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Biosynthesis of Oligosaccharides in Intact Golgi Preparations from Rat Liver

ANALYSIS OF *N*-LINKED GLYCANS LABELED BY UDP-[6-^{3}H]*N*-ACETYLGLUCOSAMINE*

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During short incubations of a Golgi apparatus-enriched subcellular fraction from rat liver with UDP-[^{3}H]GlcNAc, label is efficiently transferred to endogenous acceptors. Most of the macromolecular radioactivity is specifically released by peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase, indicating that it is mainly associated with *N*-linked oligosaccharides. The glycoprotein acceptors are resistant to proteases unless detergent is added in amounts greater than the critical micellar concentration. This shows that the acceptors are within the lumen of intact compartments, which have the correct topological orientation expected for the Golgi apparatus in intact cells. Structural characterization of the radiolabeled *N*-linked oligosaccharides shows a variety of distinct neutral and anionic species. The neutral chains include bi-, tri-, and tetra-antennary molecules with terminal β-[^{3}H] GlcNAc residues. *In vitro* sialylation shows that some of the tetra-antennary chains have β1,3-linked Gal residues on their unlabeled antennae. An unknown modification appears to block the action of β-galactosidase on these galactosylated oligosaccharides. Chasing the labeling reaction with a mixtures of UDP-Gal, CMP-Neu5Ac, and adenosine 3′-phosphate,5′-phosphosulfate causes an increase in the percent of radiolabeled anionic oligosaccharides. Most of the negative charge is due to sialic acid (Sia), and some appears to be in phosphodiester-linked [^{3}H]GlcNAc. The sialylated oligosaccharides are a mixture of bi-, tri-, and tetra-antennary species with 1–3-Sia residues, and some of the [^{3}H]GlcNAc residues are directly covered with unlabeled Gal and Sia residues.

This *in vitro* approach should recapitulate reactions that occur in the biosynthesis of *N*-linked oligosaccharides in the Golgi apparatus of the intact cell. Since the conditions during labeling do not permit inter-compartmental transport, the oligosaccharides produced should represent the biosynthetic capabilities of individual Golgi compartments. Evidence is presented for a functional association of GlcNAc transferases I, II, and α-mannosidase II, with separation from GlcNAc transferase IV and/or V. The structures also indicate co-compartmentalization of several GlcNAc transferase(s) with β-galactosyltransferase(s) and sialyltransferase(s). The compartmental organization of the Golgi apparatus is discussed in light of these findings.

The diverse *N*-asparagine-linked oligosaccharides found on many secreted and membrane bound proteins all originate from the $Glc_3Man_9GlcNAc_2$ precursor (reviewed in Refs. 1–5). Initial transfer from the high energy dolichol pyrophosphate donor to nascent polypeptides in the endoplasmic reticulum (ER)[1] is followed by remodeling ("processing") which involves both the removal and addition of monosaccharides. Following removal of 3 Glc residues and sometimes 1 Man residue in the ER, glycoproteins are transported to the Golgi apparatus, where the oligosaccharide remodeling continues on some of the chains. First, α-mannosidase I removes 4 α1,2-linked Man residues to yield $Man_5GlcNAc_2$. GlcNAc transferase I then adds 1 GlcNAc residue, making the oligosaccharide a substrate for α-mannosidase II, which removes 2 additional Man residues to yield $GlcNAc_1Man_3GlcNAc_2$. This oligosaccharide is a substrate for several glycosyltransferases including GlcNAc transferases II, III, IV, and V. Each GlcNAc residue is a substrate for UDP-Gal:GlcNAcβ1,4 galactosyltransferase (except for the "bisected" GlcNAc residue added by GlcNAc transferase III). The Gal residues can then be capped with one of several terminal monosaccharides, including α2,3- or α2,6-linked sialic acid (Sia), α1–2-linked Fuc, α1–3-linked Gal, or an extended polylactosamine chain. Oligosaccharides have also been described with Galβ1,3GlcNAc sequences instead of the much more common Galβ1,4GlcNAc linkage. This Galβ1,3GlcNAc disaccharide can be capped with α2,3-Sia but not α2,6-linked Sia. Additional modifications can include the sulfation of GlcNAc, Gal, and GalNAc residues (6–9) and the *O*-acetylation of Sia residues (10, 11). The final product is thus the result of a complex set of sequential

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[1] The abbreviations used are: ER, endoplasmic reticulum; Neu5Ac, *N*-acetylneuraminic acid; AUS, *Arthrobacter ureafaciens* sialidase; ConA, concanavalin A; ConA-I, oligosaccharides passing through ConA-Sepharose; ConA-II, oligosaccharides eluted from ConA-Sepharose by 10 mM α-methylglucopyranoside; ConA-III, oligosaccharides eluted from ConA-Sepharose by 100 mM α-methylmannopyranoside; PNGase F, peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase; α2,6-sialyltransferase, CMP-Neu5Ac:Galβ1,4GlcNAcα2,6-sialyltransferase; α2,3-sialyltransferase, CMP-Neu5Ac:Galβ1,3(4)-GlcNAcα2,3-sialyltransferase; β1,4-galactosyltransferase, UDP-Gal:Glcβ1,4-galactosyltransferase; AC, acetyl; HPLC, high performance liquid chromatography; MOPS, 4-morpholinepropanesulfonic acid; PAPS, adenosine 3′-phosphate,5′-phosphosulfate; AMP-PNP, 5′-adenylyl-β,γ-imidodiphosphate; BSA, bovine serum albumin; Ac, acetyl; PHA, phytohemagglutinin.

and concerted reactions occurring in the compartments of the Golgi apparatus.

All of these glycosyltransferase reactions occur on the lumenal side of Golgi compartments and require the translocation of donor sugar nucleotides from the cytosol into the Golgi. Specific sugar nucleotide transporters (reviewed in Refs. 12 and 13) are postulated to improve the efficiency of the glycosyltransferase reactions by concentrating the donors into the Golgi. Thus, at least four factors (sugar nucleotide, sugar nucleotide transporter, oligosaccharide acceptor, and glycosyltransferase) must be functionally co-localized in the same compartment of the Golgi apparatus for a given step in oligosaccharide biosynthesis to occur.

The biosynthetic pathway of *N*-linked oligosaccharides was first deduced by pulse labeling cells in culture with radiolabeled monosaccharides, and structurally characterizing the resulting radiolabeled oligosaccharides (14, 15). However, since transport of glycoproteins through the Golgi is very fast, less abundant oligosaccharide structures and transient intermediates may not have been identified. A complementary approach has been to determine the substrate specifities of purified, or partially purified glycosyltransferases (16). Composite results of such studies from several systems have produced rules that predict the order of action of the GlcNAc transferases (2). However, it is not known if the substrate specificities of the purified enzymes are faithfully retained in the specialized environment of the intact Golgi apparatus.

It has been previously shown that incubating Golgi-enriched preparations with radioactive sugar nucleotides results in the transfer of radiolabeled monosaccharides to endogenous proteins (17). The nature of the oligosaccharide acceptors and their topological orientation was not investigated in this study. We reasoned that if factors known to be necessary for inter-compartmental transport (18) are not added, and if the Golgi compartments remain intact during such incubations, the radiolabeled oligosaccharides should represent actual biosynthetic intermediates. Thus, characterization of these oligosaccharides should allow (*a*) the identification of transient intermediates and previously undescribed structures present in low abundance; (*b*) comparison of the structures with those predicted by the *in vitro* enzymology; and (*c*) understanding of the co-localization of different glycosyltransferases within a single functional compartment of the Golgi apparatus. Co-localization could be inferred if the transferred radiolabeled monosaccharide is directly "covered" by an additional unlabeled sugar, of if the non-labeled antennae of the same oligosaccharide are shown to bear such residues.

To address these issues, a Golgi apparatus-enriched subcellular fraction from rat liver was incubated with UDP-[^{3}H] GlcNAc, and the resulting labeled *N*-linked oligosaccharides were released, purified, fractionated, and structurally characterized. While most of the structural intermediates predicted by the established model of *N*-linked oligosaccharide biosynthesis and Golgi organization were noted, some unexpected or novel structures were also found.

EXPERIMENTAL PROCEDURES

Materials—The following materials were obtained from the sources indicated: Amberlite MB3, Sephacryl S-200, Sephadex G-10, G-15, and QAE-Sephadex A-15, Pharmacia LKB Biotechnology Inc. and Sigma; Bio-Beads SM-2, AG 3-X4a (free base), and AG 50 (H^+ form), Bio-Rad; concanavalin A (ConA)-Sepharose, Pharmacia; E_4-PHA-agarose, EY Labs; MicroPak AX-5 HPLC column, Varian; TSK-55 DEAE HPLC columns, TosoHaas; Jack bean β-hexosaminidase was from Sigma (10 milliunits/μl) or V-Labs (0.5 units/μl); Jack bean β-galactosidase (6.3 milliunits/μl), Sigma; bovine testicular β-galactosidase and *Diplococcus pneumoniae* β-hexosaminidase (1 milliunit/μl), Boehringer Mannheim. Peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase (PNGase F) was purified by one of two methods (19, 20). CMP-Neu5Ac:Galβ1,4GlcNAcα2,6-sialyltransferase (α2,6-sialyltransferase) and CMP-Neu5Ac:Galβ1,3(4)GlcNAcα2,3-sialyltransferase (α2,3-sialyltransferase) (21–23) were generously provided by Drs. Mark Williams and James Paulson, Cytel. Bovine colostrum UDP-Gal:Glcβ1,4-galactosyltransferase (β-galactosyltransferase) was obtained from Sigma, and autogalactosylated as previously described (24). *Arthrobacter ureafaciens* sialidase (AUS) was obtained as a lyophilized powder from Calbiochem, resuspended to a concentration of 4 units/ml in 100 mM sodium citrate, pH 4.6, and stored at −20 °C. Endo-β-galactosidase from *Escherichia freundii* (1 unit/ml) was kindly provided by Dr. Michiko Fukuda, La Jolla Cancer Research Foundation. UDP-[6-^{3}H]GlcNAc (15 Ci/mmol) was from ARC, and UDP-[6-^{3}H]Gal (10 Ci/mmol) was synthesized as previously described (25). Sialylated bi-, tri-, and tetra-antennary oligosaccharide standards, structurally verified by NMR, were obtained from Dionex and labeled at the reducing terminus with NaB[^{3}H]H_4. Additional oligosaccharide standards were obtained by desialylating these with AUS and/or degalactosylating with Jack bean β-galactosidase. [^{3}H]Galβ1,4GlcNAc and [^{3}H]Galβ1,4GlcNAc$_2$ were prepared by incubating UDP-[^{3}H]Gal and β-galactosyltransferase with GlcNAc and chitobiose, respectively, under conditions used for assaying the enzyme (see below). The products were purified by sequential passage over 2-ml columns of AG 1-X8 (equilibrated in 5% sodium borate), AG 3-X4a (free base), and AG 501-X8 (H^+ form). Each column was prewashed with 10 or more column volumes of water prior to loading the sample and eluting in water. The elute was lyophilized and the product confirmed by paper chromatography on Whatman No. 1 paper developed in pyridine:ethyl acetate:acetic acid:water (5:5:1:3). All other reagents were from commercial sources and were of the highest grade available.

Isolation of Golgi Apparatus-enriched Subcellular Fraction—A Golgi apparatus-enriched subcellular fraction (hereafter called "Golgi-enriched fraction") was obtained from rat liver essentially as previously described by Leelavathi (26) and modified by Tabas and Kornfeld (27). Male Harlan Sprague-Dawley rats (3–4 months of age, from Charles River) were fasted overnight, anesthetized with ether, decapitated with a guillotine, and the blood allowed to drain from the body. The livers were excised and placed on ice until use. Fibrous connective tissue was dissected out, and the livers were finely minced. Aliquots of ~7 g were placed in 30-ml Sorvall centrifuge tubes, 20 ml of 0.5 M sucrose in 50 mM sodium maleate, 5 mM $MgCl_2$, pH 6.5 (maleate/mg buffer), added, and the tissue homogenized with a Beckman Polytron fitted with a PT10–35 probe (15 s at a setting between 3 and 3.5). Intact cells, fibrous material, and nuclei were removed by centrifugation for 10 min at 600 × g_{max} in an SS-34 rotor. The post-nuclear supernatants was removed from each tube, pooled, and an aliquot saved. The post-nuclear supernatant (16 ml each tube) was layered on top of 8-ml cushions of 1.3 M sucrose and centrifuged in a Beckman Ti-50.2 rotor at 66,000 × *g* for 2 h. The "crude smooth" membranes were carefully removed from the top of the sucrose pad, pooled, the sucrose concentration adjusted to 1.1 M (determined with a refractometer), and an aliquot saved. The final step is a sucrose density gradient through which the light membranes "float" from a region of higher density to lower density. A cushion of 8 ml of 1.25 M sucrose in maleate/Mg buffer was placed in the bottom of a centrifuge tube compatible with a Beckman SW-28 rotor. This was overlaid with 20 ml of crude smooth, 7 ml of 1.0 M sucrose in maleate/Mg buffer, and finally with approximately 4 ml of 50 mM maleate/Mg buffer. After centrifugation for 1.5 h at 70,000 × *g* in a SW-28 rotor, the Golgi apparatus-enriched fraction was removed from the interface between the 1.0 M sucrose and the buffer, adjusted to approximately 0.37 M sucrose, and used directly for labeling. Thus, the membranes were never pelleted at any stage during preparation.

Marker Enzyme Assays—UDP-Gal:GlcGalβ1,4-galactosyltransferase was assayed in a final volume of 50 μl containing 400 μM UDP-galactose (~0.1 μCi of UDP-[^{3}H]Gal) 0.4% (w/v) Triton X-100, 86 mM 2-mercaptoethanol, 4 mM ATP, 40 mM $MnCl_2$, and 100 mM sodium cacodylate, pH 6.5, in the presence and absence of 80 mM GlcNAc as acceptor. After incubation at 37 °C for 20 min, assays were quenched with 1 ml of ice-cold 10 mM EDTA. Samples were applied to individual 1-ml columns of AG 1-X8 previously equilibrated with 5% (w/v) sodium tetraborate and washed with at least 5 column volumes of water. The run through and water washes of 1 ml × 1, 2 ml × 4 were collected into scintillation vials. The only radioactive molecule that can pass through the column is the enzymatic product, [^{3}H]Galβ1,4GlcNAc. The background obtained in the absence of GlcNAc acceptor was subtracted. Glucose-6-phosphatase was assayed

as described (28). Control incubations contained Man-6-phosphate instead of glucose 6-phosphate. 5'-Nucleotidase (29) and β-hexosaminidase (30) were assayed essentially as previously described.

Incorporation of [^{3}H]GlcNAc into Endogenous Macromolecules—A Golgi-enriched fraction was prepared as described above and labeled with UDP-[^{3}H]GlcNAc. For the studies reported in this paper, three aliquots (samples 1, 2, and 3) were labeled with 35–50 μCi of UDP-[^{3}H]GlcNAc (0.41 μM final) with 1 mM $MnCl_2$ added in the case of sample 3. After a 15-min incubation at room temperature, the labeling reactions were chased by adding excess nonradioactive nucleotides. Sample 1 was chased by adding UDP-GlcNAc at a final concentration of 10 μM. Samples 2 and 3 were chased by adding UDP-GlcNAc, UDP-Gal, CMP-Neu5Ac, Ac-CoA, and PAPS to a final concentration of 10 μM each. These final concentrations were chosen to be in the range of the apparent K_m values of the sugar nucleotide transporters, which are reported to be 2–20 μM (12, 13). The incubations were continued for 20 min, and the samples then centrifuged at 100,000 × *g* for 30 min. The supernatants was removed and saved, and the pellets were solubilized in ~4.5 ml of 4% (w/v) SDS, 10 mM HEPES, pH 7.0, with heating to 80 °C for ~20 min.

In a separate labeling, 5 ml of a Golgi-enriched fraction was preincubated with swainsonine (10 μg/ml final) and $MnCl_2$ (1 mM final) for 15 min at room temperature, UDP-[^{3}H]GlcNAc was added (50 μCi; 0.66 μM final), and the incubation continued for 15 min at room temperature. The labeling reaction was chased by adding UDP-GlcNAc, UDP-Gal, and CMP-Neu5Ac to 20 μM each and continuing the incubation for 15 min. The samples were then treated as described above.

Isolation of [^{3}H]GlcNAc-labeled N-Linked Oligosaccharides—The solubilized material was applied to a column (1.4 × 44 cm) of Sephacryl S-200 equilibrated with 20 mM HEPES, 0.2% (w/v) SDS, pH 7.6, and the column eluted with 0.93 ml/min of the same buffer. The labeled macromolecules eluting in the void volume were pooled, adjusted to 90% (v/v) acetone, and placed at 4 °C overnight. The precipitated macromolecules were collected by centrifugation at 3100 × *g* for 10 min. Less than 1% of the radioactivity remained in the acetone supernatant. The pellets were solubilized in 800 μl of 20 mM HEPES, 1% (w/v) SDS, 20 mM 2-mercaptoethanol, pH 8.2, with heating at 85 °C for 30 min. After mixing with 3.2 ml of 20 mM HEPES, 1.25% (v/v) Nonidet P-40, 20 mM 2-mercaptoethanol, pH 8.2, 15 μl of PNGase F was added, and the mixture incubated at 37 °C for 21 h. After adding another 10 μl of PNGase F, the incubation was continued for 3 h, and stopped by heating at 90 °C for 20 min. The mixture was reapplied to the S-200 column originally used to isolate the labeled macromolecules. The included fractions containing the released *N*-linked oligosaccharides were pooled, saturated KCl was added to 2% (v/v) final, and the mixture placed at 4 °C overnight. The precipitated potassium dodecyl sulfate was removed by centrifugation at 2000 rpm in an IEC centrifuge. The supernatant was collected, and residual detergent removed with a 10-ml column of Bio-Beads SM-2 prewashed with at least 10 column volumes of water. The sample was loaded, washed with 5 column volumes of water, and the run through and washings collected. The recovery of oligosaccharides from the KCl precipitation and Bio-Beads SM-2 was 94–97%. The oligosaccharides were lyophilized, resuspended in 1 ml of water, and desalted on Sephadex G-10 (1.5 × 16 cm) equilibrated in water. The desalted oligosaccharides were fractionated into neutral and anionic species by ion exchange chromatography. A 2-ml column of QAE-Sephadex was washed with at least 10 column volumes of 2 mM Tris base, pH~ 9.5. The purified oligosaccharides were adjusted to 2 mM Tris base, applied to the column, and the flow-through and a 20-ml wash of 2 mM Tris base collected into a single tube. The anionic oligosaccharides were eluted with 10 ml of 1 M NaCl in 2 mM Tris base. The neutral and anionic oligosaccharides were lyophilized and desalted on Sephadex G-10 as described above. The QAE-Sephadex fractionation of the neutral and oligosaccharides had to be repeated once to recover >97% anionic oligosaccharides from the mixture.

Desalting Oligosaccharides—Anionic oligosaccharides were desalted on Sephadex G-10 or G-15 in water. Neutral oligosaccharides were desalted either in this manner, or with tandem cation and anion exchange columns (2 ml each of AG-50 (H^+ form) and AG 3-X4a (free base), each prewashed with at least 10 column volumes of water).

Effect of Triton X-100 on Incorporation of [^{3}H]GlcNAc into Macromolecules—Aliquots (100 μl) of the Golgi-enriched fraction were adjusted to 1 mM final $MnCl_2$, mixed with 100 μl of 2 × Triton X-100 in maleate/Mg buffer containing 0.25 M sucrose, and incubated on ice for 20 min. The final concentration of the Triton X-100 was 0, 0.001, 0.005, 0.01, 0.02, 0.05, 0.1, or 0.5% (v/v). UDP-[^{3}H]GlcNAc was added (0.2 μCi) and the mixture was incubated at room temperature for 25 min. The reactions were stopped with 800 μl of 8% (v/v) perchloric acid and placed on ice. The precipitated macromolecules were collected by centrifugation, the pellets surface washed twice with 8% (v/v) perchloric acid, dissolved in 0.5 ml of 1 M NaOH with heating, neutralized with 0.5 ml of 1 M HCl, and counted. Duplicate incubations were used for each Triton X-100 concentration. The background was determined using 10 mg/ml BSA in place of the Golgi-enriched fraction. Control incubations prelabeled with UDP-[^{3}H]GlcNAc and then treated with Triton X-100 were used to determine if the detergent had an affect on the ability to precipitate the radiolabeled macromolecules.

Protection of the Radiolabeled Endogenous Acceptors from Added Proteases—Ten ml of Golgi-enriched fraction was labeled with ~20 μCi of UDP-[^{3}H]GlcNAc at room temperature for 16 min in the presence of 1 mM $MnCl_2$. The reaction was quenched by adding UDP-GlcNAc to a final concentration of 50 μM, and split into 6 aliquots of 270 μl. Paired aliquots were mixed with 30 μl of maleate/Mg buffer, 0.25 M sucrose containing 1% (v/v) Triton X-100, 0.1% (v/v) Triton X-100, or no detergent. One of each pair was mixed with 100 μl of 10 mg/ml Pronase, the other adjusted to 1 mg/ml BSA. Following 0, 0.5, and 16 h of incubation at room temperature, duplicate aliquots of 30 μl were removed from each sample, and the radioactive macromolecules precipitated with 4% perchloric acid as described above.

Exoglycosidase Digestions—Exoglycosidase digestions were performed in either sodium citrate, pH 4.6, sodium cacodylate, pH 5.8, sodium acetate, pH 5.5, or the buffer provided by the manufacturer. Digestions were typically incubated overnight at 37 °C using enzyme concentrations of 0.5–2 units/ml. The specific conditions of each reaction are listed in the figure legends.

Paper Chromatography—This was carried out at room temperature in different solvent systems. System A, the release of [^{3}H]GlcNAc from oligosaccharides by exoglycosidases or mild acid treatment was quantitated by descending paper chromatography on Whatman No. 1 in pyridine:ethyl acetate:acetic acid:water (5:5:1:3). Radioactivity on 1-cm strips was determined after soaking in 0.4 ml of water and adding 4 ml of scintillation fluid. The radioactivity remaining at the origin after 15–18 h (oligosaccharides) and released monosaccharides (R_F of 0.5–0.7) was determined. System B, GlcNAc and GalNAc were separated on borate-impregnated papers as previously described (31) with 1-butanol:pyridine:water (6:4:3) as the solvent. System C, the intactness of the UDP-[^{3}H]GlcNAc was determined with ethanol, 1 M ammonium acetate, pH 3.8 (7:3), as the solvent.

Radiochemical Composition of [^{3}H]GlcNAc-labeled Oligosaccharides—Aliquots (4 ml) of a Golgi-enriched fraction were incubated with UDP-[^{3}H]GlcNAc (~6 μCi) and $MnCl_2$ (1 mM final) in the presence or absence of 0.1 mM AMP-PNP (a competitive inhibitor of pyrophosphatases, that can hydrolyze UDP-[^{3}H]GlcNAc). After incubation at room temperature for 20 min, the membranes were pelleted at 100,000 × *g* for 30 min, and the supernatant saved. The pellets were surface washed with maleate/Mg buffer, the membranes resuspended in the same buffer, and pelleted. The second supernatants were discarded and the pellets were resuspended in 4 ml of maleate/Mg buffer containing 0.02% (w/v) saponin. The saponin-permeabilized membranes (10) were again pelleted. The supernatant was saved, the membranes resuspended in 1% (w/v) SDS, and the macromolecules isolated by S-200 chromatography as described above. The *N*-linked oligosaccharides were released with PNGase F and purified as described above. The purified oligosaccharides were treated for 18 h at 37 °C with Jack bean β-hexosaminidase (Sigma, 0.32 unit), Jack bean β-galactosidase (Sigma, 0.13 unit), and AUS (0.02 unit) in 100 mM sodium acetate, pH 5.5, 0.02% (w/v) NaN_3 in a final volume of 200 μl. The release of radiolabeled monosaccharides was determined by paper chromatography as described above. The original 100,000 × *g* supernatants were hydrolyzed under mildly acidic conditions to release the radiolabeled monosaccharide from the sugar nucleotide/sugar phosphate. To 200 μl of supernatant was added 200 μl of 100 mM maleic acid adjusted to pH 1.86 with NaOH (final pH 2.2), and the mixture was heated to 100 °C for 60 min. The released monosaccharide was desalted using the mixed bed resin, Amberlite MB3. In each case, paper chromatography on borate-impregnated paper (described above) was used to determine if the released monosaccharide was [^{3}H]GlcNAc or [^{3}H]GalNAc, by comparison with standards.

Lectin Affinity Chromatography—Lectin affinity chromatography was performed essentially as described (32). A 2-ml column of ConA-Sepharose at room temperature was washed with at least 10 column volumes of ConA buffer consisting of 10 mM HEPES, 150 mM NaCl,

1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.02% (w/v) NaN_3, pH 8.1. The sample (25–500 μl) was diluted to 2 ml with ConA buffer, and applied to the column. The column was washed with 4 × 2 ml of ConA buffer (ConA-I fraction) and eluted first with 5 × 2 ml of ConA buffer containing 10 mM α-methylglucopyranoside (ConA-II fraction), and then with 5 × 2 ml of ConA buffer containing 100 mM α-methylmannopyranoside, which was prewarmed to 60 °C (ConA-III fraction). Oligosaccharides fractionated on a preparative scale using ConA-Sepharose were desalted on Sephadex G-15 in water.

A column of E_4-PHA-agarose (0.7 × 14 cm) at 4 °C was washed with ConA buffer. Oligosaccharide samples (25 μl) were adjusted to 0.5 ml with ConA buffer and [^{14}C]ManNAc was added to mark the position of unretained molecules. The sample was applied to the column and the flow rate ~0.2 ml/min was maintained using a syringe pump. Fractions were collected every 2 min and the ^{3}H and ^{14}C in each fraction determined by double label scintillation counting.

High Pressure Liquid Chromatography—Neutral oligosaccharides were fractionated/analyzed using a MicroPak AX-5 HPLC column eluted with a gradient of decreasing acetonitrile in water (33). The column was equilibrated in acetonitrile:water (65:35) and the sample was injected. A gradient of 65:35 to 30:70 acetonitrile:water developed over 70 min was immediately started. The flow rate was 1 ml/min and fractions were collected every 0.3–1.0 min, depending on the fractionation range required. Anionic oligosaccharides were fractionated on a DEAE HPLC anion exchange column. The column was equilibrated in water and the sample was injected. The column was washed with water for 5 min after which a gradient of 0–100 mM NaCl was developed over 70 min. The flow rate was 0.6 ml/min and fractions were collected every 1 min.

RESULTS

Characterization of the Golgi-enriched Fraction—A subcellular fraction enriched in the Golgi apparatus (Golgi-enriched fraction) was prepared from rat liver as described under "Experimental Procedures." The procedure is essentially as previously described (27) except that the final salt wash and pelleting steps were omitted. Although this final step typically gives an additional 2-fold enrichment of the Golgi marker β1,4-galactosyltransferase, it was deliberately omitted to avoid any possible damage to Golgi compartments (none of the previous steps involve pelleting or resuspension). As shown in Table I, β1,4-galactosyltransferase was enriched an average of 45-fold relative to the post-nuclear supernatant, with an average recovery of 28%. The small amount of contamination by other subcellular components does not complicate the analysis, since transfer of [^{3}H]GlcNAc to endogenous acceptors can only occur in intact functional compartments with the correct topological orientation that also have the UDP-GlcNAc transporter and the GlcNAc transferases (see below).

Incorporation of [^{3}H]GlcNAc into Macromolecules Requires the Golgi Compartments to be Intact and of the Correct Topological Orientation—If the transfer of [^{3}H]GlcNAc from UDP-[^{3}H]GlcNAc to endogenous acceptors *in vitro* reflects normal Golgi glycosylation reactions *in vivo*, then the acceptors should be on the lumenal side of intact membrane-bound compartments. The sugar nucleotide transporters are known to be capable of concentrating the nucleotides 20–50-fold in the Golgi apparatus (12, 13). Since the K_m of many glycosyltransferases is an order of magnitude higher than that of the transporters, concentrating the nucleotides should serve to increase the efficiency of glycosylation reactions. Disruption of the Golgi membrane should therefore abolish the concentration gradient and *decrease* the transfer of label to endogenous macromolecular acceptors. If, on the other hand, [^{3}H]GlcNAc is being transferred to acceptors on broken or inside-out vesicles, detergent should *increase* the incorporation of label by stimulating the action of the transferases and improving their access to solubilized acceptors. Fig. 1 shows that below its critical micellar concentration of ~0.015%, the detergent Triton X-100 had no effect upon the incorporation of [^{3}H]GlcNAc into acid-insoluble macromolecules. At concentrations above the critical micellar concentration, the incorporation was markedly reduced. While Triton X-100 at high concentrations can also nonspecifically inhibit acid precipitation, this effect was not apparent at 0.05%, where incorporation was already reduced by 89%. These results suggest that detergent disrupts the Golgi membrane and abolishes a concentration gradient of UDP-[^{3}H]GlcNAc, and indicate that the transfer reaction is taking place in intact membrane-bound compartments.

Analysis of the labeled material by SDS-polyacrylamide gel electrophorsis showed a variety of labeled glycoproteins (data not shown). If the isolated Golgi compartments have the correct topological orientation, then these labeled products of transfer should be resistant to protease degradation. A Golgi-enriched fraction was labeled with UDP-[^{3}H]GlcNAc, chased with excess nonradioactive sugar nucleotide, and aliquots treated with or without Triton X-100 (at 0.01 and 0.1% based on the result in Fig. 1). Each sample was divided into 2 aliquots, and Pronase (2.5 mg/ml final) or BSA (1 mg/ml final) was added. The proteolytic degradation of the [^{3}H]GlcNAc-labeled proteins was determined by following acid-insoluble radioactivity, after incubation at room temperature for either 0.5 or 16 h. As shown in Fig. 2, this high concentration of Pronase could not digest the [^{3}H]GlcNAc-labeled macromolecules in 0 or 0.01% (v/v) Triton X-100, even after a 16-h incubation. In contrast, if the detergent was added at 0.1% (v/v), there was a 69% decrease in the acid-precipitation of macromolecules at 16 h (the remaining acid-resistant ma-

TABLE I

Purification and recovery of various cellular membranes in the Golgi-enriched fraction

Enzyme markers specific for various subcellular fractions were assayed in the Golgi apparatus-enriched fractions as described under "Experimental Procedures." Enrichment and recovery were calculated relative to the post-nuclear supernatant. The average values from four preparations is shown.

Enzyme assayed	Subcellular fraction	Enrichment	Recovery
		-fold	%
β1,4-Galactosyltransferase	Golgi	45	28
Glucose-6-phosphatase	ER	0.88	0.4
β-Hexosaminidase	Lysosomes	3.6	2.3
5′-Nucleotidase	Plasma membrane	4.2	2.2

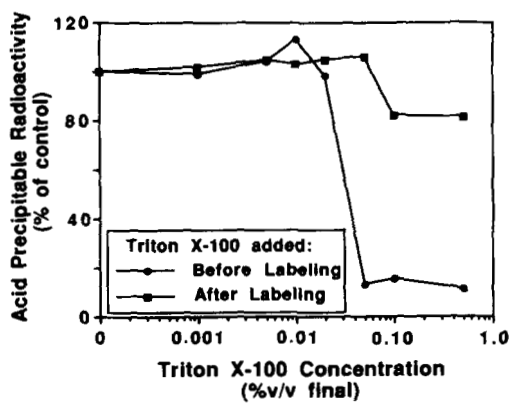

FIG. 1. **Effect of Triton X-100 on the incorporation of [^{3}H]GlcNAc into macromolecules.** Aliquots of a freshly isolated Golgi-enriched fraction were preincubated with increasing concentrations of Triton X-100, incubated with UDP-[^{3}H]GlcNAc, and the radioactivity incorporated into macromolecules was determined as described under "Experimental Procedures." Control incubations were first labeled and then treated with the same concentrations of detergent.

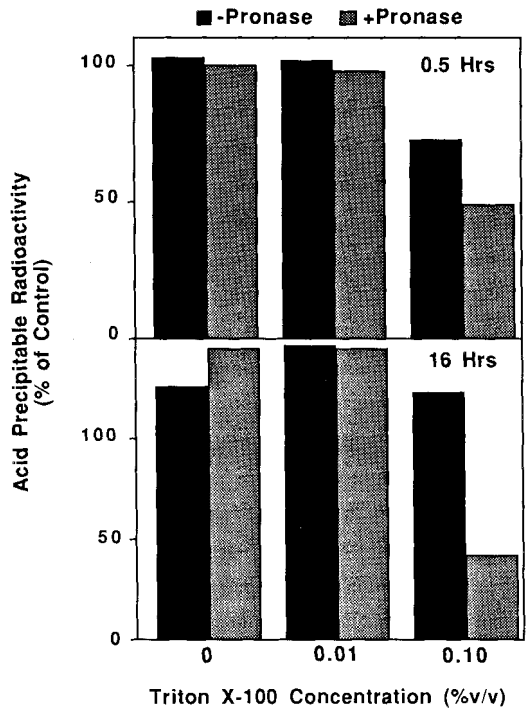

FIG. 2. **Protection of the UDP-[^{3}H]GlcNAc labeled endogenous acceptors from proteases.** Aliquots of a freshly isolated Golgi-enriched fraction were incubated with UDP-[^{3}H]GlcNAc in the presence of 1 mM $MnCl_2$, and the reactions were chased with excess nonradioactive UDP-GlcNAc (50 μM final). Triton X-100 was then added at the indicated concentrations, the mixtures incubated with Pronase (2 mg/ml final) for the indicated times, and the acid-precipitable radioactivity determined as described under "Experimental Procedures." The value for 100% of control is defined as the radioactivity precipitated after a 0.5-h incubation in the absence of added detergent or protease.

terial may be in acid-precipitable glycopeptides, glycosaminoglycans, or glycolipids). These results indicate that only sealed membranes of the appropriate topological orientation can transfer [^{3}H]GlcNAc from UDP-[^{3}H]GlcNAc to macromolecular acceptors. Thus, the labeling of such Golgi-enriched preparations should reflect the glycosylation reactions that normally occur *in vivo*.

Radiochemical Purity of the [^{3}H]GlcNAc-labeled Oligosaccharides—Although UDP-[^{3}H]GlcNAc can be epimerized to UDP-[^{3}H]GalNAc in the intact cell, this reaction requires the cytosolic enzyme UDP-GlcNAc 4′-epimerase, along with a source of NAD^+ (34). To confirm that this reaction was not significant in our experiments, a Golgi-enriched fraction was incubated with UDP-[^{3}H]GlcNAc and the ratio of [^{3}H]GlcNAc to [^{3}H]GalNAc determined in the incubation medium and in the labeled *N*-linked oligosaccharides (see "Experimental Procedures"). To deliberately exaggerate the possibility for epimerization, incubations were also done in 0.1 mM AMP-PNP, a competitive inhibitor of pyrophosphatases that hydrolyze the sugar nucleotide. Regardless of whether AMP-PNP was added, only 2–3% of the label in the medium was converted to [^{3}H]GalNAc. When the labeled *N*-linked oligosaccharides released by PNGase F (see below), were treated with a mixture of β-hexosaminidase, β-galactosidase, and sialidase, 93–94% of the label was released, and ~95% of this was in [^{3}H]GlcNAc. These results indicate that epimerization is not a significant concern in the analysis of the labeled *N*-linked oligosaccharide structures.

Preparation of Radiolabeled Endogenous N-Linked Oligosaccharides—It is known that Mn^{2+} can stimulate GlcNAc transferases I, II, III, IV, and β1,4-galactosyltransferase (but not GlcNAc transferase V) (16). Initial experiments using Golgi-enriched fractions incubated with UDP-[^{3}H]GlcNAc showed that $MnCl_2$ caused a dose-dependent increase in incorporation of label into macromolecules, and that the addition of other sugar nucleotides could alter the *N*-linked oligosaccharide structures produced (data not shown). The final concentration of $MnCl_2$ used (1 mM) gave a ~2-fold increase in incorporation of [^{3}H]GlcNAc into macromolecules. Higher concentrations of $MnCl_2$ (>20 mM) were avoided because they have been reported to cause fusion of elements of the Golgi apparatus (35). Furthermore, higher concentrations were not necessary, since even 40 mM $MnCl_2$ increased the incorporation of label only ~2-fold above that seen with 1 mM $MnCl_2$ (data not shown). Preparative labelings were carried out in the presence or absence of $MnCl_2$. Different "chase" conditions with unlabeled sugar nucleotides were also used to study the effects on oligosaccharide branching, and to see if the [^{3}H]GlcNAc could be covered by other monosaccharide residues such as Gal. For the experiments presented here, three different labeling conditions were employed, varying Mn^{2+} and additional sugar nucleotides. The nomenclature is summarized in Table II. Sample 1 was labeled without $MnCl_2$ and chased with UDP-GlcNAc alone; sample 2 was labeled without $MnCl_2$ and chased with UDP-GlcNAc, UDP-Gal, CMP-Neu5Ac, Ac-CoA, and PAPS. This combination of unlabeled nucleotides is hereafter referred to as ALL for convenience. Sample 3 was labeled in the presence of 1 mM $MnCl_2$ and chased with ALL.

After the labeling and chase reactions, the membranes were collected by centrifugation and solubilized with SDS. Radiolabeled macromolecules were isolated by gel permeation chromatography from the void volume of an Sephacryl S-200 column. An example of the elution profile (sample 2) is shown in Fig. 3*A*. Samples 1 and 3 gave similar profiles (data not shown). The radiolabeled macromolecules were pooled and the *N*-linked oligosaccharides were released with PNGase F, an enzyme that releases almost all known *N*-linked oligosaccharides from glycoproteins (36). The released oligosaccharides were separated from macromolecules by rechromatography on the same column originally used to isolate the macromolecules. As shown in Fig. 3*C*, the oligosaccharides elute in the included volume, separated from PNGase F-resistant material. In a control without PNGase F, the radioactivity quantitatively eluted in the void volume, indicating that the released oligosaccharides were free of radiolabeled contaminants (see Fig. 3*B*). The percent of the macromolecule-associated radioactivity released from samples 1, 2, and 3 was 76, 73, and 83%, respectively (see Table II). Retreatment

TABLE II

PNGase F release and QAE-Sephadex fraction of N-linked oligosaccharides

Freshly isolated Golgi-enriched preparations were incubated with UDP-[^{3}H]GlcNAc and the *N*-linked oligosaccharides were prepared as described under "Experimental Procedures." The percent of label in anionic oligosaccharides was determined by QAE-Sephadex. ALL refers to a mixture of UDP-GlcNAc, UDP-Gal, CMP-Neu5Ac, Ac-CoA, and PAPS (10 μM each final concentration).

Sample	Labeling conditions	Chase conditions	Release by PNGase F	Anionic
			%	%
1	UDP – [^{3}H]GlcNAc – $MnCl_2$	UDP-GlcNAc	76	15
2	UDP – [^{3}H]GlcNAc – $MnCl_2$	ALL	73	24
3	UDP-[^{3}H]GlcNAc + 1 mM $MnCl_2$	ALL	83	26

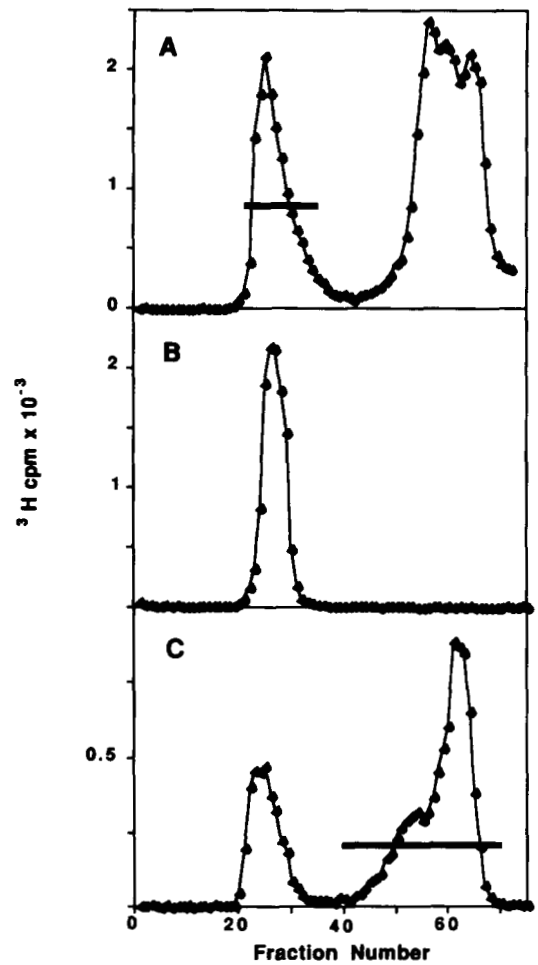

FIG. 3. **Isolation of PNGase F-released [^{3}H]GlcNAc labeled *N*-linked oligosaccharides.** This was done by gel permeation chromatography on Sephacryl S-200 column equilibrated and run in SDS, as described under "Experimental Procedures." *Panel A* shows the initial isolation of the macromolecular material from the void volume of the column. The fractions indicated by the *solid bar* were pooled, acetone precipitated, and redissolved. Aliquots were incubated with and without PNGase F and re-chromatographed on the same column. *Panel B* shows the control incubation and *panel C* the enzyme incubation. The released oligosaccharides were pooled as indicated by the *solid bar* in *panel C*. These *N*-linked oligosaccharides are the subject of study in the rest of this paper.

of the macromolecules released small amounts of additional radioactivity bringing the total PNGase F-releasable material to 86, 88, and 85% for samples 1, 2, and 3, respectively. This indicates that the majority of the acceptors are *N*-linked oligosaccharides.

QAE-Sephadex Fractionation of the Released Oligosaccharides—Oligosaccharides were fractionated into neutral and anionic species by anion exchange chromatography on QAE-Sephadex (see Table II for summary). The anionic substituents most commonly found on *N*-linked oligosaccharides are sialic acids, sulfate, and phosphate. If the labeling reaction was chased with UDP-GlcNAc alone, 15% of the label was associated with anionic oligosaccharides. However, if CMP-Neu5Ac and PAPS were also present during the chase, the proportion of anionic oligosaccharides increased to 23–26%. This indicates that a portion of the labeled oligosaccharides are further modified during the chase. Since factors known to be essential for vesicular transport (18) are not added during the incubation, the anionic modifications must be occurring in the same Golgi compartments where the initial addition of [^{3}H]GlcNAc residues occurred. While addition of 1 mM $MnCl_2$ stimulated overall incorporation of [^{3}H]GlcNAc into macromolecules by ~2-fold, it had little or no effect on either the percent released by PNGase F, or on the proportion in anionic oligosaccharides.

Exoglycosidase Digestion of the Neutral Oligosaccharides—The β-hexosaminidase from Jack bean releases all terminal β-GlcNAc residues from *N*-linked oligosaccharides, irrespective of their linkage (37). This treatment releases ~95% of the label from each sample. The addition of Jack bean β-galactosidase did not increase the percent release. Digestion with endo-β-galactosidase which cleaves polylactosamines released <1% of the label as a di- or trisaccharides. These results indicate that virtually all of the [^{3}H]GlcNAc in the neutral chains is β-linked and terminal (not covered by other monosaccharides) and is not associated with polylactosamine units.

Sialylation of the Neutral [^{3}H]GlcNAc-labeled Oligosaccharides in Vitro Detects β-Linked Gal Residues—Sialyltransferases of defined specificity were used to determine if there were β-Gal residues on other (unlabeled) antennae of these oligosaccharides. After incubation with CMP-Neu5Ac and different sialyltransferases, sialylation was determined by QAE-Sephadex. As shown in Table III, <3% of the neutral oligosaccharides became anionic after α2,6-sialyltransferase treatment, indicating a general absence of β1,4-linked Gal. In contrast, α2,3-sialyltransferase treatment converted 16–26% of the neutral oligosaccharides into anionic species. The α2,6-sialyltransferase is specific for Galβ1,4GlcNAc acceptors, whereas the α2,3-sialyltransferase can sialylate both Galβ1,4GlcNAc and Galβ1,3GlcNAc (38). Therefore, the appearance of QAE-binding only after α2,3-sialyltransferase treatment indicates the presence of Galβ1,3GlcNAc residues on a significant fraction of the neutral oligosaccharides. These sialylated oligosaccharides were analyzed by DEAE HPLC to determine the number of negative charges acquired during the sialylation reaction. Of the neutral oligosaccharides from sample 3, 72% were unretained by the column (corroborates with the data in Table III), whereas the remainder eluted in peaks consistent with monosialylated (20%), disialylated (7%), or trisialylated (1%) species (data not shown). Thus, while the majority of the galactosylated oligosaccharides probably had only a single Galβ1,3 residue, about one in four had 2 or more Galβ1,3 residues. While β1,3-linked Gal residues have been described on several bovine and rat serum proteins (39–44), they are far less common than Galβ1,4 residues. These data suggest that Galβ1,3 residues may be more common in the rat liver than previously thought. They also

TABLE III

In vitro enzymatic sialylation of the neutral oligosaccharides

The neutral oligosaccharides were incubated either with α2,6-sialyltransferase (2.4 milliunits) and 1.8 mM CMP-Neu5Ac in 25 μl of 80 mM MOPS, pH 6.8, at 37 °C for 2 h, or with α2,3-sialyltransferase (15.4 milliunits) and 1 mM CMP-Neu5Ac in 30 μl of 50 mM sodium cacodylate, 0.75 mg/ml BSA, 0.5% (w/v) Triton CF-54, pH 5.8, at 37 °C for 3 h. The reactions were diluted to 2 ml with 2 mM Tris base, pH ~ 9.5, and analyzed by QAE-Sephadex as described under "Experimental Procedures." The positive control for both enzymes was [^{3}H]Galβ1,4GlcNAc.

Sample	%bound by QAE-Sephadex	
	α2,6-Sialyltransferase	α2,3-Sialyltransferase
1	<3	20
2	<3	16
3	<3	26
[^{3}H]Galβ1,4GlcNAc	95	100

highlight the ability of this approach to detect unexpected or novel structures.

Size Analysis of the Neutral Oligosaccharides—The mixture of neutral oligosaccharides was analyzed by AX-5 HPLC as described under "Experimental Procedures." This column separates neutral oligosaccharides on the basis of size, with smaller oligosaccharides eluting first (33). As shown in Fig. 4, each sample gave several poorly resolved peaks that were smaller than $Man_9GlcNAc_2$itol indicating that none had extended polylactosamine chains. The elution profiles from the three samples were qualitatively similar, although the relative size of some peaks varied.

Lectin Affinity Chromatography of the Neutral Oligosaccharides—Preparative fractionation on ConA-Sepharose was used to facilitate subsequent analyses. The specificity of ConA under the conditions employed is well known (Ref. 32, and references therein). Triantennary, tetra-antennary, and bisected oligosaccharides do not bind (ConA-I), biantennary chains with 2 Gal residues, or with 1 Gal residue on the α1,6 arm elute with 10 mM α-methylglucopyranoside (ConA-II), and hybrid oligosaccharides or biantennary chains with or without a single Gal on the α1,3 arm elute with 100 mM α-methylmannopyranoside (ConA-III). As shown in Table IV, the neutral oligosaccharides from samples 1 and 2 gave virtually identical fractionation on ConA, indicating that the addition of UDP-Gal and CMP-Neu5Ac during the chase had little, if any, effect on the branching patterns of the oligosaccharides. The molecules from sample 3, which was labeled in 1 mM $MnCl_2$ and chased with ALL, showed an increase in ConA-I. This indicates that $MnCl_2$ may stimulate an increase in the number of antennae on the oligosaccharides. The most likely explanation is that GlcNAc transferase III or IV adds a GlcNAc residue to more of the biantennary oligosaccharides (GlcNAc transferase V does not require Mn^{2+} *in vitro*).

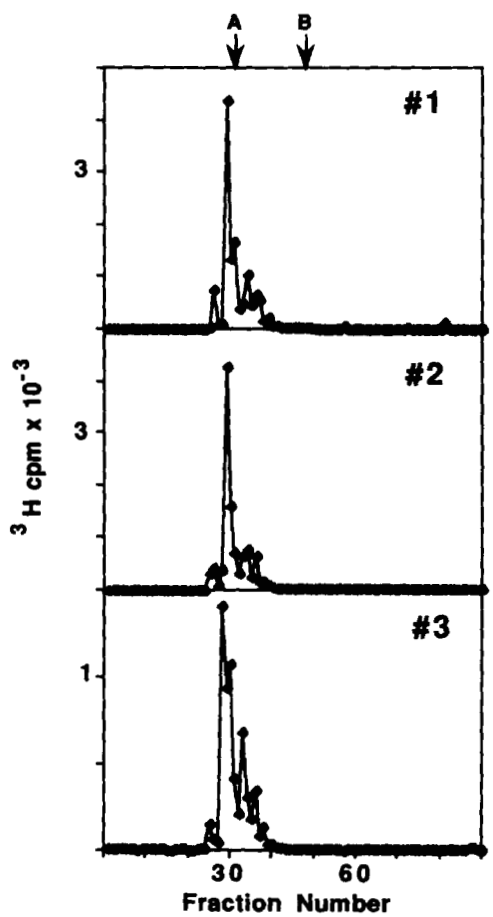

FIG. 4. **HPLC size analysis of total neutral [^{3}H]GlcNAc-labeled *N*-linked oligosaccharides.** The neutral oligosaccharides were analyzed by HPLC on a MicroPak AX-5 column as described under "Experimental Procedures." Fractions (1 min) were monitored for radioactivity. The standards *A* and *B* are $Man_5GlcNAc_2$itol and $Man_9GlcNAc_2$itol, respectively.

TABLE IV

Fractionation of radiolabeled oligosaccharides on ConA-Sepharose

The radiolabeled *N*-linked oligosaccharides were fractionated by ConA-Sepharose as described under "Experimental Procedures." The percent of radiolabel that was either unretained or eluted from the column is shown.

Sample	%total radioactivity in the sample		
	Unretained (ConA-I)	10 mM α-Me-Glu (ConA-II)	100 mM α-Me-Man (ConA-III)
1 Neutral	56	16	28
Anionic	63	14	23
2 Neutral	52	14	33
Anionic	72	12	16
3 Neutral	65	8	26
Anionic	72	15	13

Bisected oligosaccharides produced by GlcNAc transferase III are very uncommon in the rat liver because of a low amount of this enzyme in this organ (45, 46). However, it is possible that during the 10–20 min *in vitro* incubations used here such minor structures might be amplified. This possibility was addressed by lectin affinity chromatography with E_4-PHA-agarose, which binds bisected galactosylated oligosaccharides. The neutral [^{3}H]GlcNAc-labeled oligosaccharides were treated with β1,4-galactosyltransferase and the [^{3}H]GlcNAc became completely resistant to β-hexosaminidase indicating that the galactosylation had gone to completion (data not shown). The galactosylated oligosaccharides that passed through ConA-Sepharose were then analyzed on an E_4-PHA-agarose column. The elution profile was similar to that of a tetra-galactosylated tetra-antennary standard, and little, if any, radioactivity co-migrated with an authentic bisected oligosaccharide standard (data not shown). Based on the profiles obtained, it was calculated that <2% of the neutral oligosaccharides were bisected.

Structural Analysis of the ConA-fractionated Neutral Oligosaccharides—Each ConA fraction from samples 1–3 was desalted and the effects of exoglycosidase digestion were monitored by AX-5 HPLC. As an example, ConA-III oligosaccharides from sample 3 are shown in Fig. 5. The position of the single major peak is not affected by α-mannosidase treatment. Similar results were obtained from samples 1 and 2 (data not shown). The known specificity of ConA indicates that the most likely structure of this oligosaccharide is $GlcNAc_2Man_3GlcNAc_2$. This structural assignment was corroborated by partial Jack bean β-hexosaminidase digestion to generate $GlcNAc_1Man_3GlcNAc_2$, which in turn was sensitive to α-mannosidase digestion, generating a small new peak (see Fig. 5). The incomplete digestion by α-mannosidase seen in the figure is not surprising, since this enzyme is well known to have difficulty in removing the last accessible Man residue from hybrid structures (47).

The presumed $GlcNAc_2Man_3GlcNAc_2$ oligosaccharide was further characterized by treatment with β1,4-galactosyltransferase, α2,6-sialyltransferase, and their respective sugar nucleotide donors. The oligosaccharides that became anionic (>90%) were purified on QAE-Sephadex, desalted, and analyzed by DEAE HPLC. As shown in Fig. 6, 18% of the label

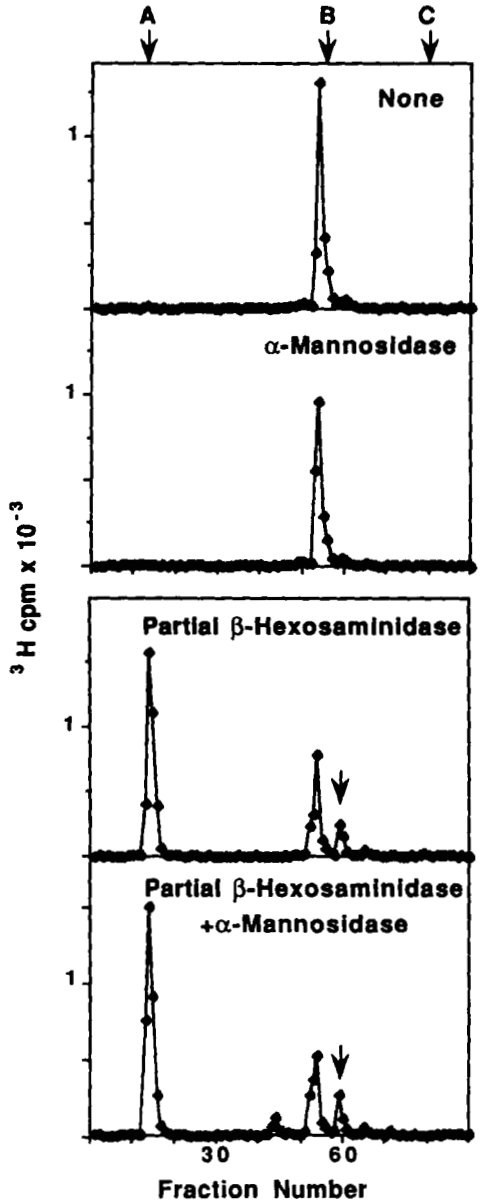

FIG. 5. **HPLC size analysis of neutral ConA-III oligosaccharides.** The oligosaccharides were analyzed without treatment, following α-mannosidase digestion, following *partial* β-hexosaminidase digestion, or following *partial* β-hexosaminidase digestion and complete α-mannosidase digestion. Jack bean α-mannosidase (0.22 unit) was used overnight at 37 °C in 75 μl of 50 mM sodium cacodylate, 0.02% (w/v) NaN_3. Partial digestion with Jack bean β-hexosaminidase was carried out with the incubation conditions described in Table III, and aliquots were removed at various times to determine when about half of the [^{3}H]GlcNAc was released. Such partially digested oligosaccharides were desalted with mixed anion and cation exchange resins, and treated with and without Jack bean α-mannosidase as described above. All samples were boiled for 5 min, diluted with 70% (v/v) acetonitrile in water, and analyzed by AX-5 HPLC. Fractions (0.5 min) were monitored for radioactivity. The standards indicated for the *upper two panels* are *A*, *B*, and *C* are GlcNAc, $Man_5GlcNAc_2$itol, and $Man_9GlcNAc_2$itol, respectively. The runs on the *lower two panels* were done on a different day, when column performance had changed slightly. The *arrow* indicates the position of elution of the untreated material on that day.

co-eluted with a monosialylated biantennary oligosaccharide standard, 79% with disialylated species, and none were more highly charged. Since the original oligosaccharide gave a single peak, this result presumably represents incomplete galactosylation and/or sialylation. The number of antennae can be

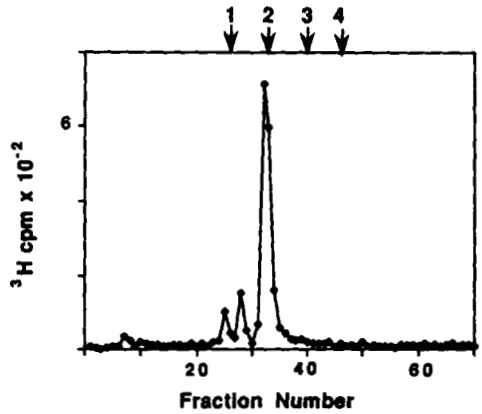

FIG. 6. **DEAE HPLC analysis of neutral ConA-III oligosaccharides after treatment with galactosyltransferase and sialyltransferase.** The oligosaccharides were incubated with β1,4-galactosyltransferase (10 milliunits), 3.2 mM UDP-Gal, α2,6-sialyltransferase (9.5 milliunits), and 4 mM CMP-Neu5Ac in 25 mM sodium cacodylate, pH 5.8, for 2.5 h at 37 °C. The resulting anionic oligosaccharides were purified with QAE-Sephadex, desalted on Sephadex G-10, and analyzed by DEAE HPLC as described under "Experimental Procedures." The standards *1–4* are Neu5Acα2,6[^{3}H]Galβ1,4GlcNAc, and disialylated biantennary, trisialylated triantennary, and tetrasialylated tetra-antennary oligosaccharides, respectively.

inferred from the *maximum* number of negative charges acquired with sialyltransferase. Negative charge was not attained without added β1,4-galactosyltransferase, confirming that the oligosaccharide did not already have β1,4Gal residues that could be sialylated. These results, together with data presented above, confirm that the oligosaccharide in ConA-III is the following.

[^{3}H]GlcNAcβ1,2Manα,1,6
Manβ1,4GlcNAcβ1,4GlcNAc
[^{3}H]GlcNAcβ1,2Manα1,3

STRUCTURE 1

The oligosaccharides in ConA-II were similarly analyzed. As shown in Fig. 7, the AX-5 HPLC elution profiles from samples 1–3 are very similar. Peak *b* comigrates with the major oligosaccharide of ConA-III, and is likely to be $GlcNAc_2Man_3GlcNAc_2$ that eluted from the ConA column prematurely. Peak *a* migrates with the $GlcNAc_1Man_3GlcNAc_2$ derived from the limited β-hexosaminidase digestion of $GlcNAc_2Man_3GlcNAc_2$. The structures of the purified peaks *a* and *b* were confirmed by the acquisition of appropriate numbers of negative charges upon treatment with β1,4-galactosyltransferase and α2,6-sialyltransferase (data not shown). The minor peak *c* from samples 1 and 2 is relatively broad, and that from 3 is a doublet. In every case, it was resistant to α-mannosidase digestion (data not shown), indicating that it is probably not $GlcNAcMan_4GlcNAc_2$, and is likely to represent partially galactosylated biantennary species. Since this peak represents only a minor fraction of the total label (1–2%), it was not further characterized.

When the neutral ConA-I species were analyzed by AX-5 HPLC, all three samples gave 4 peaks, labeled *d-g* (see Fig. 8). The distribution of radioactivity among the peaks was virtually identical in samples 1 and 2, whereas there was an increase in proportion of peak *d* in sample 3 (see Table V). There was no change in the AX-5 elution profiles following treatment with α-mannosidase (data not shown). Based on the known specificity of ConA, these peaks should therefore represent tri- and tetra-antennary oligosaccharides. The in-

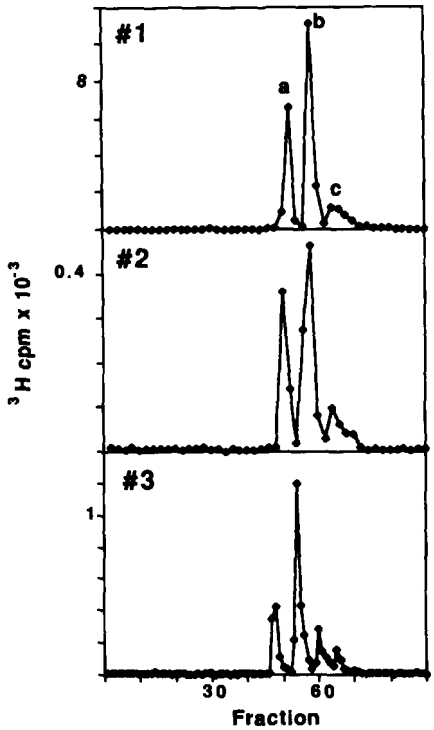

FIG. 7. **HPLC size analysis of neutral ConA-II oligosaccharides.** The oligosaccharides were analyzed by HPLC on an AX-5 column as described under "Experimental Procedures." Fractions (0.5 min) were monitored for radioactivity. Note that the peaks from sample 3 eluted somewhat earlier because of deterioration of column performance (determined with authentic standards, data not shown).

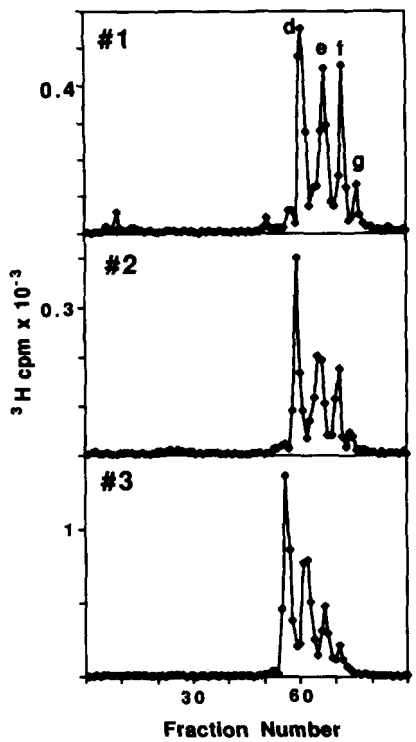

FIG. 8. **HPLC size analysis of neutral ConA-I oligosaccharides.** The oligosaccharides unretained by ConA-Sepharose were analyzed by HPLC on an AX-5 column as described under "Experimental Procedures." Fractions (0.5 min) were monitored for radioactivity.

TABLE V

Distribution of the radioactivity in HPLC peaks in neutral ConA-I oligosaccharides

The neutral oligosaccharides unretained by ConA-Sepharose (ConA-I) were fractionated by HPLC on an AX-5 column (see Fig. 8). The radioactivity in peaks *d-g* was determined by summing the radioactivity in the individual fractions associated with these peaks.

Sample	%oligosaccharides in ConA-I			
	Peak[d]	Peak[e]	Peak[f]	Peak[g]
1	38	37	19	6
2	37	38	20	5
3	45	33	16	6

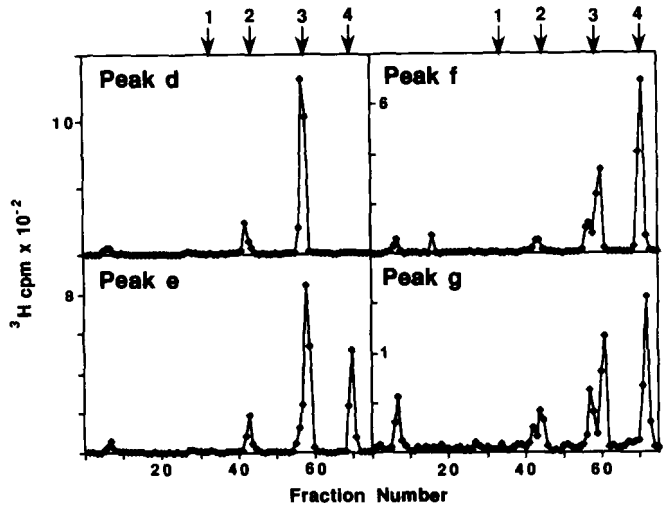

FIG. 9. **DEAE HPLC analysis of oligosaccharides galactosylated and sialylated *in vitro*.** The purified peaks *d-g* (Fig. 8, Table V) were treated with 50 milliunits of β1,4-galactosyltransferase in 40 μl of 25 mM sodium cacodylate, pH 5.8, 20 mM $MnCl_2$, 4 mM ATP, 1.25 mM UDP-Gal, 0.02% (w/v) NaN_3 for 20 h at 37 °C. The oligosaccharides were desalted by anion and cation exchange chromatography and concentrated by lyophilization. The galactosylated oligosaccharides in 8 μl of water were mixed with 12 μl of 12.5 mM sodium cacodylate, 1.25% (w/v) Triton CF-54, 1.8 mg/ml BSA, pH 6.0, 4 μl of 10 mM CMP-Neu5Ac, 0.5 μl of α2,6-sialyltransferase (2.4 milliunits), and 14 μl of α2,3-sialyltransferase (19.6 milliunits). The mixture (39 μl final) was incubated at 37 °C for 15 h, boiled, diluted with 500 μl of water, and analyzed by DEAE HPLC as described in the legend to Fig. 6. The standards *1–4* are Neu5Acα2,6[^{3}H]Galβ1,4GlcNAc, and disialylated biantennary, trisialylated triantennary, and tetrasialylated tetra-antennary oligosaccharides, respectively.

dividual peaks *d-g* were purified by HPLC and treated with β1,4-galactosyltransferase and a mixture of α2,3- and α2,6-sialyltransferase. As stated earlier, incomplete sialylation is not unexpected, and thus the species with the highest number of negative charges indicates the number of antennae. As shown in Fig. 9, peak *d* is converted almost completely into a trisialylated species. Similar treatments of peaks *e, f,* and *g* gave mixtures of di-, tri-, and tetrasialylated species indicating that each of these peaks is a tetra-antennary oligosaccharide. The extent of difference in their elution positions indicates that they likely vary from each other by additional monosaccharide residues.

As described above, 16–26% of the total neutral oligosaccharides already had β1,3-linked Gal residues (Table III). Based on the analyses above, most or all of the β1,3-linked Gal residues must occur in the ConA-I fraction. This possibility was addressed in two ways. First, the mixture of ConA-I oligosaccharides was treated with the α2,3-sialyltransferase. As shown in Fig. 10, this resulted in the formation of mono-,

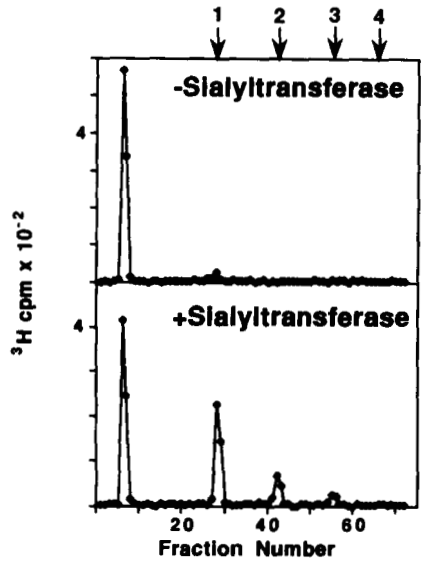

FIG. 10. **DEAE HPLC analysis of ConA-I oligosaccharides with and without prior sialylation.** The neutral ConA-I oligosaccharides from sample 3 were incubated with and without 11 μl of α2,3-sialyltransferase (15 milliunits) in 12.5 μl of 12.5 mM sodium cacodylate, 1.25% (w/v) Triton CF-54, 1.8 mg/ml BSA, pH 6.0, and 3 μl of CMP-Neu5Ac in a final volume of 19 μl at 37 °C for 19 h. The mixtures were boiled, diluted with 500 μl of water, and analyzed by DEAE HPLC as described in the legend to Fig. 6. The standards *1–4* are Neu5Acα2,6[^{3}H]Galβ1,4GlcNAc, and disialylated biantennary, trisialylated triantennary, and tetrasialylated tetra-antennary oligosaccharides, respectively.

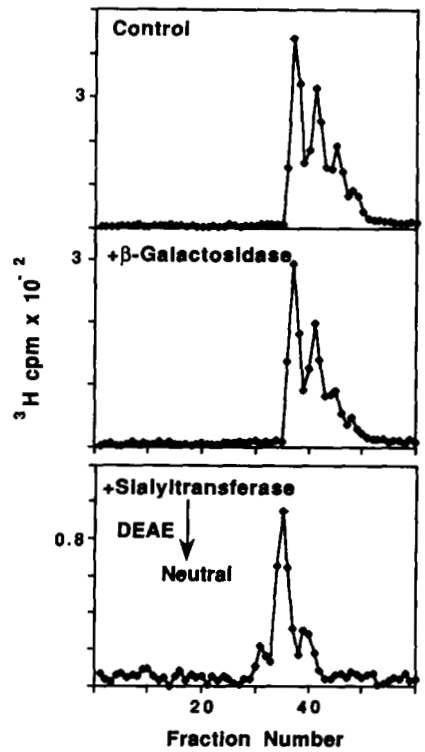

FIG. 11. **HPLC size analysis of the neutral ConA-I oligosaccharides after β-galactosidase or α2,3-sialyltransferase treatment.** Neutral ConA-I oligosaccharides from sample 3 were incubated with and without 3.5 milliunits of bovine testicular β-galactosidase in 10 μl of 50 mM sodium cacodylate, 0.02% (w/v) NaN_3 overnight at 37 °C. Reactions were boiled for 5 min, diluted to 500 μl with 70% (v/v) acetonitrile, and analyzed on an AX-5 column as described under "Experimental Procedures." Another aliquot of the same oligosaccharide was treated with α2,3-sialyltransferase as described in the legend to Fig. 10. The radioactivity that remained neutral after this treatment (passed through the DEAE column) was analyzed on the AX-5 column. Fractions (0.75 min) were monitored for radioactivity. The run in the *lower panel* of this figure was done on a different day, accounting for the slightly earlier elution of the first major peak. Also note that the *upper panel* is similar to the lower panel of Fig. 8, except that column performance had deteriorated somewhat, giving poorer resolution of peaks, and that the fraction sizes were larger.

di- and trisialylated species, which represented 45% of this fraction (*i.e.* 29% of the total neutral oligosaccharides). This is sufficient to account for all the [^{3}H]GlcNAc-labeled neutral oligosaccharides that were sialylated in the total mixture as described in Table III. The distribution of radioactivity in the original peaks *d-g* (45, 33, 16, and 6% of ConA-I, see Table V) also correlates with the charge distribution following α2,3-sialyltransferase treatment (55% neutral, 31% monosialylated, 10% disialylated, and 4% trisialylated). To determine which oligosaccharides remained neutral after α2,3-sialyltransferase treatment, these were re-analyzed by AX-5 HPLC. As shown in Fig. 11, peak *d* was apparently not sialylated, while the majority of peak *e* and most of peaks *f* and *g* were removed by the DEAE column. These data indicate that peak *d*, *e*, *f*, and *g* are probably $GlcNAc_3Man_3GlcNAc_2$, $Gal_1GlcNAc_4Man_3GlcNAc_2$, $Gal_2GlcNAc_4Man_3GlcNAc_2$, and $Gal_3GlcNAc_4Man_3GlcNAc_2$, respectively. These structural assignments further support the notion that the increase in ConA-I with added $MnCl_2$ results from the addition of more GlcNAc residues to biantennary oligosaccharides (see Tables IV and V).

To confirm the presence of the β-Gal residues, the mixture of ConA-I oligosaccharides was treated with the broad-spectrum bovine testicular β-galactosidase, which removes all β1–3 and β1–4 Gal residues under the conditions used. Surprisingly, there was no change in the elution profile following β-galactosidase treatment for any of the samples (see Fig. 11, for an example), indicating that either there are no Gal residues, or that the Gal residues were resistant to the β-galactosidase. However, the ability to sialylate these oligosaccharides was reproducible, and was dependent on addition of both the cloned sialyltransferase and the donor CMP-Neu5Ac (data not shown). This indicates that the negative charge is indeed due to sialylation, and that these oligosaccharides must have β-Gal residues. The Gal residues were therefore apparently resistant to the β-galactosidase (the same batch of enzyme quantitatively removed label from [^{3}H]Gal-labeled oligosaccharides). This indicates that these oligosaccharides are further modified in a way that blocks the action of the β-galactosidase, but still leaves them available for sialylation. Similar difficulties in exoglycosidase digestion of *N*-linked oligosaccharides have been previously reported in $P388D_1$ cells (48), and in CPAE cells (49). Because of the closeness of the peaks *d-g* on the HPLC size profiles, this modification must also be relatively small and is unlikely to be a fucose residue. The nature of this modification is the subject of ongoing investigations.

The Gal residues that were substrates for the α2,3-sialyltransferase could not be sialylated by the α2,6-sialyltransferase, indicating that they are likely to be β1,3-linked. In support of this, the accompanying article (50) shows that much of the [^{3}H]Gal transferred from UDP-[^{3}H]Gal to *N*-linked oligosaccharides in the same system is linked β1,3. Of course, it is possible that some of the galactosidase-resistant residues are β1,4-linked and that the unknown modification blocks the α2,6-sialyltransferase, but not the α2,3-sialyltransferase. If so, this modification could affect the natural sialylation pattern of oligosaccharides. It is noteworthy that most,

if not all, of the neutral [^{3}H]GlcNAc-labeled oligosaccharides that have β1,3-linked Gal residues are tetra-antennary. Since >95% of the [^{3}H]GlcNAc from neutral oligosaccharides was released by β-hexosaminidase, the [^{3}H]GlcNAc residues themselves could not been galactosylated. The simplest explanation is that the [^{3}H]GlcNAc on these molecules was added to already galactosylated triantennary oligosaccharides. The *in vitro* specificity of GlcNAc transferases also predicts that this [^{3}H]GlcNAc must be transferred by either GlcNAc transferase IV or V (2, 16). Although the enzymology predicts that neither of these transferases efficiently adds GlcNAc residues to digalactosylated oligosaccharides, di- and trigalactoslyated species (peaks *f* and *g*) are observed. Thus, either the rat liver enzymes have somewhat different specifities than the same enzymes from hen oviduct (2, 16), or additional factors in the intact Golgi modulate the specifities of the enzymes. Alternatively, the oligosaccharides may become galactosylated following the initial [^{3}H]GlcNAc labeling reaction. This is somewhat unlikely because all of the [^{3}H]GlcNAc remains β-hexosaminidase sensitive, and peaks *e-g* occur even in the absence of added $MnCl_2$ and UDP-Gal. In any event, it appears that there is a functional co-compartmentalization of β1,3-galactosyltransferase and one or more GlcNAc transferases, most likely GlcNAc transferase IV or V.

Effects of Swainsonine on the Oligosaccharides Synthesized—In contrast to the ConA-I oligosaccharides there was little, if any, β-Gal present on the molecules that bound to ConA. This indicates that there may be little co-compartmentalization of GlcNAc transferase I and II with β-galactosyltransferase(s). However, as described in an accompanying article (50), a significant fraction of both the neutral and anionic UDP-[^{3}H]Gal-labeled oligosaccharides bound to ConA and the majority of this [^{3}H]Gal was linked β1,3. To further investigate this issue, a Golgi-enriched fraction was preincubated with swainsonine (10 μg/ml) and then labeled with UDP-[^{3}H]GlcNAc in the presence of 1 mM $MnCl_2$ as described under "Experimental Procedures." The reactions were chased with UDP-GlcNAc, UDP-Gal, and CMP-Neu5Ac (20 μM each), and the labeled *N*-linked oligosaccharides were obtained exactly as described above. Swainsonine is a membrane-permeant inhibitor of α-mannosidase II (51, 52). If it causes a decrease in the percent of label in ConA-I, this would mean that α-mannosidase II, GlcNAc transferase II, and GlcNAc transferase IV and/or V are at least partially co-compartmentalized. Alternatively, if no change in the ConA-Sepharose profile occurs, this would indicate that these activities are likely to be in separate compartments. In fact, no difference was seen in PNGase F release, percentage of anionic oligosaccharides, or in the ConA-Sepharose profiles. As shown in Fig. 12, there was also little difference in the elution profiles of ConA-I neutral oligosaccharides on AX-5 HPLC. Even the distribution of radioactivity in peaks *d-g* was very similar to that observed earlier. In contrast, the structures of the ConA-bound oligosaccharides are quite different in the presence and absence of added swainsonine. The single α-mannosidase-resistant chain previously shown to be $GlcNAc_2Man_3GlcNAc_2$ is partially replaced by a larger α-mannosidase-sensitive peak (see Fig. 12). Based on the known action of swainsonine and the above data, this new peak very likely represents $GlcNAc_1Man_5GlcNAc_2$ (the lack of complete removal of Man is probably because of the relative α-mannosidase-resistance of the third Man residue). These results indicate that the [^{3}H]GlcNAc transfer to biantennary oligosaccharides in the absence of swainsonine was mediated by both GlcNAc transferase I and GlcNAc transferase II. While it is not possible to determine if both of these transferase

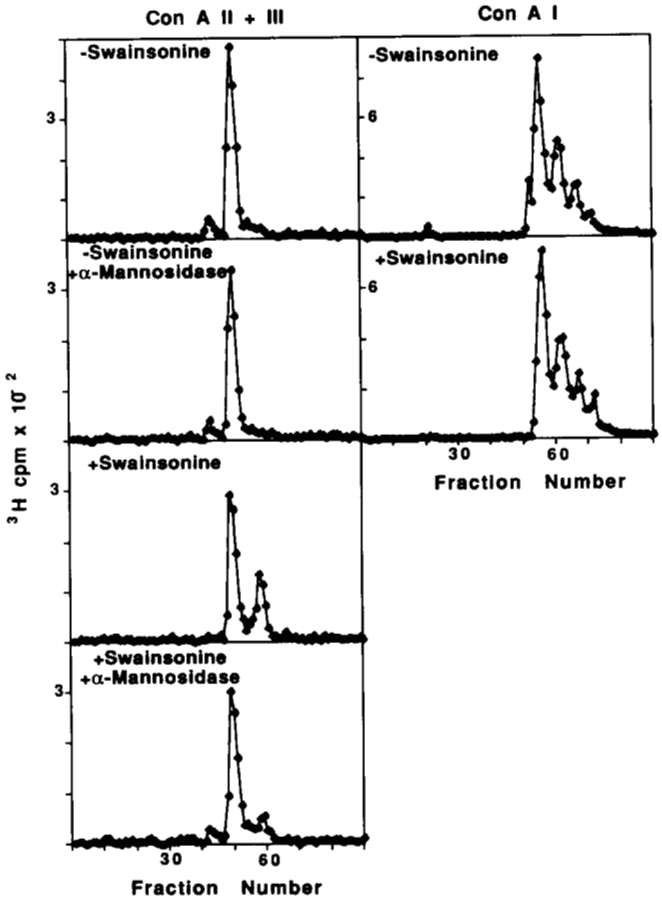

FIG. 12. **HPLC size analysis of the neutral ConA-fractionated oligosaccharides synthesized in the presence or absence of swainsonine.** The oligosaccharides were labeled, released, purified, and fractionated with QAE-Sephadex and ConA-Sepharose as described under "Experimental Procedures," except that ConA-II and -III were pooled together. Oligosaccharides were analyzed by HPLC on the AX-5 column as described under "Experimental Procedures." Fractions (0.5 min) were monitored for radioactivity. The neutral oligosaccharides bound by ConA were pretreated either with or without Jack bean α-mannosidase (V-Labs; 11.5 milliunits) overnight at 37 °C in 80 μl of 50 mM sodium cacodylate, 1.6 mM $ZnCl_2$, 0.02% NaN_3, pH 5.8.

have added [^{3}H]GlcNAc to the very same oligosaccharide, the results indicate that GlcNAc transferase II and α-mannosidase II must be functionally co-localized to a single Golgi compartment. On the other hand, the lack of change in the ConA-Sepharose profiles with swainsonine indicate that the GlcNAc transferase II/α-mannosidase II compartment is likely to be separate from the GlcNAc transferase IV and/or GlcNAc transferase V compartment.

Analysis of the Anionic [^{3}H]GlcNAc-labeled Oligosaccharides—Given prior dogma about the localization of the GlcNAc transferases (53), it was surprising to find any anionic oligosaccharides labeled by UDP-[^{3}H]GlcNAc. The most common negatively charged substituents on *N*-linked oligosaccharides are sialic acids, sulfate, and phosphate. When CMP-Neu5Ac and PAPS were added during the chase reaction, more of the labeled oligosaccharides become anionic (see Table II), indicating that at least a portion of the negative charge on these oligosaccharides is acquired during the chase. To determine the nature of the anionic substitutents, these oligosaccharides were treated with sialidase, alkaline phosphatase, or mild acid. The latter should also release GlcNAc linked through a phosphodiester bond to mannose (48), the

precursor of the Man-6-PO_4 lysosomal enzyme recognition signal. As shown in Table VI, sialidase neutralizes 56, 63, and 75% from samples 1, 2, and 3, respectively. Thus, additional sialic acid is added during the chase period when CMP-Neu5Ac is provided. Mild acid treatment released 37, 24, and 17% for samples 1, 2, and 3, respectively, as free [^{3}H]GlcNAc, which was presumably linked through a phosphodiester bond. There were no oligosaccharides with phosphomonoesters as the only negatively charged substituent. Approximately 10% of the anionic oligosaccharide was resistant to all these treatments, indicating that either there was more than one anionic substituent on these oligosaccharides, or that the oligosaccharides were sulfated. In any event, these resistant anionic oligosaccharides represent only ~2% of the total labeled *N*-linked oligosaccharides and were not analyzed further.

The anionic oligosaccharides were preparatively fractionated on ConA-Sepharose (see Table IV). There were minimal differences in the elution profile among the three samples. The individual ConA fractions were analyzed by DEAE HPLC to determine their charge distribution (see Table VII). As shown in Fig. 13, those in ConA-I had 1–3 charges and were quantitively neutralized by sialidase. These are presumably tri- and tetra-antennary partially sialylated molecules. Those in ConA-II contained 1–2 negative charges, and sialidase treatment neutralized about half of them, indicating some sialylated biantennary chains. In contrast, those in ConA-III have one or two negative charges, and a major fraction is not neutralized by sialidase treatment. These are likely to be high mannose-type oligosaccharides with one and two phosphodiesters, respectively (48). Most, if not all, of the sialidase-resistant anionic oligosaccharides binding to ConA can be accounted for by the mild acid labile (phosphodiester linked) [^{3}H]GlcNAc (data not shown). The exact nature of the small fraction of mild acid and sialidase-resistant species was not pursued.

Exoglycosidase Digestion of the Anionic Oligosaccharides—To determine if the anionic oligosaccharides carried [^{3}H]GlcNAc directly covered by other residues, the individual ConA fractions were digested with combinations of β-hexosaminidase, β-galactosidase, and sialidase and the percent [^{3}H]GlcNAc released determined by paper chromatography. As shown in Table VIII, very little of the [^{3}H]GlcNAc on ConA-III oligosaccharides was released by a combination of all enzymes. This is not surprising, since this fraction contains the majority of the phosphodiester-linked [^{3}H]GlcNAc. From ConA-II fractions, β-hexosaminidase releases ~40–60% of the [^{3}H]GlcNAc, presumably from phosphorylated hybrids or sialylated biantennary oligosaccharides. If β-galactosidase and sialidase were also added to the incubations, an additional 10–20% was released, indicating that at least a portion of the [^{3}H]GlcNAc was covered by Gal and possibly by sialic acid. Based on the known specificity of ConA, it can be assumed that such oligosaccharides are biantennary in nature. Thus, at least a portion of the GlcNAc transferase I and/or II in the Golgi apparatus is functionally colocalized with one or more galactosyltransferase(s) and sialyltransferase(s). In support of this, some of the ConA-II oligosaccharides themselves were neutralized by sialidase treatment (see Fig. 13 and Table VII). In contrast, it was shown above that little, if any, of the neutral ConA-II oligosaccharides had terminal Gal residues. The implication is that once β-Gal is added to such oligosaccharides, it is rapidly sialylated. Data presented in the accompanying article (50) with UDP-[^{3}H]Gal labeling support this view.

Digestion of ConA-I anionic oligosaccharides with β-hexosaminidase alone released 65, 45, and 39% of the label from samples 1, 2, and 3, respectively. Thus, the major fraction of the [^{3}H]GlcNAc on the tri- and tetra-antennary chains was not covered by other monosacccharides, and was probably added by either GlcNAc transferase IV and V to oligosaccharides that were already sialylated or became sialylated soon after the addition of the labeled GlcNAc. The commonest group of these should have the composite structure shown below (with variable numbers of Gal and Sia residues).

Siaα2(3)6Galβ1,(3)4GlcNAcβ1,2Manα1,6
Manβ1,4GlcNAcβ1,4GlcNAc
Siaα2(3)6Galβ1,(3)4GlcNAcβ1,2Manα1,3
4
[^{3}H]GlcNAcβ1

STRUCTURE 2

The addition of [^{3}H]GlcNAc to such di- and trisialylated oligosaccharides further indicates that the substrate specific-

TABLE VI

Analysis of the negative charge on the anionic [^{3}H]GlcNAc-labeled oligosaccharides

The anionic [^{3}H]GlcNAc-labeled oligosaccharides were either treated with AUS (10 milliunits) overnight at 37 °C in 100 μl of 10 mM sodium acetate 0.02% (w/v) NaN_3, pH 5.5, or with alkaline phosphatase (9 milliunits) for 2 h at 37 °C in 17 μl of 100 mM Tris-HCl, pH 9.1. The reactions were diluted to 2 ml with 2 mM Tris base, pH ~ 9.5, and fractionated into neutral and anionic species as described under "Experimental Procedures." The oligosaccharides were also treated with 1 ml of 10 mM HCl at 100 °C for 45 min. These mixtures were lyophilized and analyzed by paper chromatography to determine the release of [^{3}H]GlcNAc.

Sample	Neutralized by		[^{3}H]GlcNAc released by mild acid
	Sialidase	Alkaline phosphatase	
	%		%
1	56	1	37
2	63	0	24
3	75	0	17

TABLE VII

Charge distribution of the ConA-fractionated anionic oligosaccharides

The anionic oligosaccharides were fractionated on ConA-Sepharose and analyzed by DEAE-HPLC with and without prior sialidase treatment as described in the legend to Fig 13. The radioactivity associated with each charged species was determined and expressed as a percent of the total.

Sample	Sialidase	Net negative charge				Sialidase resistant
		0	−1	−2	−3	
		%				%
ConA-I						
1	−	7	56	22	15	
	+	97	1	2	0	3
2	−	5	50	32	14	
	+	98	1	1	0	2
3	−	5	49	35	12	
	+	100	0	0	0	0
ConA-II						
1	−	0	74	26		
	+	15	61	24		85
2	−	3	59	38		
	+	27	53	21		73
3	−	3	53	44		
	+	51	39	10		49
ConA-III						
1	−	6	50	44		
	+	13	42	45		87
2	−	0	58	42		
	+	22	38	40		78
3	−	7	61	32		
	+	42	33	26		58

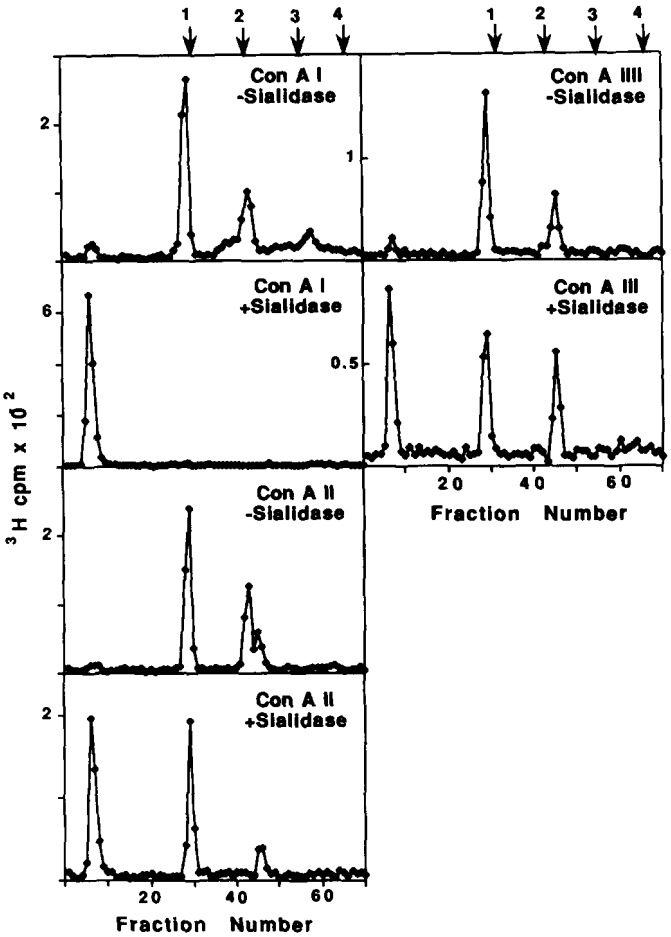

FIG. 13. **DEAE HPLC of the ConA-fractionated anionic oligosaccharides.** The anionic oligosaccharides from sample 3 were fractionated on ConA, desalted, and treated either with or without AUS (20 milliunits) in 100 mM sodium acetate, 0.02% (w/v) NaN_3, pH 5.5, at 37 °C for 9 h. The samples were heated to 90 °C for 5 min, diluted with 500 μl of water, and analyzed by DEAE HPLC as described in the legend to Fig. 6. Fractions (1 min) were monitored for radioactivity. The standards *1–4* are Neu5Acα2,6[^{3}H]Galβ1,4GlcNAc, and disialylated biantennary, trisialylated triantennary, and tetrasialylated tetra-antennary oligosaccharides, respectively.

TABLE VIII

Exoglycosidase digestion of ConA-fractionated anionic oligosaccharides

The ConA-fractionated anionic oligosaccharides were treated overnight at 37 °C with jack bean β-hexosaminidase (V-Labs; 150 milliunits), bovine testicular β-galactosidase (1.75 milliunits) and *Arthrobacter ureafaciens* sialidase (4 milliunits) in 50 mM sodium cacodylate, 0.02% (w/v) NaN_3, pH 5.8. α-Mannosidase was inadvertently added to each sample, including the minus enzyme controls but this by itself did not cause any release of [^{3}H]GlcNAc. Following treatment, each reaction was directly spotted and chromatographed on Whatman No. 1 paper as described under "Experimental Procedures" to determine release of [^{3}H]GlcNAc.

Fraction	Sample	β-Hexosaminidase	β-Galactosidase	Sialidase	Release of [^{3}H]GlcNAc
					%
ConA-I	1	−	−	−	0
		+	−	−	65
		+	+	−	68
		+	+	+	92
	2	−	−	−	0
		+	−	−	45
		+	+	−	43
		+	+	+	78
	3	−	−	−	0
		+	−	−	39
		+	+	−	40
		+	+	+	77
ConA-II	1	−	−	−	0
		+	−	−	42
		+	+	+	65
	2	−	−	−	0
		+	−	−	58
		+	+	+	67
	3	−	−	−	0
		+	−	−	38
		+	+	+	56
ConA-III	1	−	−	−	0
		+	+	+	13
	2	−	−	−	0
		+	+	+	12
	3	−	−	−	0
		+	+	+	22

ity of GlcNAc transferase IV and/or V from rat liver is either different from that in hen oviduct, or is modulated by unknown factors present in the intact Golgi apparatus. Of course, it is possible that the rules established for the action of the GlcNAc transferases are not strictly applicable when many of the Gal residues are linked β1,3 rather than β1,4. Alternatively, the Gal and Sia residues may have been added exclusively during the chase period.

There was no increase in [^{3}H]GlcNAc release from these anionic oligosaccharides if the broad spectrum β-galactosidase from bovine testicle was included in the digestion with β-hexosaminidase. However, if sialidase was also added, the release was increased to 92, 78, and 77% from samples 1, 2, and 3, respectively. These data indicate that some of the [^{3}H]GlcNAc is directly covered not only by β-Gal residues, but also by sialic acid residues, *i.e.* in the sequence Siaα2(3)6Galβ1,(3)4[^{3}H]GlcNAcβ1(2,4,6)Man. This further demonstrates a functional co-localization of one or more GlcNAc transferases with galactosyltransferase(s) and sialyltransferase(s). It is noteworthy that the [^{3}H]GlcNAc associated with tri- and tetra-antennary oligosaccharides was still not quantitatively released by β-hexosaminidase despite the fact that sialic acid is the only anionic substituent present. The lack of complete release may be due to the unknown modification noted above on the neutral oligosaccharides.

DISCUSSION

We have presented here a novel approach to studying the biosynthetic function and organization of the Golgi apparatus. The approach originates from previously well known studies of the uptake and concentration of sugar nucleotides by intact Golgi compartments (12, 13, 54, 55), and others in which radioactive sugar nucleotides were added to microsomal preparations, with analysis of labeled products (17, 56, 57). In the former studies, acid-insoluble products were noted, but not analyzed. In the latter, the topological orientation and intactness of the system were not investigated. We found that radiolabeled monosaccharide from the sugar nucleotide is very efficiently transferred to endogenous acceptors in the rat liver Golgi apparatus, and that the major products labeled are *N*-linked oligosaccharides which can be specifically released, purified, and analyzed. Transfer requires intactness of the compartments, and the radiolabeled glycoprotein products are completely resistant to exogenously added protease. Thus, it is reasonable to assume that the reactions are taking place within the lumen of intact compartments that are very similar to those of the Golgi apparatus in the intact cell. Since

vesicular transport cannot occur during the incubations, the structures of the labeled oligosaccharides produced should represent the biosynthetic capability of the individual compartments present in the Golgi apparatus-enriched fraction. Thus, this *in vitro* approach provides a "freeze frame" view of the normally rapid and dynamic process of *N*-linked oligosaccharide biosynthesis. Of course, while the oligosaccharides produced by the isolated Golgi preparations are likely to reflect structures produced *in vivo*, the relative quantities of the individual species may not exactly correspond to those produced in the intact cell, wherein the transit time through the Golgi apparatus may also affect the final structures produced.

It is also noteworthy that this system defines itself, *i.e.* only intact compartments of the correct topological orientation can take up, concentrate, and transfer label from sugar nucleotides. Thus, extreme purity of the Golgi-enriched subcellular fraction is not required (and is actually not desired, because of the risk of discarding important subcomponents). Indeed, mixtures of compartments can be studied simultaneously. A further advantage is that the rate-limiting factor is the K_m of the transporters for the sugar nucleotide donors (in the 2–20 μM range), rather than the K_m values of the transferases for the donors (100–500 μM). Thus, labeling reactions can be saturated with reasonable amounts of commercially available sugar nucleotides, which can be used directly, without diluting the specific activity. Most of the biosynthetic intermediates predicted by the accepted model of *N*-linked oligosaccharide biosynthesis were found by this approach. Structures that are usually minor components in rat liver-derived proteins, *e.g.* bisected chains were not detected, indicating that amplification of minor pathways is probably not occurring during the 10–20-min incubations. On the other hand, some unexpected and potentially novel structures were encountered.

Label from UDP-[^{3}H]GlcNAc was transferred to several distinct proteins (data shown in the accompanying article (58), see also Ref. 17). Although the nature of these endogenous acceptors is not clear, they are likely to be plasma membrane proteins, lysosomal proteins, resident Golgi apparatus proteins, or secreted proteins. We have chosen to study the oligosaccharides on the entire mixture of labeled proteins rather than on a single protein. This is because a single protein may not provide a complete picture of all possible reactions occurring in the compartments of the Golgi apparatus, while the mixture of proteins should represent the full spectrum of biosynthetic reactions. In the long run, identification of the individual endogenously labeled glycoproteins is of interest as well.

When intact cells are metabolically labeled with [^{3}H]$GlcNH_2$, substantial epimerization to [^{3}H]GalNAc and conversion to other sugars can be expected (59, 60). However, in this *in vitro* system, epimerization was practically undetectable. While the [^{3}H]GlcNAc associated with neutral oligosaccharides was essentially all β-linked and terminal, we also found evidence for Gal residues on antennae other than those that were labeled. Surprisingly, these residues appeared to be mainly β1,3-linked and are found almost exclusively on tetraantennary oligosaccharides. Evidence is also presented for an unknown modification of *N*-linked oligosaccharides that blocks the action of β-galactosidase on these residues. Based on the evidence, it can be predicted that the modification must be small, and may involve either the β-Gal or the β-GlcNAc residue. Based on prior studies indicating acid lability of similar modifications (48, 49), one possibility to consider is a plasmal (cyclic acetal) substituent, such as recently described on the glycolipid, galactosylceramide (61).

Contrary to the current dogma about the localization of GlcNAc transferases in relation to galactosyltransferases and sialyltransferases (53), a significant fraction of the [^{3}H]GlcNAc label was associated with galactosylated and/or sialylated oligosaccharides (see Table IX). The majority of the negative charge was due to sialic acid residues. The percent of sialylated oligosaccharides increased if UDP-Gal and CMP-Neu5Ac were added during the quench reaction. This, together with arguments made above, indicates that one or more GlcNAc transferases functionally co-localizes in the Golgi apparatus with one or more of the galactosyltransferase(s) and sialyltransferase(s). Evidence is presented here for the compartmental overlap of GlcNAc transferase I and or II with galactosyltransferase(s) and sialyltransferase(s). Specific arguments are also made that the GlcNAc transferase IV and V must overlap more with galactosyltransferase(s) and sialyltransferase(s) than do the earlier GlcNAc transferases. These observations go against the popularly held view that the addition of GlcNAc, Gal, and sialic acid each occur in completely separate compartments of the Golgi apparatus (17, 53, 62–67). They support the minority view that, while different glycosyltransferases are more concentrated in specific regions, they may be broadly distributed across several compartments of the Golgi apparatus (68–72). Other evidence in the literature supports this minority view. When Chinese hamster ovary cells are transfected with α2,6-sialyltransferase, the enzyme itself can be easily detected throughout the Golgi apparatus (73). When CPAE endothelial cells were metabolically labeled in the presence of brefeldin A, there was only a partial decrease in the sialylation of *N*-linked oligosaccharides, indicating that at least a portion of the sialyltransferase(s) are located in a compartment of the Golgi apparatus proximal to the *trans*-Golgi network (49). A resident Golgi apparatus protein in rat brain neurons (MG160) that has been localized to the *medial* Golgi apparatus is apparently sialylated (74). Another resident Golgi apparatus protein localized to the *cis-medial* Golgi elements of normal rat kidney cells ($GIMP_c$) is also reported to be sialylated (75). However, it is possible that these proteins are not sialylated in the compartments in which they finally reside, but that they are sialylated in a later compartment and recycled back to the earlier compartments. Likewise, it is possible that in this study, some of the endogenous acceptors labeled with [^{3}H]GlcNAc had

TABLE IX

Summary: presence of other substituents on [^{3}H]GlcNAc-labeled N-linked oligosaccharides

The percent of [^{3}H]GlcNAc associated with sialylated oligosaccharides was determined by multiplying the percent of anionic oligosaccharides completely neutralized with sialidase, by the % of anionic oligosaccharides. This represents a minimum estimate because some oligosaccharides may have had sialic acids and some other negatively charged substituent, and would have been omitted from these calculations. The percent of galactosylated oligosaccharides was determined by multiplying the percent of neutral oligosaccharides that became anionic following treatment with α2,3-sialyltransferase by the percent of total neutral oligosaccharides, and adding the percent of sialylated oligosaccharides. Again, this is a minimum estimate. The percent associated with phosphorylated oligosaccharides was derived by multiplying the percent of [^{3}H]GlcNAc released from anionic oligosaccharides by mild acid treatment by the percent of total anionic oligosaccharides.

Sample	Total [^{3}H]GlcNAc-labeled *N*-linked oligosaccharides		
	Galactosylated	Sialylated	Phosphorylated
		%	
1	20	10	6
2	35	19	6
3	35	22	4

recycled back from later compartments (76).

The argument could be raised that the compartmental overlap of GlcNAc transferases with galactosyltransferase(s) and sialyltransferase(s) described in this report is an artifact that results from nonspecific mechanical fusion of Golgi compartments during homogenization and purification. Several lines of evidence are against this possibility. If such fusion were truly nonspecific, one might also expect fusion of Golgi elements with other subcellular compartments. Hepatocytes comprise ~70% of the cells in the liver but ~93% of the total volume of the liver (77). In the rat hepatocyte, the Golgi apparatus contains ~6% of the total membrane while the ER contains ~63% of the total membrane (~10 times that of the Golgi apparatus). Thus, if nonspecific mixing had occurred in the crude homogenate, a significant portion of the Golgi compartments should have mixed with the ER. However, as seen in Table I when 28% of the Golgi marker was recovered, it was contaminated with only 0.04% of the ER. Thus, the extent of mixing could not have been greater than 1.4% (0.4/28 × 100). It could be argued that the nonspecific mixing is restricted to adjacent stacks of the Golgi that happen to be directly apposed to one another at the exact instant of homogenization. However, if the addition of GlcNAc, Gal, and sialic acid occurred in separate but adjacent compartments in the intact cell, then this type of localized nonspecific mixing should equally affect each of the adjacent compartments. In fact, a wide range of compartmental overlap was observed in this study and in the accompanying paper (50). Thus, only ~35% of the [^{3}H]GlcNAc was associated with galactosylated oligosaccharides and ~20% with sialylated oligosaccharides if UDP-Gal and CMP-Neu5Ac was added during the chase reaction. However, as described in an accompanying article (50), ~90% of β1,4-linked [^{3}H]Gal from UDP-[^{3}H]Gal was directly covered by sialic acid if CMP-Neu5Ac was added during a chase. In striking contrast, only 2% or less of the [^{3}H]Neu5Ac added to *N*-linked oligosaccharides from CMP-[^{3}H]Neu5Ac was *O*-acetylated even if adequate Ac-CoA was added during the chase reaction. The latter occurs despite the fact that 30–40% of the sialic acid residues on *N*-linked oligosaccharides in rat liver are *O*-acetylated (11). Also, direct covering of labeled residues with unlabeled monosaccharides such as galactose and sialic acid occurs even without exogenous addition of the appropriate donor nucleotide. This indicates that some endogenous pools of sugar nucleotide are preserved within the isolated compartments. If nonspecific breakage of compartments was occurring extensively during the homogenization, one might have expected such internal pools to be depleted by dilution with the large volume of the total homogenate. Finally, it has recently been demonstrated that purified Golgi membranes do not fuse with each other during *in vitro* incubations (78). For all of these reasons, nonspecific fusion is not likely to be a major problem during the preparation of the Golgi-enriched preparation.

In the final analysis, while this approach has some potential limitations, it serves to complement many other approaches that are taken to understanding the structure, organization, and function of the Golgi apparatus, particularly with regard to its role in oligosaccharide biosynthesis. In addition, it can generate clues regarding the existence of novel and unexpected structures. In the accompanying articles (50, 58), this approach is extended to other radiolabeled nucleotide donors.

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