol of UDP-GalNAc (14,000 cpm/nmol) and 1.0 mU of N-acetylgalactosaminyltransferase. Reaction mixture (Table I) contained 20 nmol of UDP-GalNAc (14,000 cpm/nmol) and 1.0 mU of the fucosyltransferase. Acceptors were Fucα1 → 2 transferrin (A), and NeuAcα2 → 6 transferrin (O). D, α-N-acetylgalactosaminidase α2 → 3 fucosyltransferase. Reaction mixtures (Table II) contained 30 nmol of GDP-fucose (11,700 cpm/nmol) and 2.7 mU of fucosyltransferase. Acceptors were asialotransferrin (O), Fucα1 → 2 transferrin (C), GalNAcα1 → 3 (Fucα1 → 2) transferrin (A), and NeuAcα2 → 6 transferrin (O). E, β-galactoside α2 → 6 sialyltransferase. Reaction mixtures (Table II) contained 139 nmol of CMP-NeuAc (10,150 cpm/nmol) and 6.0 mU of sialyltransferase. Acceptors were asialotransferrin (O) and Fucα1 → 2 transferrin (C). F, (fucosyl α1 → 2) galactoside α1 → 3 N-acetylglactosaminyltransferase. Reaction mixtures (Table I) contained 20 nmol of UDP-GalNAc (14,000 cpm/nmol) and 1.0 mU of the transferase. Acceptors were Fucα1 → 2 antifreeze glycoprotein (V), Fucα1 → 2 transferrin (C), and NeuAcα2 → 6(Fucα1 → 2) antifreeze glycoprotein (W)."

"Fig. 4. Proposed biosynthetic pathway for oligosaccharides formed by the sequential actions of the N-acetylgalactosaminidase α2 → 6 sialyltransferase (I), the β-galactoside α2 → 3 sialyltransferase (II), the β-galactoside α1 → 2 fucosyltransferase (III), and the Fucα1 → 2 galactoside α1 → 3 N-acetylgalactosaminyltransferase (IV). The solid bars indicate that the reaction cannot proceed, and the hatched bars indicate that the reaction proceeds very slowly."

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Glycosylation Pathways in Glycoprotein Biosynthesis

The accumulation of incompletely glycosylated chains as involved in the synthesis of the oligosaccharide chains of A blood group positive submaxillary mucin as outlined in Fig. 5. Results shown in Table I, it is possible to predict the reactions major secretory product of the gland. Thus, based on the enzyme suggests that its major role may be in ganglioside biosynthesis (8). If this is the case, a physical separation of

Each of the four glycosyltransferases discussed above were purified from porcine submaxillary glands, and in this tissue three of the four are involved in the synthesis of mucin, the major secretory product of the gland. Thus, based on the results shown in Table I, it is possible to predict the reactions involved in the synthesis of the oligosaccharide chains of A blood group positive submaxillary mucin as outlined in Fig. 5. Because of the substrate specificities of the individual transferases, formation of the completely glycosylated pentasaccharide product must proceed by the sequence of reactions shown in the center. Branching from this pathway results in the accumulation of incompletely glycosylated chains as indicated in the figure. Schachter et al. (37) have shown that the α-N-acetylgalactosaminide β1 → 3 galactosyltransferase will not use the acceptor NeuAcα2 → 6GalNAcThr/Ser. Thus, the prior action of the α-N-acetylgalactosaminide α2 → 6 sialyltransferase blocks further elongation. Similarly, as shown in Table I, the transfer of N-acetylgalactosamine by the (fucosyl α1 → 2) galactoside α1 → 3 N-acetylgalactosaminyltransferase prevents the incorporation of sialic acid, resulting in the accumulation of the product GalNAcα1 → 3 (Fucα1 → 2)Galβ1 → 3GalNAcThr/Ser. The occurrence of these "dead end" products in native mucin (3) suggests that the transferases are not constrained to act in a specific sequence. Indeed, synthesis by these alternate pathways may account for much of the heterogeneity in the mucin. The occurrence of sialyl α2 → 6 galactose linkages has been reported in both A' H+ (38) and A' (39) porcine submaxillary mucin. However, since this sialyltransferase activity has not been demonstrated, it was not possible to determine how the formation of oligosaccharides with this structure fit into the scheme shown in Fig. 5.

Using the human milk (fucosyl α1 → 2) galactoside α1 → 3 N-acetylgalactosaminyltransferase under conditions different from those used in this study, Takasaki et al. (40) were unable to demonstrate incorporation into the acidic oligosaccharides of type O human erythrocytes which have the structure Fucα1 → 2Galβ1 → 3(NeuAcα2 → 6)GalNAcThr/Ser. However, as shown above, the N-acetylgalactosaminyltransferase from porcine submaxillary gland can transfer to this same acceptor structure on fucosyl α1 → 2 (sialyl α2 → 6) antifreeze glycoprotein, although at only 10% the rate at which it transfers to the corresponding neutral triasaccharide on fucosyl α1 → 2 antifreeze glycoprotein (Fig. 3F). Thus it is not clear whether there is actually a difference in the acceptor specificities of the human and porcine transferases, or if this apparent discrepancy simply reflects a difference in the reaction conditions used with the two enzymes.

The product of the β-galactoside α2 → 3 sialyltransferase has not been detected in the oligosaccharide chains of porcine submaxillary mucin (3, 39), despite the fact that asialomucin is a good substrate in vitro (7). At present there is no satisfactory explanation for these observations although several possibilities can be considered. The substrate specificity of the enzyme suggests that its major role may be in ganglioside biosynthesis (8). If this is the case, a physical separation of

![Fig. 5. Proposed pathway for the biosynthesis of the oligosaccharide chains of A⁺ porcine submaxillary mucin. The solid bars indicate that the reaction cannot proceed, and the hatched bars indicate that the reaction proceeds very slowly.](http://www.jbc.org/)

Mucin Polypeptide

NeuAcα2 → 6GalNAcThr/Ser

Galβ1 → 3GalNAcThr/Ser

Galβ1 → 3

NeuAcα2 → 6

Fucα1 → 2Galβ1 → 3GalNAcThr/Ser

GalNAcα3

Galβ1 → 3

NeuAcα2 → 6

Fucα1 → 2

NeuAcα2 → 6

GalNAcα3

Galβ1 → 3

NeuAcα2 → 6

Fucα1 → 2

NeuAcα2 → 6

GalNAcα3

Galβ1 → 3

NeuAcα2 → 6

Fucα1 → 2

NeuAcα2 → 6

GalNAcα3

Galβ1 → 3

NeuAcα2 → 6

Fucα1 → 2

NeuAcα2 → 6

GalNAcα3

Galβ1 → 3

NeuAcα2 → 6

Fucα1 → 2

NeuAcα2 → 6

GalNAcα3

Galβ1 → 3

NeuAcα2 → 6

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GalNAcα3

Galβ1 → 3

NeuAcα2 → 6

Fucα1 → 2

NeuAcα2 → 6

GalNAcα3

Galβ1 → 3

NeuAcα2 → 6

Fucα1 → 2

NeuAcα2 → 6

GalNAcα3

Galβ1 → 3

NeuAcα2 → 6

Fucα1 → 2
this enzyme in a subcellular compartment or in a cell type distinct from the site of glycoprotein biosynthesis may exist such that the mucin is never available as a substrate. Alternatively, since the prior action of the other transferases either blocks or inhibits the action of the $\alpha_2 \rightarrow 3$ sialyltransferase, the absence of this product in the mucin may simply be due to an unfavorable competition for common acceptor sites. Resolution of this question will ultimately depend on more precise localization of these enzymes within the cell.

Fig. 6 summarizes the biosynthetic interactions of the $\beta$-galactoside $\alpha_1 \rightarrow 2$ fucosyltransferase (III), the (fucosyl $\alpha_1 \rightarrow 2$) galactoside $\alpha_1 \rightarrow 3$ N-acetylgalactosaminyltransferase (IV), the N-acetylglucosaminide $\alpha_1 \rightarrow 3$ fucosyltransferase (V), and the $\beta$-galactoside $\alpha_2 \rightarrow 6$ sialyltransferase (VI) with asialotransferrin, which contains typical complex type asparagine-linked oligosaccharide chains. As reported previously (21), the actions of the sialyltransferase and the $\alpha_1 \rightarrow 3$ fucosyltransferase are mutually exclusive. The $\beta$-galactoside $\alpha_2 \rightarrow 6$ sialyltransferase (VI) cannot use structure (b) as an acceptor, and the N-acetylglucosaminide $\alpha_1 \rightarrow 3$ fucosyltransferase (V) cannot use structure (c) as an acceptor. The absence of the product (n) in naturally occurring oligosaccharides is in accord with these specificities. Recently, a structure similar to (n) with the sequence NeuAca2 $\rightarrow 3$Gal$\beta_1 \rightarrow 4$(Fuc$\alpha_1 \rightarrow 3)$GlcNAc$\beta$... has been reported in glycolipids (41) and in the major fucoprotein of rat brain (42). Preliminary experiments indicate that NeuAco2 $\rightarrow 3$Gal$\beta_1 \rightarrow 4$Glc is not an acceptor for the $\beta$-N-acetylgalactosaminide $\alpha_1 \rightarrow 3$ fucosyltransferase, suggesting that synthesis of this structure may well proceed by the initial attachment of fucose followed by the sialic acid. To date the sialyltransferase responsible for forming the sialyl $\alpha_2 \rightarrow 3$ galactose linkage in acceptors with the nonreducing terminal sequence Gal$\beta_1 \rightarrow 4$GlcNAc has not been described.

The other monosialyl-monofucosyl transferrin derivative, containing the structure NeuAco2 $\rightarrow 6$(Fuc$\alpha_1 \rightarrow 2$)Gal$\beta_1 \rightarrow 3$GalNAc$\cdots$ (o) can only be synthesized by one of the two possible pathways since structure (k) is not an acceptor for the $\beta$-galactosidase $\alpha_1 \rightarrow 2$ fucosyltransferase (III). Transfer of sialic acid to structure (m) is very slow, which may explain the absence of this trisaccharide in the reported structures of naturally occurring glycoproteins.

The sequence NeuAco2 $\rightarrow 6$(Fuc$\alpha_1 \rightarrow 2$)Gal$\beta_1 \rightarrow 3$GalNAc has been reported in both A H and A' porcine submaxillary mucin (38, 39). The substrate specificity of the $\beta$-galactosidase $\alpha_1 \rightarrow 2$ fucosyltransferase suggests that to synthesize this structure, the transfer of fucose should precede that of sialic acid. Unfortunately, the sialyltransferase which forms this linkage has not been described, hence this hypothesis cannot be tested directly.

The difucosylated transferrin structure (p) can only be synthesized if the fucosyl $\alpha_1 \rightarrow 2$ galactose linkage is formed first, since structure (l) is not a substrate for the $\alpha_1 \rightarrow 2$ fucosyltransferase. The action of the $\alpha_1 \rightarrow 3$ fucosyltransferase is enhanced somewhat by the presence of the $\alpha_1 \rightarrow 2$-linked fucose in structure (m) so that the difucosylated product is easily formed. This structure has been reported in both glycoproteins (43) and glycolipids (44). Of the three potential acceptors for the $\alpha$-N-acetylgalactosaminyltransferase, only the monofucosylated derivative (m) is active. As reported previously (33), the presence of the $\alpha_1 \rightarrow 3$-linked fucose in structure (p) prevents transfer of the N-acetylgalactosamine. The pentasaccharide sequence (f) can be formed, however, if the N-acetylgalactosaminyltransferase is transferred initially to structure (m) followed by transfer of fucose by the $\alpha_1 \rightarrow 3$ fucosyltransferase. Again, this structure has been reported in both glycoproteins (43) and glycolipids (45). It appears that the actions of the $\beta$-galactosidase $\alpha_2 \rightarrow 6$ sialyltransferase and the $\alpha$-N-acetylgalactosaminyltransferase are mutually exclusive since they cannot use structure (q) and (o) as acceptors, respectively. Thus, the pentasaccharide sequence NeuAco2 $\rightarrow 6$(Fuc$\alpha_1 \rightarrow 2$)GalNAca2 $\rightarrow 3$(Gal$\beta_1 \rightarrow 4$GlcNAc$\cdots$ (s) could not be formed in vitro, and it has not been described in naturally occurring oligosaccharides.

The pathways proposed in Fig. 6 are based on experiments using glycosyltransferases from three different sources, porcine submaxillary gland, bovine colostrum, and human milk. Only one of these enzymes, the $\alpha$-N-acetylgalactosaminyltransferase, has been purified sufficiently from another source to permit a comparison of the enzymic properties of the same glycosyltransferase isolated from different tissues (14, 46). Therefore, it is not possible at this time to conclude whether the substrate specificities observed here are unique to these specific transferases or are common to all mammalian enzymes that form the same glycosidic linkage, regardless of the source. However, the excellent agreement between the structures that could be synthesized in vitro and those which occur naturally in mammalian glycoproteins and glycolipids (47) suggests that the pathways proposed in Fig. 6 accurately represent a part of the general scheme of mammalian glycoprotein biosynthesis.

Glycosylation of the tri- and tetraantennary structures found linked to asparagine on many glycoproteins may be much more complex than that outlined here for the bianten-
nary oligosaccharide chains of human transferrin. It appears that the close juxtaposition of the acceptor sites in these branched structures interferes with their accessibility to the glycosyltransferases. Both the β-galactoside α1 → 2 fucosyltransferase and the N-acetylgalactosaminide α1 → 3 fucosyltransferase (12) are able to fucosylate only 50% of the potential acceptor sites on asialo-α, acid glycoprotein. While the β-galactoside α2 → 6 sialyltransferase can saturate the acceptor sites on this acceptor, transfer to the first 50% of the sites is more than 100 times faster than to the remaining 50% (21). These results suggest that substitution of one or two of the potential acceptor sites in a tetraantennary structure can drastically influence subsequent incorporation of sugars in the remaining sites. No data are available on the effects of partial substitution by one glycosyltransferase on the subsequent action of another, but similar kinds of inhibitions almost certainly occur. Thus, the scheme outlined in Fig. 6 may be an oversimplification of the concerted actions of the glycosyltransferases in the synthesis of tri- and tetraantennary oligosaccharide chains because it does not take into account the potential effect that substitution of one branch of an oligosaccharide chain might have on subsequent glycosylation of an adjacent branch.

Asparagine-linked carbohydrate chains in mammalian glycoproteins contain sialic acid in both α2 → 3 and α2 → 6 linkage to galactose. Oligosaccharides with the biantennary core structure shown in Fig. 2 have been described with both sialic acid residues in α2 → 6 linkage (23, 48), and with one in α2 → 6 linkage and the other in α2 → 3 linkage (48, 50). In the latter case, the α2 → 3-linked sialic acid has always been reported on the Man1α1 → 3Man branch and the α2 → 6 linked sialic acid on the Man1α1 → 6Man branch. Since the two sialyltransferases must be capable of transferring sialic acid to both types of branches in a biantennary acceptor, the mechanism by which the synthesis of this hybrid product is controlled seems unclear. The biphasic incorporation of sialic acid into the asialotransferrin acceptor by the β-galactoside α2 → 6 sialyltransferase (Fig. 3E) may be an important clue toward solving this problem. If the initial rapid incorporation should be preferentially into the Man1α1 → 6Man branch, then the observed structures could be explained solely by the relative amounts of the two sialyltransferases in the Golgi apparatus. Resolution of this question must await the purification and characterization of the appropriate β-galactoside α2 → 3 sialyltransferase and the structural analysis of the partially sialylated products.

The results obtained in these studies provide an enzymatic basis for the observed reciprocal relation between the sialic acid and fucose content of mammalian glycoproteins originally described by Dische (51). While several structures containing both fucose and sialic acid in the same chain could be synthesized, this appears to be the exception rather than the rule. Of the nine instances in this study where a sialylated acceptor substrate was tested with a fucosyltransferase or a fucosylated acceptor was tested with a sialyltransferase, six of the reactions were completely blocked, and two of the remaining reactions were drastically inhibited. Only the Fucα1 → 2Galα1 → 3(NeuAcα2 → 6)GalNAc structure could be easily synthesized. Thus, sialylation and fucosylation are often alternative means of chain termination in mammalian oligosaccharide biosynthesis. In addition, since the acceptor substrate specificities of the sialyl- and fucosyltransferases often overlap, the synthesis of a particular structure may require some further type of regulatory control over the expression of these enzymes.

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