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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]GALACTOSE, CMP-[9-^{3}H]*N*-ACETYLNEURAMINIC ACID, AND [*acetyl*-^{3}H]ACETYL-COENZYME A*

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When a rat liver Golgi apparatus-enriched subcellular fraction is incubated with UDP-[^{3}H]Gal, CMP-[^{3}H]Neu5Ac, or [*acetyl*-^{3}H]acetyl (Ac)-CoA, label is efficiently transferred to endogenous acceptors, which are resistant to added proteases, unless detergent is added at a sufficiently high concentration. Thus, the acceptors are within the lumen of intact compartments of correct topological orientation, which are likely to be similar to those of the Golgi apparatus in the intact cell. In each case, ~90% of the macromolecular radioactivity is specifically released by peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase digestion, as labeled *N*-linked oligosaccharides. Label from UDP-[^{3}H]Gal is transferred to several distinct *N*-linked oligosaccharides, and many of these carry sialic acid (Sia) residues. This amount increases if the transfer reaction is chased with CMP-Neu5Ac. A major fraction of the [^{3}H]Gal is directly "covered" with Sia residues, indicating that at least a portion of the β-galactosyltransferase(s) are co-localized with one or more sialyltransferases. The majority of the [^{3}H]Gal is found in a β1,3-linkage, rather than the more common β1,4-linkage. The *N*-linked oligosaccharides labeled by CMP-[^{3}H]Neu5Ac carry labeled Sia residues in either α2,3 or α2,6 linkage, and showed a range of charge distribution. The transferred [^{3}H]Neu5Ac is not *O*-acetylated even when Ac-CoA is added at saturating concentrations, implying that the sialyltransferases and the *O*-acetyltransferase(s) are not functionally co-localized. However, ~20% of label released from *N*-linked oligosaccharides by sialidase does not co-migrate with authentic Neu5Ac in high performance liquid chromatography analysis, indicating that transferred [^{3}H]Neu5Ac is modified by unknown enzymes in the Golgi. Most of the [^{3}H]acetate transferred from [*acetyl*-^{3}H]Ac-CoA to *N*-linked oligosaccharides is on Sia residues that are exclusively α2,6-linked, and is enriched on tri- and tetra-antennary chains that do not appear to carry any 2,3-linked Sia residues. These data indicate a restricted substrate preference of the *O*-acetyltransferase(s). About one-quarter of the [^{3}H]acetate transferred is sialidase-resistant, indicating either transfer to monosaccharides other than sialic acid, or to sialidase-resistant sialic acids. While most of these sialidase-resistant oligosaccharides remain negatively charged, about 10% are neutralized by sialidase, confirming transfer of [^{3}H]acetate to monosaccharides other than sialic acid.

Monosaccharide residues at the nonreducing termini of *N*-linked oligosaccharides can be functionally important for the glycoproteins to which they are attached. For example, desialylation of circulating glycoproteins decreases their half-life because the newly exposed β1,4-linked Gal residues bind to the asialoglycoprotein receptor on hepatocytes (1–5). Upon binding, such proteins are endocytosed, delivered to the lysosome, and degraded. Thus, terminal β1,4-linked Gal is believed to play a role in glycoprotein clearance. Terminal sialic acids are involved in cell adhesion mediated by the selectins, which recognize specific sialylated ligands (reviewed in Refs. 6–8). Likewise, many microorganisms bind to specific terminal sialic acid residues on cell surfaces (9, 10).

Covalent modifications can modulate the biological activity of terminal monosaccharides. Influenza A and B virus binding is blocked if sialic acids are *O*-acetylated (11), while influenza C binding requires 9-*O*-acetylated sialic acids (12). Sialic acid *O*-acetylation has also been implicated in the development of neuroectodermal tissues. A gradient of 9-*O*-acetylated G_{D3}[1] ganglioside is found in the developing mouse retina (13), in the face of uniform expression of unmodified G_{D3}. Furthermore, transgenic mice expressing a viral 9-*O*-acetyl sialic acid esterase in retina and adrenal gland show abnormal development in both of these organs (14). Several pituitary hormones have biantennary *N*-linked oligosaccharides that terminate with 4-sulfated GalNAc residues instead of sialic acid. These GalNAc-SO_4 residues can accelerate clearance of these hor-

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[1] The abbreviations used are: G_{D3}, Siaα2,8Siaα2,3Galβ1,4Glcβ1,1′Cer; Sia, general abbreviation for sialic acids; Neu5Ac, *N*-acetylneuraminic acid; $Neu5,9Ac_2$, 9-*O*-acetyl-*N*-acetylneuraminic acid; $Neu5,7Ac_2$, 7-*O*-acetyl-*N*-acetylneuraminic acid; DFP, diisopropyl fluorophosphate; ConA, concanavalin A; ConA-I, oligosaccharides passing through ConA-Sepharose; ConA-II, oligosaccharides eluted from ConA-Sepharose by α-methylglucopyranoside; ConA-III, oligosaccharides eluted from ConA-Sepharose by 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; BSA, bovine serum albumin; PAPS, adenosine 3′-phosphate,5′-phosphosulfate; MES, 4-morpholinepropanesulfonic acid; HPLC high performance liquid chromatography; NDVS, Newcastle disease virus sialidase; Ac, acetyl; PHA, phytohemagglutinin.

mones via a specific receptor on endothelial and Kuppfer cells of the liver, and hence modulate their bioactivity (3, 15, 16).

In the preceding article (17), we describe an approach to studying the "biosynthetic capability" of the Golgi apparatus. When Golgi-enriched fractions are incubated with UDP-[^{3}H] GlcNAc, label is transferred to endogenous acceptors in intact compartments of the correct topological orientation. The majority of the label occurs in *N*-linked oligosaccharides, which include most of the intermediates predicted by the established model of *N*-linked oligosaccharide biosynthesis. The present study takes a similar approach to studying terminal glycosylation of *N*-linked oligosaccharides. While many expected structures were found, some unexpected observations were also made regarding specific oligosaccharides sequences and concerning the co-localization of biosynthetic activites.

EXPERIMENTAL PROCEDURES

Most of the "Experimental Procedures" are as described in the preceeding article (17), with the following additions and changes.

Materials—Bio-Gel P-4 was from Bio-Rad. UDP-[6-^{3}H]galactose (10 Ci/mmol) was synthesized as previously described (18). CMP-[9-^{3}H]Neu5Ac (10 Ci/mmol) and [*acetyl*-^{3}H]Ac-CoA (2.28 Ci/mmol) was synthesized as previously described (19, 20). β-Hexosaminidase from *Diplococcus pneumoniae* (0.03 unit/200 μl in 50 mM sodium cacodylate, pH 5.8), and α-galactosidase from coffee bean (20 units/ml) were from Oxford Glycosystems. Newcastle disease virus sialidase (2.5 units/ml) was purified as previously described (9). Endo-β-galactosidase from *Escherchia freundii* (1 unit/ml) was generously provided by Dr. Michiko Fukuda, La Jolla Cancer Research Foundation. Trifluoroacetic acid (sequencing grade) was from Pierce Chemical Co. All other reagents were obtained from commercial sources and were of the highest quality available.

Incorporation of [^{3}H]Gal into Endogenous Macromolecules—A subcellular fraction enriched in the Golgi apparatus was obtained as previously described (17). To 21 ml of the freshly prepared chilled Golgi fraction, 1.2 mCi of UDP-[^{3}H]galactose was added (5.5 μM final). The mixture was immediately split into three equal aliquots that were labeled samples 4, 5, and 6, respectively (see Table I). The sample numbering was chosen to follow on those of the similarly prepared samples labeled with UDP-[^{3}H]GlcNAc described in the accompanying article (17). $MnCl_2$ (1 mM final) was added to samples 5 and 6, the three aliquots were warmed to room temperature, and the incubation was continued for 18 min. The reaction was chased by addition of nonradioactive sugar nucleotides. Sample 5 was chased with UDP-Gal at a final concentration of 15 μM, whereas samples 4 and 6 were chased with 20 μM UDP-GlcNAc, 15 μM UDP-Gal, 20 μM CMP-Neu5Ac, 20 μM Ac-CoA, and 20 μM PAPS (this mixture is referred to as ALL). These final concentrations were chosen to be greater than or equal to the K_m values of the sugar nucleotide transporters, which have apparent K_m values of 2–20 μM (21, 22). The incubation was allowed to proceed for an additional 12 min. The samples were centrifuged at 100,000 × *g* for 30 min, the supernatant removed, and the pellet resuspended in 2 ml of 2% (w/v) SDS containing 40 mM 2-mercaptoethanol. Sonication and heating to 90 °C for 10 min was needed to fully solubilize the pellet.

Isolation of [^{3}H]Gal-labeled N-Linked Oligosaccharides—The [^{3}H] Gal-labeled *N*-linked oligosaccharides were released, purified, and fractionated on QAE-Sephadex as previously described (17) except that ~12.5 milliunits of PNGase F was used, and the final volume of the PNGase F digest was 3 ml.

Incorporation of [^{3}H]Neu5Ac and [acetyl-^{3}H]Ac-CoA into Endogenous Macromolecules—A subcellular fraction enriched in the Golgi apparatus was obtained as previously described (17). To 15 ml of the Golgi fraction, 15 μl of 1 M diisopropyl fluorophosphate (DFP, an irreversible inhibitor of serine active site esterases, dissolved in isopropyl alcohol) and 15 μl of 1 M $MnCl_2$ was added. The mixture was incubated on ice for 15 min, and then divided into 4 equal aliquots of 3.2 ml that were called samples 7, 8, 9, and 10 (see Table I). Samples 7 and 8 were incubated with 15 μCi of CMP-[^{3}H]Neu5Ac (0.47 μM final), whereas samples 9 and 10 were incubated with 15 μCi of [*acetyl*-^{3}H]Ac-CoA (2.1 μM final). The mixtures were incubated at room temperature for 12 min, and the reactions were chased by the addition of nonradioactive nucleotides. Sample 7 was chased with 20 μM CMP-Neu5Ac; sample 8 with 20 μM UDP-GlcNAc, 20 μM UDP-Gal, 20 μM CMP-Neu5Ac, and 20 μM Ac-CoA. Likewise, sample 9 was chased with 20 μM Ac-CoA and sample 10 with 20 μM UDP-GlcNAc, 20 μM UDP-Gal, 20 μM CMP-Neu5Ac, and 20 μM Ac-CoA. The incubation was allowed to proceed for an additional 12 min. The samples were centrifuged at 100,000 × *g* for 30 min, the supernatants removed, and the pellets disolved in 0.5 ml of 20 mM MES, 1% (w/v) SDS, pH 6.2, with heating at 50 °C for 15–30 min.

Isolation of [^{3}H]Neu5Ac- and [^{3}H]Acetate-labeled N-Linked Oligosaccharides—The [^{3}H]Neu5Ac and [^{3}H]acetate-labeled *N*-linked oligosaccharides were released, purified, and fractionated on QAE-Sephadex as previously described (17) except that ~2.5 milliunits of PNGase F was used, the final volume of the PNGase F digest was 0.5 ml, and the Sephacryl S-200HR column was equilibrated in MES, pH 6.2, instead of HEPES, pH 7.6, to minimize loss of *O*-acetyl esters.

Protection of Radiolabeled Endogenous Acceptors from Proteases—A Golgi-enriched fraction was isolated as described above. A 1.4-ml aliquot was incubated with ~13 μCi of UDP-[^{3}H]Gal at room temperature for 12 min. The reaction was chased by adding UDP-Gal to a final concentration of 0.5 mM and the incubation continued for an additional 12 min. The mixture was split into 6 aliquots of 0.2 ml. To two aliquots 25 μl of 1% (v/v) Triton X-100 in 50 mM maleate, 5 mM $MgCl_2$, 0.25 M sucrose, pH 6.5, was added; two others received 25 μl of 0.1% (v/v) Triton X-100 in the same buffer, and the last two aliquots received buffer alone. One of each pair was treated with 25 μl of 20 mg/ml Proteinase K (2 mg/ml final), the other was incubated with 1 mg/ml BSA (final). Duplicate aliquots of 30 μl were removed from each of these samples following 0, 0.5, and 16 h of incubation at room temperature. The acid-precipitable macromolecules were measured by adding 8% (v/v) perchloric acid (final) as previously described (23).

Similar experiments were performed to determine if the CMP-[^{3}H] Neu5Ac and [*acetyl*-^{3}H]Ac-CoA-labeled endogenous acceptors were protected from Proteinase K. The conditions were exactly the same except that the initial labeling was chased with 0.24 mM CMP-Neu5Ac or 1.5 mM Ac-CoA. Also, the volumes of the aliquots were approximately half the size of those used for the [^{3}H]Gal-labeled endogenous acceptors, although the final concentrations were the same.

Exoglycosidase Digestions—All exoglycosidase digestions were performed in either sodium citrate, pH 4.6, sodium cacodylate, pH 5.8, or 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 described in the legends.

Radiochemical Composition of [^{3}H]Gal-labeled Oligosaccharides—Desalted oligosaccharides (20 μl in water) were placed in a pre-cleaned reactivial and 1 ml of 2 M trifluoroacetic acid was added. The tubes were flushed with N_2, capped, and placed in a vacuum oven preheated to 121 °C. After 1 h the vials were cooled and the sample diluted to 10 ml with water and lyophilized. The samples were further dried in a vacuum dessicator containing NaOH and phosphorus pentoxide. The dried samples were resuspended in 100 μl of water, nonradioactive Gal and Glc were added as internal standards, and analyzed by Dionex HPLC using a Carbo-Pac PA-1 chromatography column, equilibrated in and eluted with 3 mM NaOH, at 1 ml/min (24). The nonradioactive Gal and glucose were detected with a pulsed amperometric detector and the radiolabeled monosaccharides were detected by liquid scintillation counting. For the latter, fractions were collected every 0.33 min and neutralized with 10 μl of glacial acetic acid before counting.

Preparative Desialylation of Anionic [^{3}H]Gal-labeled Oligosaccharides—Anionic [^{3}H]Gal-labeled oligosaccharides were desialylated with 20 milliunits of AUS in 75 μl of 50 mM sodium citrate, pH 4.6, at 37 °C for 15 h. The reactions were heated to 95 °C for 15 min, 1 ml of 2 mM Tris base added and the samples fractionated into neutral and anionic species on QAE-Sephadex as described (17). The neutral oligosaccharides were then desalted by sequential anion and cation exchange chromatography as described (17).

Lectin Affinity Chromatography—Lectin affinity chromatography on ConA-Sepharose and E_4-PHA-agarose was performed essentially as previously described (17, 25).

Paper Chromatography—The release of [^{3}H]Gal from oligosaccharides by exoglycosidases was quantitated by paper chromatography as described (17).

High Pressure Liquid Chromatography (HPLC)—Neutral oligosaccharides were fractionated/analyzed with a bonded-amine, MicroPak AX-5, HPLC column developed with a gradient of decreasing acetonitrile in water as described (17, 26). Anionic oligosaccharides were fractionated on the basis of charge with 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, at which time a gradient of increasing NaCl concentration was begun. For the analysis of [^{3}H]Gal-labeled oligosaccharides, the gradient was 0–100 mM NaCl over 70 min. For the analysis of [^{3}H]Neu5Ac-labeled oligosaccharides the gradient was 0–150 mM NaCl over 85 min. The flow rate was 0.6 ml/min and fractions were collected every 1 min.

RESULTS

The accompanying articles (17, 27) demonstrate that the structural characterization of radiolabeled oligosaccharides synthesized *in vitro* by Golgi-enriched fractions yields important information about *N*-linked oligosaccharide biosynthesis and provides a new approach for discovering novel oligosaccharide structures. This approach is extended here using UDP-[^{3}H]Gal, CMP-[^{3}H]Neu5Ac, and [*acetyl*-^{3}H]Ac-CoA to study the transfer of the terminal monosaccharides Gal and sialic acid, and the acetylation of oligosaccharides. The resulting labeled oligosaccharides should represent later events in the normal biosynthesis of *N*-linked oligosaccharides, and have a precursor-product relationship with those labeled by UDP-[^{3}H]GlcNAc (17). Additionally, previously undescribed oligosaccharide structures might be found.

Protection of Endogenous Acceptors from Proteases—As described in the accompanying articles (17, 27), the endogenous glycoprotein acceptors of UDP-[^{3}H]GlcNAc and UDP-[^{3}H]GalNAc are protected from exogenously added proteases, demonstrating that the transfer of label occurs only to endogenous acceptors in intact vesicles of the correct topological orientation. Proteins free in solution or those on inside-out vesicles are not labeled to a significant extent. Thus, the glycosylation reactions in these Golgi-enriched fractions should be similar to those that occur in the intact Golgi apparatus *in vivo*. Similar experiments were performed for the [^{3}H]Gal, [^{3}H]Neu5Ac, and [^{3}H]acetate-labeled acceptors. A Golgi-enriched fraction was incubated with either UDP-[^{3}H]Gal, CMP-[^{3}H]Neu5Ac, or [*acetyl*-^{3}H]Ac-CoA and the transfer reactions chased by adding excess nonradioactive UDP-Gal, CMP-Neu5Ac, or Ac-CoA, respectively. The labeled fractions were then treated with combinations of proteases and/or detergents for varying periods of time, and the macromolecular acid-precipitable radioactivity determined, exactly as described in the preceding paper (17). The results obtained with UDP-[^{3}H]Gal and CMP-[^{3}H]Neu5Ac (data not shown) were very similar to those reported for UDP-[^{3}H]GlcNAc (17). Protease treatment did not decrease the amount of acid-precipitable radioactivity derived from these nucleotides, if Triton X-100 was omitted, or added at low concentrations (0.01%; less than the critical micellar concentration). In contrast, if the detergent was added at a final concentration above the critical micellular concentration (0.1%, v/v), there was a marked decrease in acid-precipitable radioactivity, indicating that the radiolabeled endogenous acceptors became susceptible to proteolytic degradation. These results indicate that the endogenous acceptors for UDP-[^{3}H]Gal and CMP-[^{3}H]Neu5Ac are located inside sealed vesicles of correct topological orientation.

A similar experiment was performed with [^{3}H]acetate-labeled macromolecules (see Fig. 1). Interpretation of the results is complicated by endogenous esterases in the Golgi apparatus, which are capable of removing the labeled *O*-acetyl groups from sialic acids (23). To minimize this problem, the Golgi fraction was pretreated with 1 mM DFP, an irreversible inhibitor of many serine esterases, including those that can de-*O*-acetylate sialic acids (28). Consistent with prior reports (23), there was still some time-dependent decrease in the acid-precipitable radioactivity. This indicates that either the DFP

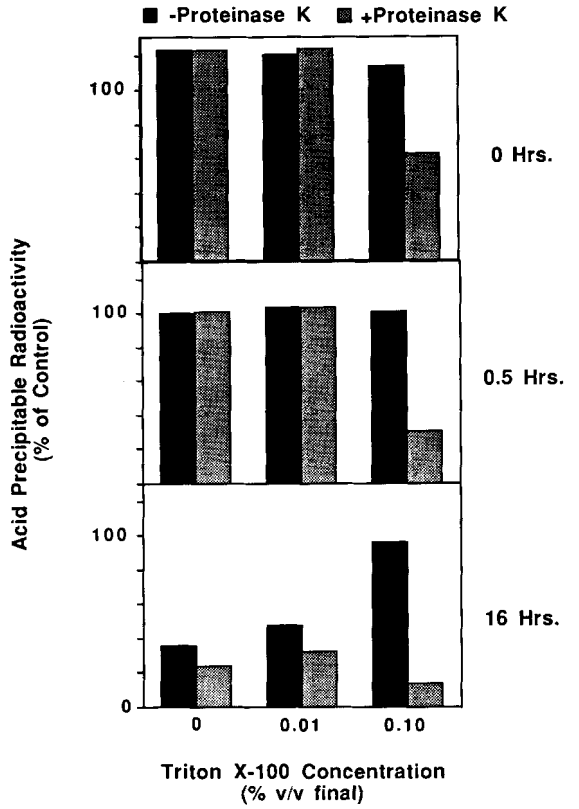

FIG. 1. **Protection of the [^{3}H]acetate-labeled acceptors from proteases.** A Golgi-enriched fraction was incubated with [*acetyl*-^{3}H]Ac-CoA in the presence of 1 mM DFP and 1 mM $MnCl_2$, chased with excess nonradioactive Ac-CoA, incubated with or without 2 mg/ml Proteinase K or Triton X-100, and the acid-precipitable radioactivity determined as described under "Experimental Procedures." The 100% control is defined as the radioactivity precipitated following a half-hour incubation in the absence of added detergent or protease.

concentration or the time of treatment were insufficient to completely inhibit esterases, or that DFP-resistant esterases are present. The esterase activity is especially evident following a 16-h incubation where the addition of 0.1% (v/v) Triton X-100 led to an *increase* in the acid-precipitable radioactivity (see Fig. 1). The simplest explanation of this result is that when membrane compartments are solubilized by the detergent, this markedly dilutes both the esterase and the [^{3}H]acetate-labeled molecules, such that the esterase is less effective. However, regardless of the esterase activity, the data in Fig. 1 show that the [^{3}H]acetate-labeled macromolecules are resistant to proteolytic degradation in the absence of high concentrations of detergent, and are therefore located in sealed compartments of the correct topological orientation.

Isolation of Radiolabeled Endogenous Acceptor Oligosaccharides—A Golgi-enriched fraction was incubated with UDP-[^{3}H]Gal, CMP-[^{3}H]Neu5Ac, or [*acetyl*-^{3}H]Ac-CoA as described under "Experimental Procedures." Based on preliminary experiments, three different conditions were used for labeling with UDP-[^{3}H]Gal and two for labeling with either CMP-[^{3}H]Neu5Ac or [*acetyl*-^{3}H]Ac-CoA. The sample nomenclature, labeling, and chase conditions are summarized in Table I. Continuing the nomenclature developed in the preceeding article (17), the [^{3}H]Gal-labeled samples are called 4, 5, and 6, respectively, the CMP-[^{3}H]Neu5Ac-labeled samples are 7 and 8, and the [*acetyl*-^{3}H]Ac-CoA-labeled samples are 9 and 10.

Sample 4 was labeled with UDP-[^{3}H]Gal without added $MnCl_2$ and chased with UDP-GlcNAc, UDP-Gal, CMP-

TABLE I

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

The labeling and isolation of macromolecules, release of *N*-linked oligosaccharides by PNGase F and QAE-Sephadex fractionation of the released oligosaccharides were performed as described under "Experimental Procedures." ALL refers to UDP-GlcNAc, UDP-Gal, CMP-Neu5Ac, AcCoA, and PAPS. The [^{3}H]Neu5Ac- and [^{3}H]acetate-labeled oligosaccharides were quantitatively retained by a DEAE HPLC column (see text) indicating that the QAE-Sephadex column was not fully efficient in binding anionic oligosaccharides.

Sample	Labeling conditions	Chase	Release by PNGase F	Anionic
			%	%
4	UDP-[^{3}H]Gal − $MnCl_2$	ALL	86	51
5	UDP-[^{3}H]Gal + 1 mM $MnCl_2$	UDP-Gal	85	33
6	UDP-[^{3}H]Gal + 1 mM $MnCl_2$	ALL	84	52
7	CMP-[^{3}H]Neu5Ac + 1 mM $MnCl_2$	CMP-Neu5Ac	89	95
8	CMP-[^{3}H]Neu5Ac + 1 mM $MnCl_2$	ALL, except PAPS	90	95
9	[*acetyl*-^{3}H]Ac-CoA + 1 mM $MnCl_2$	AcCoA	87	91
10	[*acetyl*-^{3}H]Ac-CoA + 1 mM $MnCl_2$	ALL, except PAPS	87	91

Neu5Ac, Ac-CoA, and PAPS. This combination of nonradioactive sugar nucleotides, Ac-CoA, and PAPS is termed ALL for convenience. Sample 5 was labeled with 1 mM $MnCl_2$ and chased with UDP-Gal alone. Sample 6 was labeled with 1 mM $MnCl_2$ and chased with ALL. Since Mn^{2+} stimulates UDP-Gal:GlcNAcGalβ1,4-galactosyltransferase (β1,4-galactosyltransferase), the addition of $MnCl_2$ to the Golgi apparatus fraction caused a dose-dependent increase in [^{3}H]Gal incorporation into macromolecules (not shown). The final concentration used for the experiments reported here (1 mM $MnCl_2$) gave a 2–5-fold increase in incorporation of [^{3}H]Gal into macromolecules. High concentrations of $MnCl_2$ (>20 mM) were avoided because they can cause fusion of elements of the Golgi apparatus (29). In any event, higher concentrations of $MnCl_2$ would have been of limited value, since even 40 mM $MnCl_2$ increased the incorporation of [^{3}H]Gal into macromolecules only ~2-fold above that seen with 1 mM $MnCl_2$. The different chase conditions were also chosen to study the effects of the other sugar nucleotides on oligosaccharide branching, and to see if the [^{3}H]Gal could be "covered" by other monosaccharide residues such as Neu5Ac. Samples 7 and 8 were labeled by CMP-[^{3}H]Neu5Ac with 1 mM $MnCl_2$ and chased with either CMP-Neu5Ac alone or All (less PAPS), respectively. Samples 9 and 10 are analogous to 7 and 8 except that [*acetyl*-^{3}H]Ac-CoA was used instead of CMP-[^{3}H]Neu5Ac and the reactions are chased with Ac-CoA or All (less PAPS), respectively. The addition of 1 mM $MnCl_2$ had no effect on the incorporation of label from [*acetyl*-^{3}H]Ac-CoA or CMP-[^{3}H]Neu5Ac into macromolecules (data not shown). However, it was added to maximize the transfer of Gal and GlcNAc from UDP-Gal and UDP-GlcNAc, respectively, during the chase.

After the reactions, membranes were collected and solubilized in SDS, and the radiolabeled macromolecules isolated from the void volume region of an S-200 gel filtration column. The *N*-linked oligosaccharides were specifically released with PNGase F (30) and purified by gel filtration on the same column, as described in the preceding article (17). In the absence of PNGase F, the radioactivity quantitatively elutes with the column void volume in each case (data not shown). Most of the macromolecule-associated radioactivity was released by PNGase F from each sample (see Table I), indicating that the majority of the acceptors are *N*-linked oligosaccharides. The addition of $MnCl_2$ during labeling had no effect on the percent of radioactivity released by PNGase F.

QAE-Sephadex Fractionation of the Released Oligosaccharides—*N*-Linked oligosaccharides from each sample were fractionated into neutral and anionic species by QAE-Sephadex as described (17). As shown in Table I, the percent of [^{3}H]Gal associated with anionic oligosaccharides increases from 33 to 52% if the initial labeling is chased with CMP-Neu5Ac and PAPS. As described below, 95% of these molecules are neutralized by sialidase, indicating that sialic acid accounts for virtually all of the negative charge on the [^{3}H]Gal-labeled anionic oligosaccharides. Since factors known to be required for vesicular transport are not added to the incubations, these data imply that one or more β-galactosyltransferases are located in the same compartment of the Golgi apparatus as one or more sialyltransferases. This will be discussed in more detail in later sections. The addition of $MnCl_2$ during the labeling reaction had no effect on the percent of [^{3}H]Gal associated with anionic oligosaccharides. Note that the QAE-Sephadex is not completely efficient, since only 95% of the [^{3}H]Neu5Ac-labeled oligosaccharides from samples 7 and 8 bind to it on a single pass (see DEAE HPLC analyses below).

In previous studies, a similarly prepared Golgi-enriched fraction incorporated label from [*acetyl*-^{3}H]Ac-CoA into endogenous acceptors, mostly to the C-7, C-8, or C-9 hydroxyl groups of sialic acids (23). Analysis of samples 9 and 10 on QAE-Sephadex shows that ~90% of the [^{3}H]acetate is associated with anionic oligosaccharides. However, on DEAE HPLC, the radioactivity was quantitatively retained (see below) showing that all of the oligosaccharides are negatively charged.

Radiochemical Composition of the [^{3}H]Gal-labeled Oligosaccharides—UDP-[^{3}H]Gal can be converted into UDP-[^{3}H]Glc by a cytosolic 4′-epimerase which requires NAD^+ (31). However, when neutral and anionic oligosaccharides from samples 4–6 were hydrolyzed and the resulting monosaccharides analyzed as described under "Experimental Procedures," >95% of the radioactivity in monosaccharides co-elutes with nonradioactive Gal, with little, if any, identifiable [^{3}H]Glc (data not shown).

Lectin Affinity Chromatography—Both the neutral and anionic oligosaccharides from samples 4–10 were analyzed by concanavalin A (ConA)-Sepharose. The results are shown in Table II. The specificity of ConA is well known (25, 34). Triantennary, tetra-antennary, and bisected oligosaccharides do not bind (ConA-I), biantennary chains with 2 Gal residues, or 1 Gal residue on the α1,6 arm elute with 10 mM α-methylglucopyranoside (ConA-II), and hybrid oligosaccharides and biantennary chains with a single Gal on the α1,3 arm elute with 100 mM α-methylmannopyranoside (ConA-III).

The [^{3}H]Gal-labeled oligosaccharides from samples 4–6 give nearly identical ConA elution profiles, showing that the addition of $MnCl_2$ does not alter the branching pattern of the oligosaccharides. However, differences in ConA elution profiles are seen between the neutral and anionic oligosaccharides of all three samples: while the percent in ConA-I was the same, a larger percent of the anionic oligosaccharides eluted in ConA-II and a smaller fraction in ConA-III (Table II). The simplest explanation is that a portion of the [^{3}H]Gal is added to biantennary oligosaccharide with a single sialic acid residue

TABLE II

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 by, or eluted from the column is shown. Note that all of the oligosaccharides labeled with CMP-[^{3}H]Neu5Ac and [*acetyl*-^{3}H]Ac-CoA were anionic.

Sample	Percent of total radioactivity		
	ConA-I	ConA-II	ConA-III
		%	
4 Neutral	69	9	22
Anionic	69	19	13
5 Neutral	63	13	24
Anionic	60	27	13
6 Neutral	65	11	25
Anionic	61	26	13
7 Anionic	62	28	10
8 Anionic	62	29	9
9 Anionic	76	21	3
10 Anionic	79	18	3

on the α1,3 branch and an exposed GlcNAc residue on the other branch. The resulting anionic digalactosylated biantennary oligosaccharide would elute in ConA-II. Alternatively, the [^{3}H]Gal may be added to a neutral oligosaccharide and subsequently sialylated (see below). The [^{3}H]Gal-labeled oligosaccharides eluting in ConA-III can have only 1 Gal residue. Since sialic acid is the only anionic species on these molecules, the sialic acid must be linked directly to the radiolabeled Gal residue. Thus, the sialyltransferase(s) and galactosyltransferase(s) responsible for generating these structure(s) must be located in the same Golgi compartment. Since such structures can be generated even without added CMP-Neu5Ac, there must be an endogenous pool of this sugar nucleotide available to the sialyltransferase(s) in the Golgi-enriched fraction.

The difference in the percent of the neutral and anionic oligosaccharides in ConA-II and ConA-III implies a precursor-product relationship between the two, since the more mature biantennary oligosaccharides (*i.e.* those that have 2 Gal residues) are found in the anionic fraction. This notion is further supported by comparing the ConA elution profiles of the neutral [^{3}H]Gal-labeled oligosaccharides with that of the [^{3}H]GlcNAc-labeled oligosaccharides described in the preceding paper (17). The two profiles are more similar than are the profiles of the neutral and anionic [^{3}H]Gal-labeled oligosaccharides. Additionally, the ConA elution profiles of the anionic [^{3}H]Gal-labeled oligosaccharides are similar to those seen with the [^{3}H]Neu5Ac-labeled samples 7 and 8 (see Table II). The ConA elution profiles of the [^{3}H]acetate-labeled oligosaccharides showed an increase in the percent of ConA-III and a decrease in ConA-II relative to the [^{3}H]Gal- or [^{3}H]Neu5Ac-labeled molecules. This implies that the acetyltransferase(s) prefer more highly branched sialylated acceptors.

The neutral and anionic [^{3}H]Gal-labeled oligosaccharides were also analyzed by E_4-PHA-agarose, which retards bisected galactosylated oligosaccharides. Since sialic acid linkages can complicate the interpretation of the E_4-PHA elution profile (32), the anionic oligosaccharides were desialylated with AUS prior to analysis. In all of the samples, >95% of the [^{3}H]Gal-labeled oligosaccharides co-eluted with [^{14}C]ManNAc used to mark unretained species, indicating the almost complete absence of bisected oligosaccharides (data not shown). These results are in agreement with those obtained with the [^{3}H]GlcNAc-labeled oligosaccharides (17), and are consistent with the low level of GlcNAc transferase III in the rat liver (33, 34). The [^{3}H]Neu5Ac and [^{3}H]acetate-labeled oligosaccharides were not analyzed on E_4-PHA-agarose.

Exoglycosidase Digestion of the Neutral [^{3}H]Gal-labeled Oligosaccharides—These oligosaccharides were treated with exoglycosidases of different specificities to determine the linkage of [^{3}H]Gal residues and to ascertain if they were terminal or covered by another neutral monosaccharide. As shown in Table III, only 2–6% of the [^{3}H]Gal is released by α-galactosidase, and <2% by endo-β-galactosidase. Ninety percent or more is released from the neutral oligosaccharides of samples 4–6 by bovine testicular β-galactosidase, which can cleave Gal-linked β1,3, β1,4, or β1,6. This indicates that the [^{3}H]Gal is mostly β-linked and is not covered by other monosaccharides. Treatment with Jack bean β-galactosidase (which releases β1,4-linked Gal preferentially but will release β1,3-linked Gal at a reduced rate) gave a reduced release of [^{3}H]Gal (Table III). Treatment with β-galactosidase from *D. pneumonieae*, which is specific for β1,4-linked Gal residues, releases less than 5% of the [^{3}H]Gal from the neutral oligosaccharides from samples 4 and 6, whereas 25% is released from sample 5. Since Galβ1,6 linkages have not been reported in rats (and in fact are immunogenic in mammalian species, see Ref. 35), these data indicate that a major fraction of [^{3}H]Gal is in β1,3 linkage. Sample 5 was not incubated with CMP-Neu5Ac during the chase. This indicates that as β1,4-linked [^{3}H]Gal residues are synthesized (see also below), they are efficiently sialylated if sufficient CMP-Neu5Ac is available either from an endogenous pool or from exogenous addition. Since the resulting sialylated oligosaccharide is anionic, the percent of [^{3}H]Gal-linked β1,4 in the neutral fraction would be reduced.

HPLC Analysis of the Neutral [^{3}H]Gal-labeled Oligosaccharides—The neutral oligosaccharides were analyzed by HPLC with an AX-5 column as described under "Experimental Procedures," in conjunction with exoglycosidase digestion, to determine the number and nature of the species present in each sample. This column separates neutral oligosaccharides on the basis of size, with smaller oligosaccharides eluting first. Total neutral oligosaccharides from samples 4–6 were treated with either endo-β-galactosidase (cleaves polylactosamine chains) or chicken liver α-fucosidase (releases α1,-2, α1,4-, and α1,6-linked fucose) and analyzed. The profiles obtained from sample 6 are shown in Fig. 2. Treatment with α-fucosidase released some of the radiolabel as a monosaccharide, indicating that there is a contaminating β-galactosidase in the enzyme preparation. However, there is no other change in the elution profile. There is also no change following endo-β-galactosidase digestion. These data indicate that there are no polylactosamine chains and probably no fucose residues anywhere on the radiolabeled oligosaccharides. This corroborates the observation that similar [^{3}H]GlcNAc-labeled oligosaccharides lack polylactosamine chains (17).

To facilitate more detailed HPLC analyses, ConA was used to fractionate the neutral oligosaccharides from samples 4–6 on a preparative scale. Since a relatively small fraction eluted with 10 mM α-methyl-Glc, this step was omitted and elution was done only with 100 mM α-methyl-Man. The oligosaccharides that bound to ConA were treated with a broad spectrum α-mannosidase or β-hexosaminidase from Jack bean, and analyzed by AX-5 HPLC. Profiles obtained from sample 6 are shown in Fig. 3. Without digestion, 2 peaks are seen, called *a* and *b*. Treatment with α-mannosidase has no effect on the profile indicating that there are no oligosaccharides with exposed mannose residues (hybrid type oligosaccha-

TABLE III

Glycosidase digestion of neutral [^{3}H]Gal-labeled oligosaccharides

Oligosaccharide were treated with 50 milliunits of Jack bean β-galactosidase, 21 milliunits of bovine testicular β-galactosidase, 14 milliunits of endo-β-galactosidase, or 100 milliunits of coffee bean α-galactosidase overnight at 37 °C in either 50 mM sodium citrate, pH 4.6, or glycosidase digestion buffer supplied by the manufacturer in a final volume of 25 μl. Digestions with *D. pneumoniae* β-galactosidase were performed overnight at 37 °C in a final volume of 20 μl with 6 milliunits of enzyme. The reactions were directly spotted and chromatographed on Whatman No. 1 paper to determine the release of free monosaccharide as previously described (17). Less than 2% of the total radioactivity was released from untreated samples, or samples treated with endo-β-galactosidase (not shown).

Sample	Jack bean β-galactosidase (β1-4 > β1,3)	Bovine testicular β-galactosidase (β1-4, β1,3)	*D. pneumoniae* β-galactosidase (β1-4)	Calculated (β1-3)	α-Galactosidase
			%		
4	25	90	3	87	6
5	43	95	25	70	2
6	24	89	5	84	2

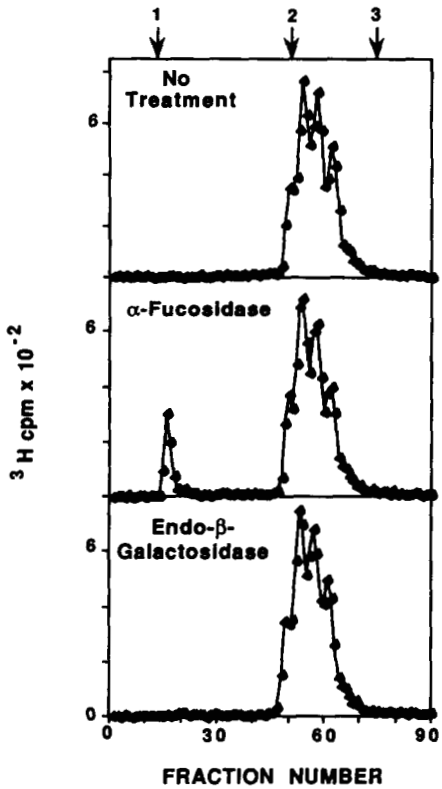

FIG. 2. **HPLC size analysis of the neutral [^{3}H]Gal-labeled oligosaccharides.** The neutral oligosaccharides from sample 6 were incubated overnight at 37 °C in 32 μl of 50 mM sodium cacodylate, pH 5.8, 0.02% NaN_3 (w/v) with 8 milliunits of chicken liver α-fucosidase, 4 milliunits of *E. freundii* endo-β-galactosidase, or no enzyme. The samples were adjusted to 400 μl with 70% acetonitrile in water and analyzed by AX-5 HPLC as described under "Experimental Procedures." Fractions (0.5 min) were monitored for radioactivity. The standards *1–3* are GlcNAc, $Man_5GlcNAc_2$itol, and $Man_9GlcNAc_2$itol, respectively.

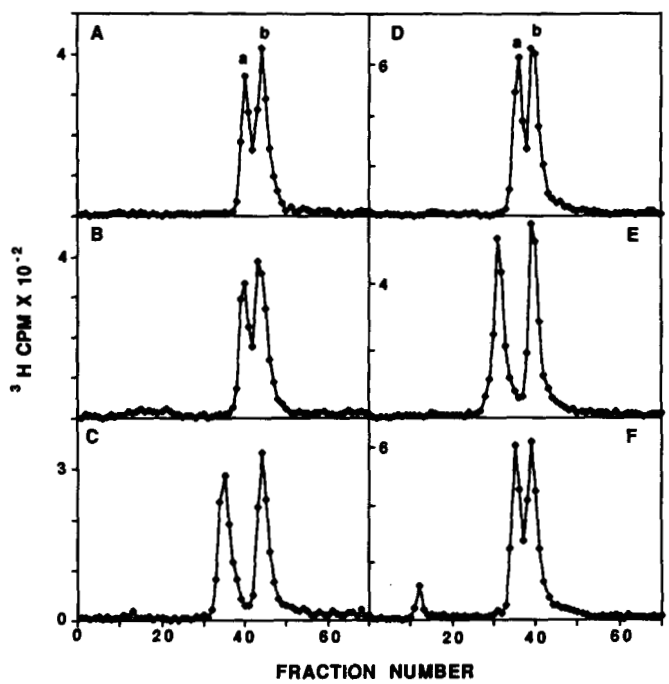

FIG. 3. **HPLC size analysis of the neutral ConA bound [^{3}H] Gal-labeled oligosaccharides.** The oligosaccharides from sample 6 were treated overnight at 37 °C with 0.46 unit of Jack bean α-mannosidase (*panel B*), 0.5 unit of Jack bean β-hexosaminidase (*panel C*), or no enzyme (*panel A*) in 50 mM sodium cacodylate, pH 5.8, 0.02% (w/v) NaN_3 in a final volume of 16 μl. $ZnCl_2$ was added to the α-mannosidase-treated samples to a final concentration of 2 mM. Alternatively, the same oligosaccharides were treated overnight at 37 °C with 16.6 milliunits of *D. pneumonieae* β-hexosaminidase (*panel E*), 2 milliunits of *D. pneumonieae* β-galactosidase (*panel F*), or no enzyme (*panel D*) in 36 μl of 50 mM sodium cacodylate, pH 5.8, 0.02% NaN_3. The samples were adjusted to 450 μl with 70% acetonitrile in water and analyzed by AX-5 HPLC as described under "Experimental Procedures." Fractions (0.5 min) were monitored for radioactivity.

rides). Treatment with Jack bean β-hexosaminidase causes a shift in peak *a* but has no affect on peak *b*. A similar shift in peak *a* but not peak *b* was obtained using β-hexosaminidase from *D. pneumonieae*, which is specific for β1,2-linked GlcNAc residues under the conditions used. Similar elution profiles were obtained with the oligosaccharides from samples 4 and 5 (data not shown). Additionally, there is no change in the elution profile after treatment with *D. pneumonieae* β-galactosidase indicating that all of the Gal residues (radiolabeled or not) are linked β1,3 rather than β1,4. The oligosaccharides from sample 4 behave similarly to sample 6 in this regard. However, 26% of the [^{3}H]Gal from the oligosaccharides from sample 5 is released by *D. pneumonieae* β-galactosidase, indicating the presence of labeled β1,4-linked Gal residues.

Based on ConA binding, exoglycosidase sensitivity, and AX-5 elution profiles, peaks *a* and *b* represent mono- and digalactosylated biantennary oligosaccharides with predominantly Galβ1,3 residues. This is supported by the results of treating the oligosaccharides with recombinant CMP-Neu5Ac:Gal β1,3(4)α2,3-sialyltransferase (α2,3-sialyltransferase). This sialyltransferase can transfer Neu5Ac to both β1,3- and β1,4-linked Gal, whereas the α2,6-sialyltransferase is specific for β1,4-linked Gal residues (36–38). Both a mixture of peaks *a* and *b* and purified peak *b* were treated with the

α2,3-sialyltransferase and analyzed for negative charge by DEAE HPLC. As shown in Fig. 4, sialylation of the mixture of peak *a* and *b* gives two anionic species with 1 and 2 negative charges, respectively, and the distribution of radioactivity is similar to that of the two peaks. A small amount of the radiolabeled oligosaccharides remains neutral, and is unretained by the column, presumably because of incomplete sialylation. Sialylation of purified peak *b* gives an almost pure disialylated species. Similar results were obtained with the oligosaccharides from samples 4 and 5 (data not shown). These results further support the notion that peak *a* is a monogalactosylated biantennary oligosaccharide, and that peak *b* is a digalactosylated biantennary oligosaccharide. The ratio of radioactivity associated with peaks *a* and *b* is approximately the same as the ratio between ConA-III and ConA-II in the original analysis (Table II). Thus, the oligosaccharides from ConA-II are probably represented by peak *b*, and those from ConA-III by peak *a*. If so, the specificity of ConA would require that the single Gal residue on peak *a* is on the α1,3 arm. Differences in the exact ratio of radioactivity in peak *a* and *b* and the ratio in ConA-II and ConA-III may be accounted for by the branch specificity of the galactosyltransferase(s). Comparison of samples 4 and 6 suggests that the addition of Mn^{2+} may alter the branch specificity of the β1,3-galactosyltransferase(s).

The neutral oligosaccharides from sample 6 that ran through ConA were similarly analyzed by AX-5 HPLC, with and without prior treatment with β-hexosaminidases from Jack bean (broad spectrum) and *D. pneumonieae* (β1–2 specific). As shown in Fig. 5, there are 4 identifiable peaks named *c-f*. β-Hexosaminidase had no affect on either peak *e* or *f* indicating that these oligosaccharides do not have exposed GlcNAc residues, and are therefore likely to be completely galactosylated tri- and tetra-antennary oligosaccharides, respectively. The identity of peaks *c* and *d* seemed less clear because a new species appear to comigrate with peak *c* after β-hexosaminidase treatment. Peak *d* may have 2 or more exposed GlcNAc residues and peak *c* no exposed GlcNAc residues. Alternatively, both peak *c* and *d* may have exposed GlcNAc residues and the new peak that comigrates with peak *c* would be the β-hexosaminidase-sensitive product of peak *d*. To address this issue, the two peaks were preparatively purified on the AX-5 column, and then subjected to β-hexosaminidase digestion. Both peaks were found to be susceptible to Jack bean β-hexosaminidase (data not shown). Furthermore, some of the exposed GlcNAc residues were also susceptible to *D. pneumonieae* β-hexosaminidase, indicating that they are β1,2-linked (data not shown). Since these oligosaccharides were unretained by ConA and radiolabeled with [^{3}H]Gal, they are most likely to be mono- and digalactosylated triantennary oligosaccharides. Similar profiles were obtained with the oligosaccharides from samples 4 and 5 (data not shown).

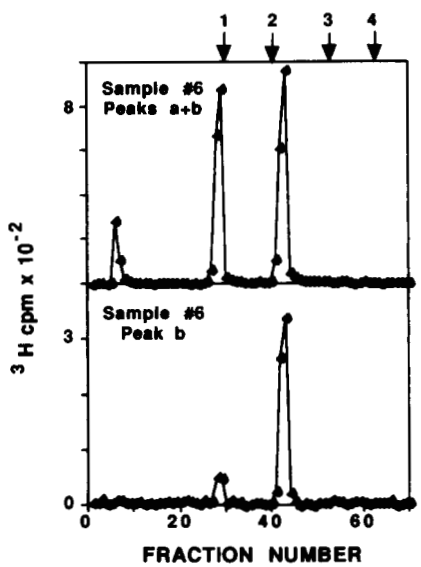

FIG. 4. **DEAE HPLC analysis of the ConA bound neutral [^{3}H]Gal-labeled oligosaccharides following treatment with α2,3-sialyltransferase.** The oligosaccharides from sample 6 were dried and resuspended in 12.5 μl of assay mixture consisting of 125 mM sodium cacodylate, 1.8 mg/ml BSA, 1.25% (v/v) Triton CF-54, pH 5.8. To this was added 14 μl of α2,3-sialyltransferase (19.6 milliunits) and 3 μl of 10 mM CMP-Neu5Ac. After overnight incubation at 37 °C, the mixture was boiled, diluted to 500 μl with water, and analyzed by DEAE HPLC as described under "Experimental Procedures." Fractions (1 min) were monitored for radioactivity. The standards *1–4* are Neu5Acα2,6[^{3}H]Galβ1,4GlcNAc, and disialylated biantennary, trisialylated triantennary, and tetra-sialylated tetra-antennary oligosaccharides, respectively.

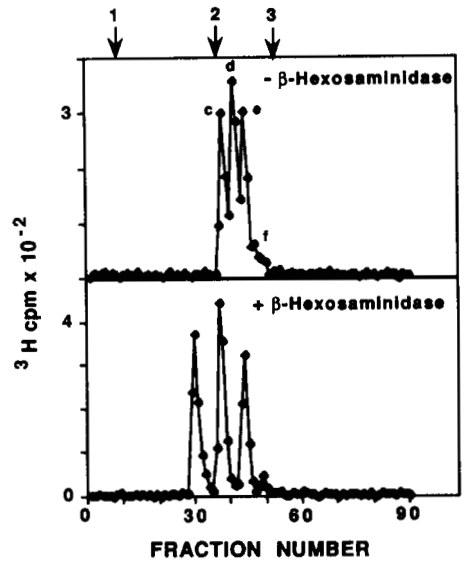

FIG. 5. **HPLC size analysis of neutral [^{3}H]Gal-labeled oligosaccharides that were not bound by ConA.** The oligosaccharides from sample 6 were treated overnight at 37 °C in 32 μl of 50 mM sodium citrate, pH 4.6, 0.02% (w/v) NaN_3, with 0.5 units of Jack bean β-hexosaminidase or no enzyme.The samples were adjusted to 450 μl with 70% acetonitrile in water and analyzed by AX-5-HPLC as described under "Experimental Procedures." Fractions (0.5 min) were monitored for radioactivity. The standards are as described in the legend to Fig. 2.

To further prove these structural assignments, purified peaks *c-f* were treated with α2,3-sialyltransferase and analyzed by DEAE HPLC. Since steric hindrance can prevent complete sialylation, the number of galactosylated antennae on each species is inferred from the *maximum* number of negative charges that can be acquired by treatment with the sialyltransferase. As shown in Fig. 6, sialylation of peak *c* gives only a monosialylated species, consistent with this being a monogalactosylated triantennary oligosaccharide. Peak *d* gives two peaks, with the majority in disialylated species, consistent with this being a digalactosylated triantennary oligosaccharide. Peak *e* gives three peaks, with the majority in trisialylated species, consistent with a trigalactosylated triantennary oligosaccharide. Sialylation of peak *f* gives three peaks. Although the majority is associated with trisialylated species, some tetrasialylated derivatives are observed, consistent with this being a tetragalactosylated tetra-antennary oligosaccharide. The completeness of the sialylation reaction is inversely proportional to the number of Gal residues present

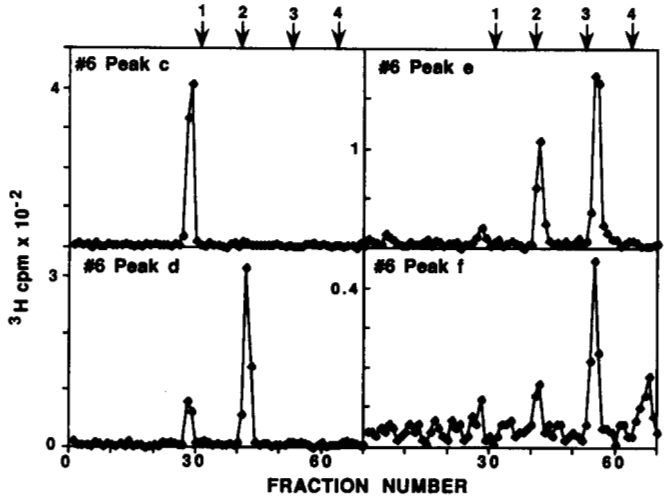

FIG. 6. **DEAE HPLC analysis of neutral [^{3}H]Gal-labeled oligosaccharides following treatment with α2,3-sialyltransferase.** The neutral oligosaccharides from sample 6 that were unretained by ConA-Sepharose were fractionated into the individual peaks *c-f* by AX-HPLC (see Fig. 7). Aliquots of the individual peaks (3.5 μl) were added 12.5 μl of assay mixture consisting of 125 mM sodium cacodylate, 1.8 mg/ml BSA, 1.25% (v/v) Triton CF-54, pH 5.8, 14 μl of α2,3-sialyltransferase (19.6 milliunits), and 6 μl of 10 mM CMP-Neu5Ac. After incubation overnight at 37 °C, the mixtures were boiled, diluted to 500 μl with water, and analyzed by DEAE HPLC as described under "Experimental Procedures." Fractions (1 min) were monitored for radioactivity. Standards are as described in legend to Fig. 4.

TABLE IV

Distribution of [^{3}H]Gal-labeled neutral oligosaccharides

The neutral oligosaccharides were fractionated by ConA-Sepharose and HPLC on an AX-5 chromatography column. The nomenclature of the individual peaks is described in the text. The percent of radioactivity in each species was determined by summing the radioactivity associated with each AX-5 peak, multiplying by the % of label in the ConA fraction, and correcting for the % associated with neutral oligosaccharides in the entire sample.

Sample	Total [^{3}H]Gal-oligosaccharides					
	Peak *a*	Peak *b*	Peak *c*	Peak *d*	Peak *e*	Peak *f*
			%			
4	8	7	14	12	8	<1
5	9	15	9	12	19	2
6	9	8	10	11	8	2

implying some degree of steric hindrance of the sialyltransferase by the more branched structures. Thus, the most likely structures of the oligosaccharides represented by peaks *c-f* are mono-, di-, and trigalactosylated triantennary and tetragalactosylated tetra-antennary oligosaccharides with most of the Gal residues being linked β1,3.

The completely galactosylated oligosaccharides, that is the digalactosylated biantennary (peak *b*) and trigalactosylated triantennary oligosaccharides (peak *e*), are more abundant in sample 5. These differences are even more pronounced if the distribution of these oligosaccharides is calculated relative to the total label in the *N*-linked oligosaccharides (see Table IV). Sample 5 was not incubated with CMP-Neu5Ac during the chase period. Since the addition of CMP-Neu5Ac gives an increase in the percent of anionic oligosaccharides, it appears that the more completely galactosylated oligosaccharides are preferentially sialylated during the chase period. Comparison of samples 4 and 6 shows that the addition of $MnCl_2$ to the labeling and chase reactions has little or no effect on the relative distribution of the different oligosaccharide species (Table IV).

Analysis of [^{3}H]Gal-labeled Anionic Oligosaccharides—Following sialidase (AUS) treatment, >95% of these oligosaccharides run thru a QAE-Sephadex column compared with <2% for undigested controls. Thus, the negative charge is almost exclusively due to sialic acids. To determine the number and linkage of the sialic acids, these oligosaccharides were treated with the broad spectrum AUS (which releases α2,3-, α2,6-, and α2,8-linked sialic acids), or with Newcastle disease virus sialidase (NDVS, which, under the conditions used, specifically releases α2,3- and α2,8-linked sialic acids), and analyzed by DEAE HPLC. Fig. 7 shows the elution profiles of the anionic oligosaccharides from sample 6. In the absence of sialidase, 75% of the oligosaccharides are mono- and disialylated, and few, if any, are tetrasialylated. Since 60–70% of these anionic oligosaccharides did not bind to ConA (indicating tri- or tetra-antennary chains), a substantial fraction of these molecules are incompletely sialylated. As expected, >95% of these oligosaccharides are "neutralized" by AUS treatment. Treatment with NDVS neutralizes 22%, indicating that the sialic acid on a minority of the oligosaccharides is exclusively α2,3- or α2,8-linked. Sialic acids in the α2,8-linkage are very rarely found on *N*-linked oligosaccharides (39), and obviously cannot be present on the monosialylated species. Although NDVS treatment gives no change in the percent of monosialylated species, there is a decrease in the di- and trisialylated species. Either the sialic acids on the

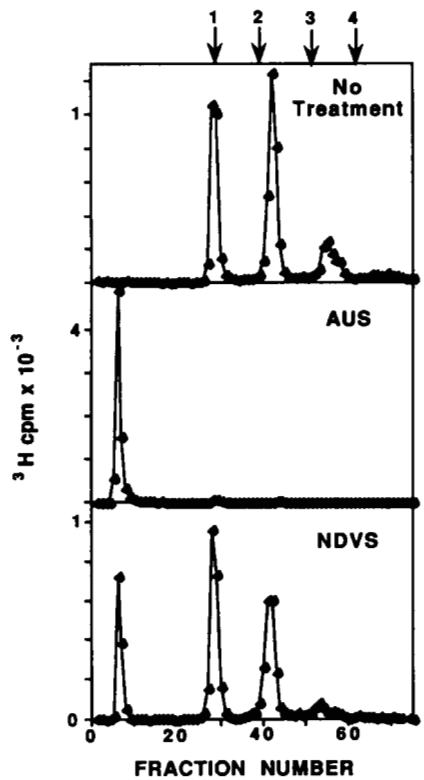

FIG. 7. **DEAE HPLC analysis of the anionic [^{3}H]Gal-labeled oligosaccharides.** Aliquots of these oligosaccharides from sample 6 were incubated overnight at 37 °C with 20 milliunits of AUS or with no enzyme, in 25 μl of 40 mM sodium cacodylate, 0.02% (w/v) NaN_3, pH 5.8. Another aliquot was treated with 12.5 milliunits of NDVS for 15 min at 37 °C in 25 μl of 40 mM sodium cacodylate, 0.02% (w/v) NaN_3, 2 mg/ml BSA, pH 5.8. The mixtures were boiled, diluted to 500 μl with water and analyzed by DEAE HPLC as described under "Experimental Procedures." Fractions (1 min) were monitored for radioactivity. The standards are as described in the legend to Fig. 4.

monosialylated oligosaccharides are exclusively α2,6-linked and some of the di- and trisialylated oligosaccharides have only α2,3-linked residues, or more likely, the mono-, di-, and trisialylated species have a mixture of α2,3- and α2,6-linked sialic acids, and treatment with NDVS causes an overall shift in the elution profile of all of the species. The anionic oligosaccharides from samples 4 and 5 give qualitatively similar profiles (data not shown). However, the omission of CMP-Neu5Ac during the chase period (sample 5) results in more monosialylated oligosaccharides and less di- and trisialylated molecules, supporting the notion that at least a portion of the oligosaccharides radiolabeled with [^{3}H]Gal are subsequently sialylated. The addition of $MnCl_2$ has no affect on the distribution of the [^{3}H]Gal in the different anionic species.

The linkage of [^{3}H]Gal in the anionic molecules was determined with different β-galactosidases as described above. The oligosaccharides were also treated with sialidase to see if some of the [^{3}H]Gal residues were directly covered by sialic acids. The results are presented in Table V. Bovine testicular β-galactosidase releases 27, 59, and 26% of the label from samples 4, 5, and 6, respectively, representing terminal [^{3}H]Gal residues linked either β1,3 or β1,4. In the absence of added CMP-Neu5Ac during the chase (sample 5), the percent of terminal [^{3}H]Gal increases. Of the total [^{3}H]Gal associated with the anionic oligosaccharides from samples 4, 5, and 6, the percent in terminal β1,4 linkage (determined by *D. pneumoniae* β-galactosidase) is 1, 26, and 2%, respectively. The percent of β1,3-linked [^{3}H]Gal is calculated by subtraction. The increase in terminal [^{3}H]Gal residues observed in the absence of added CMP-Neu5Ac is mainly due to increase in β1,4-linked [^{3}H]Gal, with a small increase in [^{3}H]Gal β1,3 residues. Total β1,4-linked [^{3}H]Gal was determined by combined AUS and *D. pneumoniae* β-galactosidase treatment, and is 48, 64, and 59% from samples 4, 5, and 6, respectively. Comparison of 4 with 5 and 6 shows an increase of 10–15% in the total β1,4-linked [^{3}H]Gal in the anionic fraction if $MnCl_2$ is added during the initial labeling reaction. The uncovered [^{3}H]Gal residues increased from 2% in the presence of added CMP-Neu5Ac during the chase period to 26% if CMP-Neu5Ac was omitted. The percent of total β1,4-linked [^{3}H]Gal that is directly covered by sialic acid can be calculated by subtraction. Thus, 90% of β1,4-linked [^{3}H]Gal is covered if CMP-Neu5Ac is present during the chase period as compared with only 34% in the absence of added CMP-Neu5Ac (see Table VI). This indicates an extensive co-localization of the Galβ1,4-galactosyltransferase and one or more sialyltransferase(s). Since [^{3}H]Galβ1,4 residues can be covered with sialic acid residues in the absence of added CMP-Neu5Ac, there is an endogenous pool of the sugar nucleotide that survived the isolation of the Golgi-enriched fraction.

Treatment of the anionic oligosaccharides with both AUS and bovine testicular β-galactosidase releases >95% of the label, indicating that virtually all of the [^{3}H]Gal associated with these molecules is β-linked (only 2–4% was released by coffee bean α-galactosidase, data not shown). The percent of [^{3}H]Gal that is β1,3-linked and covered by sialic acid is calculated by subtraction. Of the [^{3}H]Gal associated with anionic oligosaccharides 21, 0, and 16% from samples 4, 5, and 6, respectively, was both β1,3-linked and covered with sialic acid. Since sialylated [^{3}H]Galβ1,3 linkages were not found if CMP-Neu5Ac was omitted, these results indicate that the UDP-Gal:GlcNAcGalβ1,3-galactosyltransferase and the CMP-Neu5Ac:Galβ1,3(4)α2,3-sialyltransferase are at least partly colocalized within the rat liver Golgi apparatus. The overall distribution of Galβ1,3 and Galβ1,4 residues in [^{3}H]Gal transferred to *N*-linked oligosaccharides is summarized in Table VI, along with the extent of covering by sialic acids. Note that the decrease in proportion of Galβ1,3 linkages upon addition of Mn^{2+} is only relative, since there was a major increase in the total label transferred.

The anionic oligosaccharides were desialylated with AUS and the resulting neutral molecules fractionated by size on an AX-5 HPLC column (data not shown). There is a slight shift toward larger oligosaccharides in the anionic fraction compared with neutral oligosaccharides, most notably, an increase in tetragalactosylated, tetra-antennary oligosaccharides. Treatment with Jack bean β-hexosaminidase has minimal effects on the profile, with a new peak appearing that represents only 6% of radioactivity. Thus, unlike the neutral molecules the anionic oligosaccharides are nearly fully galactosylated, supporting the notion of precursor-product relationship between the two populations of [^{3}H]Gal-labeled oligosaccharides.

Analysis of the [^{3}H]Neu5Ac-labeled Oligosaccharides—The ConA-Sepharose elution profiles of the [^{3}H]Neu5Ac-labeled oligosaccharides (samples 7 and 8) were similar to those of the anionic [^{3}H]Gal-labeled oligosaccharides described above. The linkage of the sialic acid residues was determined by treatments with AUS or NDVS, and the released sialic acid residues were fractionated away from the resistant oligosaccharides by gel permeation chromatography on Bio-Gel P-4. Of the [^{3}H]Neu5Ac label, >95% is released by AUS (not shown), whereas only ~40% is released by NDVS (Table VII). Since α2,8-linked sialic acid is rarely found on *N*-linked oligosaccharides, it can be assumed that the ~40% of [^{3}H]Neu5Ac released by NDVS is α2,3-linked. The 2:3 ratio of α2,3- to α2,6-linked sialic acid is somewhat different from the 1:4 ratio predicted from the relative activities of the two sialyltransferases in the rat liver (40). This may either be due to differences in the availability of substrate in this static *in vitro* system or may reflect factors that modulate the relative activities of these two enzymes in the intact Golgi apparatus.

The oligosaccharides that elute in the void volume of the P-4 column with and without prior sialidase treatment were

TABLE V

Exoglycosidase digestion of the anionic [^{3}H]Gal-labeled oligosaccharides

Oligosaccharides were treated overnight at 37 °C with either 3.5 milliunits of bovine testicular β-galactosidase or 2 milliunits of *D. pneumoniae* β-galactosidase in the presence or absence of 8 milliunits of AUS in 70 mM sodium citrate, pH 4.6, in a final volume of 14 μl. The reactions were directly spotted and chromatographed on Whatman No. 1 paper and the percent release determined as previously described (17). Less than 2% was released from the untreated samples and from the samples treated with sialidase alone (not shown).

Sample	Bovine testicular β-galactosidase (β1,4, β1,3)	*D. pneumoniae* β-galactosidase (β1-4)	Calculated β1-3	AUS + *D. pneumoniae* β-galactosidase	Calculated sialyl-β1-4	AUS + bovine testicular β-galactosidase	Calculated sialyl-β1-3
				%			
4	27	1	26	48	47	95	21
5	59	26	33	64	38	97	0
6	26	2	24	59	57	97	16

TABLE VI

Percent distribution of the [^{3}H]Gal incorporated into N-linked oligosaccharides

The percent of the [^{3}H]Gal transfered to *N*-linked oligosaccharides in either β1,4- or β1,3-linkage is presented. The terminal [^{3}H]Gal includes β-galactosidase-sensitive label in both neutral and anionic oligosaccharides combined. The data are compiled from Tables III and V.

Sample	Terminal Gal-β1,4	Sia α2-6(3) Gal-β1,4	Total Gal-β1,4	Terminal Gal-β1,3	Sia α2,3 Gal-β1,3	Total Gal-β1,3	Total β-linked Gal
				%			
4	2	24	26	56	11	67	93
5	25	13	38	59	0	59	97
6	3	30	33	52	7	59	97

TABLE VII

Charge distribution of [^{3}H]Neu5Ac-labeled oligosaccharides

The anionic oligosaccharides were analyzed by DEAE HPLC as described under "Experimental Procedures." The radioactivity associated with each charged species is summed and expressed as a percent of the total radioactivity.

Sample	Sialidase	Sialidase released	Net charge					
			−1	−2	−3	−4	−5	>−5
		%			%			
7	None	0	10	42	23	14	7	3
7	NDVS	42	10	31	9	5	1	1
8	None	0	10	42	24	14	6	4
8	NDVS	39	10	34	10	5	1	1

analyzed by anion exchange chromatography on a DEAE HPLC column (see Table VII). The untreated oligosaccharides resolve into five distinct species having 1–5 negative charges, with a small amount of material that has greater than 5 negative charges. Since the radiolabel is in the sialic acid itself, it is difficult to determine if all of the negative charge is due to sialic acids, or if the oligosaccharides are also modified with sulfate or phosphate groups. Treatment with NDVS causes an overall shift toward oligosaccharides with a smaller number of negative charges (Table VII), indicating that several of the sialylated oligosaccharides have both α2,3- and α2,6-linked sialic acids. There is no difference in the charge distribution of the radiolabeled oligosaccharides between samples 7 and 8 (Table VII), indicating that the addition of other sugar nucleotides during the chase period has no affect on the sialylation of the oligosaccharides and that the sialylated chains are relatively mature. However, while 52% of the [^{3}H]Neu5Ac is associated with oligosaccharides having only one or two negative charges, 38% is associated with tri- and tetra-antennary oligosaccharides (as determined by ConA-Sepharose binding; see above) indicating that some of these oligosaccharides are incompletely sialylated.

Lack of O-Acetylation of the [^{3}H]Neu5Ac-labeled Oligosaccharides—We have recently shown that a large fraction (30–40%) of the sialic acid residues on the *N*-linked oligosaccharides of rat liver membranes are *O*-acetylated at the 7- or 9-positions (41). However, we have also shown that, in similarly prepared Golgi apparatus fractions, less than 3% of the total [^{3}H]Neu5Ac transferred from CMP-[^{3}H]Neu5Ac to total endogenous acceptors (glycoproteins and glycolipids combined) is *O*-acetylated, even if Ac-CoA is added (23). This is despite the fact that the *O*-acetyltransferase(s) have an apparent K_m lower than that observed for sialyltransferase(s) and a V_{max} of approximately one-third that of sialyltransferase(s) under identical conditions (42). Since none of the factors known to be required for vesicular transport are added, these results suggested that sialylation and *O*-acetylation may occur in different compartments of the Golgi apparatus. To re-examine this question specifically for *N*-linked oligosaccharides, the ^{3}H label released from the *N*-linked oligosaccharides by AUS was separated from residual oligosaccharides by gel permeation chromatography and analyzed by an AX-5 HPLC system designed to separate *O*-acetylated sialic acids from non-*O*-acetylated ones (43). As shown in Fig. 8, the majority (~80%) of the radioactivity released by both AUS and NDVS from sample 10 co-migrates with an authentic Neu5Ac standard. The sialic acids from sample 9 give a similar elution profile (data not shown). There is no increase in the percent of radiolabel in the [^{3}H]Neu5Ac peak following base treatment (de-*O*-acetylation) indicating that the [^{3}H]Neu5Ac transferred to *N*-linked oligosaccharides is not *O*-acetylated. However, there are at least four radioactive species eluting before Neu5Ac which represent ~20% of the total radioactivity released with AUS. The distribution of radiolabel associated with Neu5Ac and with these "non-Neu5Ac" peaks is presented in Table VIII. One peak in the non-Neu5Ac region becomes prominent following base treatment, apparently at the expense of some of the initial peaks (Fig. 8). Radioactivity in the non-Neu5Ac region is much less evident (<5%) in acid hydrolysates of the CMP-[^{3}H]Neu5Ac used for labeling (data not shown). The peaks have therefore arisen from reactions occurring in the Golgi-enriched preparations. This indicates that the [^{3}H]Neu5Ac transferred to *N*-linked oligosaccharides can be further modified by unknown enzymes in the Golgi apparatus and that these modifications are not *O*-acetyl groups. Since only 5–10% of the NDVS-released radioactivity was associated with non-Neu5Ac (Table VIII and Fig. 8) these unknown modifications may be relatively specific for α2,6-linked Neu5Ac, or they may make α2,3-linked sialic acids relatively resistant to NDVS. The nature of these novel modified sialic acids is a topic of current investigation.

Analysis of the [^{3}H]Acetate-labeled Oligosaccharides—The ConA-Sepharose elution profile of the [^{3}H]acetate-labeled oligosaccharides was described above. This label is more selectively associated with tri- and tetra-antennary oligosaccharides than any of the other radiolabeled monosaccharides described, and are all anionic. These molecules were treated with either AUS or NDVS, both of which can release *O*-acetylated sialic acids from oligosaccharides (44). As shown in Fig. 9, AUS released 76% of the radiolabel from sample 10, whereas NDVS released only 2–3%. Similar results were obtained with sample 9 (see Table IX). Since NDVS can release *O*-acetylated sialic acids from glycoconjugates (45), this indicates that the great majority of the [^{3}H]acetate trans-

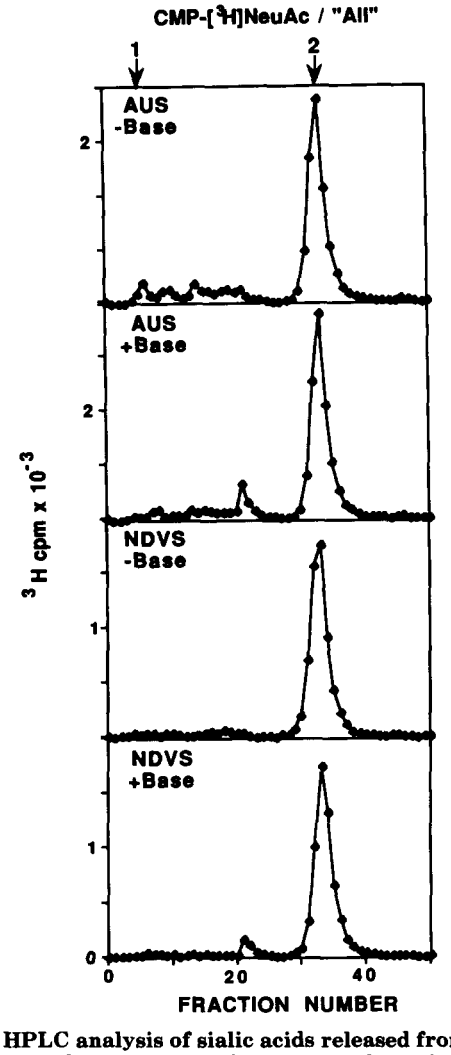

FIG. 8. **HPLC analysis of sialic acids released from the [^{3}H] Neu5Ac-labeled oligosaccharides.** The sialidase-released monosaccharides that were purified by Bio-Gel P-4 and analyzed by AX-5 HPLC column as described under "Experimental Procedures." Prior to analysis, aliquots were treated with 0.1 M NaOH for 30 min at 37 °C to hydrolyze any *O*-acetyl-esters The standards *1* and *2* are acetate and Neu5Ac, respectively.

TABLE VIII

Distribution of sialidase released material from [^{3}H]Neu5Ac-labeled oligosaccharides

The sialidase-released radioactivity was analyzed by HPLC as described in Fig. 8. The percent radioactivity that co-migrated with either authentic Neu5Ac or with peaks other than Neu5Ac is presented.

Sample	Base treatment	AUS		NDV sialidase	
		Non-Neu5Ac	Neu5Ac	Non-Neu5Ac	Neu5Ac
		%			
7	−Base	18	82	6	94
7	+Base	20	80	5	95
8	−Base	22	78	12	88
8	+Base	22	78	10	90

ferred to *N*-linked oligosaccharides is added only to α2,6-linked sialic acids, as predicted by previous reports (23).

The radiolabeled oligosaccharides eluting in the void volume of the P-4 column with and without prior sialidase treatment were analyzed by anion exchange chromatography on a DEAE HPLC column. As shown in Fig. 10, the untreated oligosaccharides resolve into five distinct species having 1–5

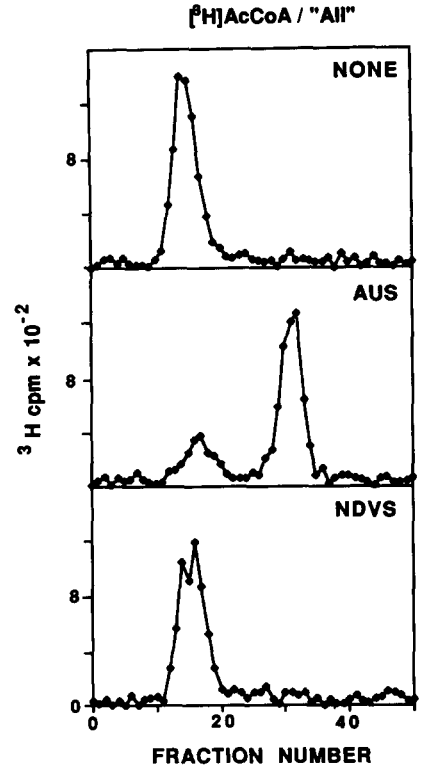

FIG. 9. **Bio-Gel P-4 chromatography of the sialidase-treated [^{3}H]acetate-labeled oligosaccharides.** The [^{3}H]acetate-labeled oligosaccharides were treated exactly the same as the [^{3}H] Neu5Ac-labeled oligosaccharides described in the legend to Table IX, and the reactions fractionated on Bio-Gel P-4 as described under "Experimental Procedures."

negative charges, with a small amount having >5 negative charges. There was no difference in the profiles between samples 9 and 10 (see Table IX). The radiolabeled oligosaccharides that eluted with the void volume of the P-4 column following AUS treatment have a reduced number of negative charges. Thus, these oligosaccharides contain, in addition to AUS-sensitive sialic acids, either AUS-resistant sialic acids or some other negative charges. Of the AUS-resistant radiolabel, 11% was associated with neutral oligosaccharides indicating that approximately 3% of the total [^{3}H]acetate transfer to *N*-linked oligosaccharides must be attached to monosaccharide residues other than sialic acid.

Even though the sialidase-sensitive [^{3}H]acetate label is only on α2,6-linked sialic acid residues, removal of unlabeled α2,3-linked residues on other antennae of the same oligosaccharides should cause a shift in the overall elution profile. Surprisingly, NDVS has a very small effect on the overall DEAE elution profile. This indicates that the [^{3}H]acetate is selectively transferred to oligosaccharides in which most, or all of the sialic acids are α2,6-linked (such molecules are very rare). This is in striking contrast to the *N*-linked oligosaccharides labeled by CMP-[^{3}H]Neu5Ac, which showed the expected mixture of α2,3- and α2,6-linked sialic acid residues on individual oligosaccharides. The charge distribution of the [^{3}H] acetate-labeled oligosaccharides is summarized in Table IX.

The sialidase-resistant [^{3}H]acetate-labeled oligosaccharides were isolated on Bio-Gel P-4 base and treated and reapplied to the same column. Fig. 11 shows the result with sample 10, and sample 9 gave similar profiles (data not shown). The radioactivity now migrated quantitatively with the included volume indicating that the [^{3}H]acetate was added to the oligosaccharide through a base-labile linkage that, by analogy

TABLE IX

Charge distribution of [^{3}H]acetate-labeled oligosaccharides

The [^{3}H]acetate-labeled oligosaccharides were treated with either AUS or NDV sialidase, and the released Sia residues fractionated away from the resistant oligosaccharides as described in Fig. 9. The sialidase-resistant oligosaccharides that eluted in the void volume of the column were pooled and analyzed by DEAE HPLC as described under "Experimental Procedures." The percent of total radioactivity either released by the sialidases or migrating with the different charged species on the DEAE column is presented.

Sample	Sialidase	Sialidase released	Net charge						
			0	−1	−2	−3	−4	−5	>−5
		%				%			
9	None	0	0	2	24	30	24	11	8
9	AUS	71	3	12	7	3	2	1	0
9	NDV	2	0	4	32	27	20	9	6
10	None	0	0	1	22	29	26	13	8
10	AUS	76	2	11	7	3	1	0	0
10	NDV	3	0	3	30	26	20	10	9

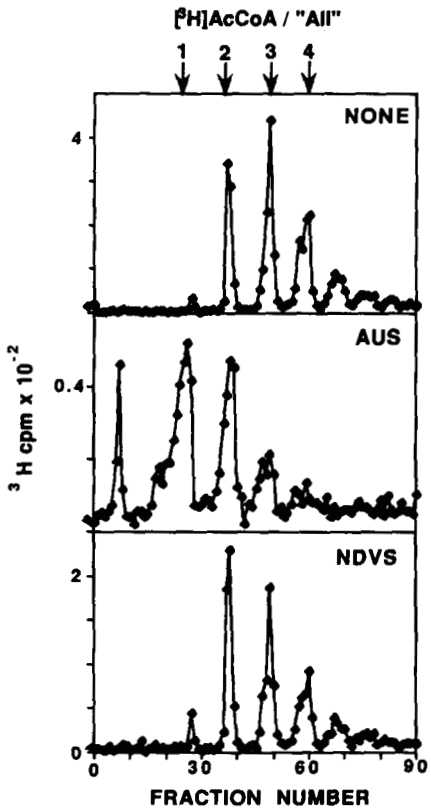

FIG. 10. **DEAE HPLC analysis of the [^{3}H]acetate-labeled oligosaccharides.** The oligosaccharides that eluted in the void volume of the Bio-Gel P-4 described in the legend to Fig. 9 were analyzed by DEAE HPLC as described under "Experimental Procedures." The standards are as in Fig. 4.

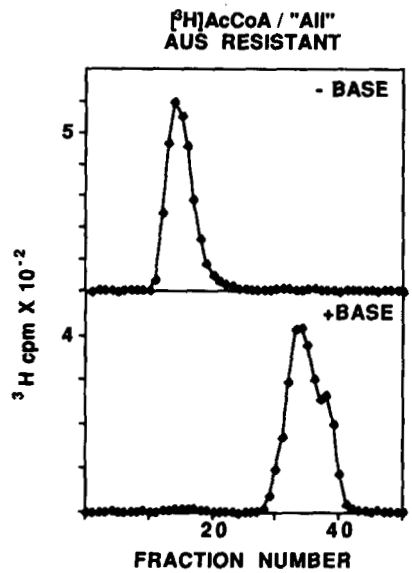

FIG. 11. **Bio-Gel P-4 chromatography of sialidase-resistant [^{3}H]acetate-labeled oligosaccharides.** The oligosaccharides continuing to elute in the void volume of the Bio-Gel P-4 column after AUS (Fig. 9) were treated with 0.1 M NaOH at 37 °C for 30 min and reapplied to the same Bio-Gel P-4 column. The shift in elution pattern indicates the release of free acetate from the oligosaccharides.

to the *O*-acetylated sialic acids, is most likely to be an ester linkage. The nature of these *O*-acetylated molecules remains unknown. They may represent sialidase-resistant *O*-acetylated sialic acids or other *O*-acetylated monosaccharides.

The sialidase-released material was also analyzed by HPLC to determine the nature of the [^{3}H]acetate-labeled sialic acids. The elution profile from sample 10 is shown in Fig. 12. Sample 9 gave a similar profile (data not shown). There are several distinct [^{3}H]acetate-labeled species. Base treatment causes all of the radioactivity to co-migrate with [^{3}H]acetate, indicating that all of the label was in base-labile linkage. This is in agreement with published observations that [^{3}H]acetate transferred from [*acetyl*-^{3}H]Ac-CoA to sialic acid is linked through an *O*-acetyl-ester bond (23, 42, 44). Based on these previous results, the different [^{3}H]acetate-labeled sialic acids seen are likely to be a mixture of (7/8/9) mono- and di-*O*-acetylated sialic acids.

DISCUSSION

This paper describes the structural characterization of the radiolabeled *N*-linked oligosaccharides synthesized by a Golgi-enriched fraction incubated with UDP-[^{3}H]Gal, CMP-[^{3}H]Neu5Ac, or [*acetyl*-^{3}H]Ac-CoA. The radiolabeled monosaccharides or acetate are transferred to endogenous glycoprotein acceptors in the Golgi apparatus by specific transferases. Although the nature of these acceptors is not clear, they are likely to be plasma membrane proteins, lysosomal proteins, resident Golgi apparatus proteins, or secreted proteins. With each donor nucleotide, ~90% of the radiolabel can be released by PNGase F, indicating that the label was predominantly associated with *N*-linked oligosaccharides. The radiolabeled glycoproteins are completely resistant to exogenously added protease in the absence of added detergent, but become protease-sensitive if Triton X-100 was added to a sufficiently high concentration (greater than the critical micellular concentration). This indicates that the labeling occurs only within the lumen of intact vesicles that are of the appropriate

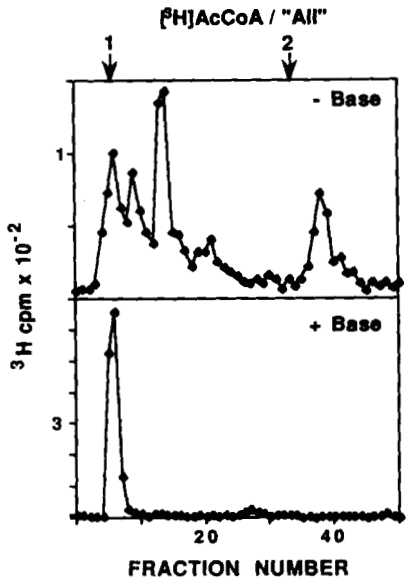

FIG. 12. **HPLC analysis of sialic acids released from the [^{3}H] acetate-labeled oligosaccharides.** Sialidase-released monosaccharides were purified by Bio-Gel P-4 as described in the legend to Fig. 9, and analyzed by HPLC on an AX-5 HPLC column as described in the legend to Fig. 8. Standards *1* and *2* are acetate and Neu5Ac, respectively.

topological orientation, and that are therefore similar to the compartments of the Golgi apparatus in the intact cell. Since none of the factors known to be essential for vesicular transport were added during the incubations, the structures of the radiolabeled oligosaccharides produced should represent the biosynthetic capability of the individual compartments present in the Golgi-enriched fraction.

Incubation with UDP-[^{3}H]Gal results in the labeling of several distinct proteins (data not shown, see also Ref. 46). Several different neutral *N*-linked oligosaccharides were found. Terminal mannose residues were not found indicating the absence of hybrid structures, and no polylactosamine chains or terminal fucose residues were noted. With the exception of the β1,3-linked Gal residues (see below), the structures found correspond to those predicted from the known pathways for *N*-linked oligosaccharide biosynthesis. A significant fraction of the *N*-linked oligosaccharides were negatively charged and bore sialic acids even in the absence of added CMP-Neu5Ac. If the transfer reaction was chased with CMP-Neu5Ac, there was a marked increase in the percent of label associated with anionic oligosaccharides. In each case, a major fraction of the [^{3}H]Gal was directly covered with sialic acid residues. Thus, at least a portion of the β-galactosyltransferase(s) are colocalized to a compartment of the Golgi apparatus with one or more sialyltransferases.

The most unexpected finding is the large amount of β1,3-linked [^{3}H]Gal. About two-thirds of the [^{3}H]Gal is linked to the underlying oligosaccharide through a β1,3-linkage, and the remaining one-third is in the more commonly expected β1,4 linkage. Addition of Mn^{2+} increases the amount of β1,3-linked [^{3}H]Gal residues, although the relative proportion decreases because the amount of β1,4 linkages increases even further. The high proportion of β1,3-linked [^{3}H]Gal found under all labeling conditions implies that this linkage may be much more common in rat liver than previously thought. It is true that the results obtained may not be truly representative of the *in vivo* situation because of the static nature of this labeling reaction, and because accessibility to acceptors may be relatively different in various compartments. However, the increases in products seen upon addition of Mn^{2+} and/or the addition of unlabeled nucleotides indicate that acceptor substrates are probably not limiting for most of the reactions studied here. Also, the gel autoradiographic patterns found under the different conditions of labeling were not substantially different (data not shown), indicating that the labeled proteins under any one set of conditions do not represent a minor subset of acceptors that could skew the results.

While the exact proportion of β1,3-linked Gal linkages in native rat liver cannot be determined from these data, it is worth considering some implications of having many of these residues. While the α2,6-sialyltransferase can only sialylate β1,4-linked Gal, the α2,3-sialyltransferase can sialylate β1,3-linked Gal as well (47), and sialylation on the 6 position of GlcNAc can only occur on Neu5Acα2,3Galβ1,3GlcNAc antennae (48, 49). Thus, the βGal linkage can determine the number and linkages of sialic acids. Recent NMR studies found that the solution conformation of α2,3- and α2,6-sialyllactose are quite different (50–52). Thus the βGal linkage can dictate the linkage of the sialic acid which in turn can affect the solution conformation of the oligosaccharide, and ultimately perhaps its biological activity. It is also noteworthy that an enzyme transferring β1,3Gal to GlcNAc could not be detected in rat liver using a detergent-solubilized assay with an exogenous acceptor (53). Thus, intactness of the Golgi may be required for detecting this activity.

Adding CMP-Neu5Ac during the chase caused an increase in percent of [^{3}H]Gal associated with anionic oligosaccharides. With added CMP-Neu5Ac, ~90% of the β1,4-linked [^{3}H]Gal was covered with a sialic acid indicating co-compartmentalization of the β1,4-galactosyltransferase and one or more sialyltransferase(s). In contrast, even with added CMP-Neu5Ac, only 12–16% of the β1,3-linked [^{3}H]Gal became sialylated. Three interpretations are possible. First, only a small percent of the β1,3-galactosyltransferase may be located in the same Golgi compartment as the α2,3-sialyltransferase. Second, the sialyltransferase may be kinetically limited and unable to sialylate all of the available β1,3-linked [^{3}H]Gal residues. Third, the β1,3-linked Gal residues may not normally be sialylated in the intact cell. If the endogenous acceptors that acquire β1,3-linked [^{3}H]Gal during the labeling are normally expressed on the cell surface of the hepatocyte, they may be significant for three reasons. First, if desialylation occurs, the resulting oligosaccharide will have mostly terminal β1,3-linked Gal. Since oligosaccharides with β1,3-linked Gal interact poorly with the asialoglycoprotein receptor compared to those with β1,4-linked Gal residues (54), high expression of the former on the cell surface may prevent unwanted cross-linking of the asialoglycoprotein receptor with other plasma membrane proteins. Second, if the β1,3-linked Gal residues are terminal, and not sialylated, they could be important in cell-cell adhesion between the different cell types of the liver. Third, as described above, Gal linkages can influence the sialic acid linkages, and ultimately the conformation of the oligosaccharide. Of course, β1,3-linked Gal has been described on several bovine and rat serum proteins (54–58), although much less commonly than the β1,4 linkage (59, 60). Further understanding of the implications of the β1,3-linked Gal requires a better insight into the identity and location of the acceptors.

Even if CMP-Neu5Ac was omitted during the chase, only ~25% of the [^{3}H]Galβ1,4 was terminal, and uncovered by sialic acids. If CMP-Neu5Ac was added, 97–98% of the [^{3}H]Galβ1,4 was covered with sialic acids. This supports the currently held minority view (61–63) that Galβ1,4-galactosyl-

transferase is co-localized with one or more sialyltransferase(s) in a single compartment of the Golgi apparatus (46, 64–70). Thus, Galβ1,4-galactosyltransferase and sialyltransferase should not be used as marker enzymes of distinct Golgi apparatus compartments, at least in the rat liver. ConA fractionation suggests a precursor-product relationship between the oligosaccharides labeled with [^{3}H]Gal, [^{3}H]Neu5Ac, and [^{3}H]acetate, respectively. Likewise, gel electrophoresis and fluorography implies that the same proteins are labeled with [^{3}H]Neu5Ac and [^{3}H]acetate (23). Among the variety of *N*-linked oligosaccharides labeled with CMP-[^{3}H]Neu5Ac, the transferred sialic acid was linked either α2,3 or α2,6 in a 2:3 ratio. Since the label is on the most terminal monosaccharide, complete analysis of the underlying oligosaccharide structure is not feasible. In agreement with previous results on total membrane-bound sialic acids (23), the transferred [^{3}H]Neu5Ac was not *O*-acetylated even if Ac-CoA was added. Since transfer of acetate from Ac-CoA to sialic acid is enriched in Golgi fractions (23, 41, 44) and the V_{max} of the *O*-acetyltransferase is more than one-third that of sialic acid transfer under similar conditions (42), the current results indicate that addition of sialic acid and *O*-acetylation may occur in different Golgi compartments. This is in striking contrast to the situation with [^{3}H]Gal transfer where extensive overlap of β-galactosyltransferase(s) and sialyltransferase(s) was observed. Taken together, these widely disparate levels of overlap also indicate that nonspecific fusion of Golgi compartments is not occurring in these subcellular fractions either during homogenization, or subsequent purification. Further evidence against nonspecific fusion is presented in the preceding paper (17).

Surprisingly, ~20% of radiolabel released from CMP-[^{3}H]Neu5Ac-labeled *N*-linked oligosaccharides by *A. ureafaciens* sialidase did not co-migrate with an authentic Neu5Ac standard in HPLC analysis. Thus, transferred [^{3}H]Neu5Ac can be further modified by unknown enzymes in the Golgi apparatus. The nature of these unexpected modifications is the subject of ongoing investigations. Finding such unexpected modifications further illustrates the power of this approach in detecting unusual or unknown molecules. With regard to [*acetyl*-^{3}H]Ac-CoA, previous reports suggest that the majority of [^{3}H]acetate donated to endogenous acceptors in the rat liver Golgi is transferred to sialic acid residues on *N*-linked oligosaccharides (23). This observation has been confirmed here by directly analyzing the purified [^{3}H]acetate-labeled *N*-linked oligosaccharides from a Golgi-enriched fraction. All of the [^{3}H]acetate transferred to sialic acids was base-sensitive, indicating an *O*-acetyl-ester linkage. The [^{3}H]acetate was transferred exclusively to α2,6-linked, and not to α2,3-linked sialic acids, indicating a substrate preference of the *O*-acetyltransferase(s). Furthermore, unlike the [^{3}H]Neu5Ac-labeled oligosaccharides, *all* of the sialic acids on the *O*-acetylated oligosaccharides appeared to be α2,6-linked, indicating that the sialate-*O*-acetyltransferase(s) may specifically recognize *N*-linked oligosaccharides with exclusively α2,6-linked sialic acid residues. The [^{3}H]acetate was also greatly enriched on oligosaccharides that were not retained by ConA-Sepharose. This indicates that either the sialate:*O*-acetyltransferase(s) specifically recognizes highly branched oligosaccharides, or that addition of [^{3}H]acetate occurs in a later compartment than the addition of [^{3}H]Neu5Ac, and the acceptors are more highly branched as a result.

Of the [^{3}H]acetate transferred to *N*-linked oligosaccharides ~25% was sialidase-resistant, despite the fact AUS can release mono-, di-, and tri-*O*-acetylated sialic acids if the acetyl group(s) are located at the C-7, C-8, and C-9 positions (45). This indicates that either the [^{3}H]acetate was transferred to monosaccharides other than sialic acids, or to AUS-resistant sialic acids (*e.g.* 4-*O*-Ac-Sia). Of the AUS-resistant [^{3}H]acetate, approximately 90% was associated with anionic oligosaccharides and their net negative charge was reduced by the sialidase treatment. The remaining 10% was associated with neutral oligosaccharides and must therefore have been transferred to monosaccharides other than sialic acid. This indicates that there may be a previously undescribed class of *O*-acetylated *N*-linked oligosaccharides.

Thus, a variety of *N*-linked oligosaccharides radiolabeled *in vitro* with [^{3}H]Gal, [^{3}H]Neu5Ac, and [^{3}H]acetate have been obtained and characterized. Almost all of the biosynthetic intermediates predicted by the accepted *N*-linked oligosaccharide biosynthetic pathway were found. The requirement that the Golgi compartments be intact for efficient labeling supports the notion that the glycosylation reactions recapitulate those that occur *in vivo*. Together, these observations further demonstrate the power of this *in vitro* radiolabeling approach to studying *N*-linked oligosaccharide biosynthetic pathways, in complementation with the *in vitro* enzymology. In addition to the predicted structures, several novel structures were also found.

Many previous reports concerning the compartmental organization of the Golgi apparatus have described the localization of only a single component needed for the efficient transfer of a monosaccharide to a newly synthesized oligosaccharide. However, at least four factors (sugar nucleotide, sugar nucleotide transporter, glycosyltransferase, and acceptor) must be present together for efficient transfer to occur. Generation of a signal in the current approach requires that all of the components must be present in the same compartment at the same time. Thus, the inferences drawn from these studies must be strongly considered in discussing the compartmentalization and organization of the Golgi apparatus

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