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The Biosynthesis of Oligosaccharides in Intact Golgi Preparations from Rat Liver

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

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Endogenous acceptors in a Golgi apparatus-enriched subcellular fraction from rat liver were labeled with UDP-[^{3}H]GalNAc. The great majority of these acceptors were protected from protease degradation in the absence of detergent. These molecules are therefore present in intact vesicles of the correct topological orientation, which are likely to be similar to the Golgi compartments of the intact cell. Several distinct glycoproteins are labeled, but most are different from those labeled with UDP-[^{3}H]GlcNAc. The enzyme peptide-N^4(*N*-acetyl-β-glucosiminyl)asparagine amidase releases label from a few specific proteins, indicating that [^{3}H]GalNAc is transferred to *N*-linked oligosaccharides. Both neutral and anionic *N*-linked oligosaccharides are found, the great majority of which do not bind to ConA-Sepharose. Most of the [^{3}H]GalNAc found in neutral oligosaccharides is terminal and β-linked. The negative charge on the anionic molecules is due to sialic acid, and phosphate. A major portion of the [^{3}H]GalNAc in this fraction is acid labile, and is released with kinetics consistent with it being in a phosphodiester linkage. These results show the existence of a whole new class of GalNAc-containing *N*-linked oligosaccharides, and demonstrates that this *in vitro* approach can detect previously undescribed structures.

***O*-Linked oligosaccharide biosynthesis was also studied in the same labeled rat liver Golgi apparatus preparations. β-Elimination releases ~95% of the peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase (PNGase F)-resistant label which, in the absence of other added nucleotides, is almost exclusively [^{3}H]GalNAcitol. If other unlabeled sugar nucleotides and adenosine 3′-phosphate,5′-phosphosulfate are added during the chase period two anionic *O*-linked oligosaccharides are synthesized, indicating that the UDP-GalNAc:peptide-*N*-acetylgalactosaminyltransferase is at least in part functionally co-localized with enzymes that extend and modify *O*-linked oligosaccharides.**

Of the many hundreds of *N*-linked oligosaccharide structures described to date (1–5), very few contain GalNAc residues. The best known examples are the biantennary oligosaccharides found on several pituitary hormones (6, 7) that have GalNAcβ1,4GlcNAcβ1- sequence in place of the more usual Galβ1,4GlcNAcβ1- motif. The GalNAc residues are added by a specific transferase that recognizes a tripeptide sequence near the glycosylated asparagine (8). While this sequence is not limited to the pituitary hormones, very few other examples have been described (9–14). The GalNAc residues are then sulfated at the 4-position in the case of the pituitary hormones, and in tissue factor pathway inhibitor (14). Alternatively, it may sialylated, *e.g.* in tissue plasminogen activator from Bowes melanoma cells (9), or remain terminal, *e.g.* in certain glycoproteins from *Schistosoma* (10, 11) and human urine (13). In the absence of sialic acid or sulfate, β-GalNAc terminated oligosaccharides in mammalian plasma would be bound by the hepatic asialoglycoprotein receptor and cleared from the circulation (15). On the other hand, addition of sulfate to GalNAc causes the recognition of the oligosaccharide by a distinct hepatic receptor (16). Essentially all of the other *N*-linked oligosaccharides known to carry GalNAc are blood group antigens. For instance, the blood group A determinant, GalNAcα1,3(Fucα1,2)Galβ1–3GlcNAc, has been described on the epidermal growth factor receptor from A431 cells (17, 18), on several fucosylated *N*-linked oligosaccharides from human small intestinal cells (19) and on the blood group A GalNAc transferase itself (20). Terminal β-linked GalNAc residues have also been described on *N*-linked oligosaccharides of urinary Tamm-Horsfall glycoprotein as part of the Cad, or Sda antigen with the sequence GalNAcβ1,4(Siaα2,3)Galβ1- (12, 21). With the exception of the α-linked blood group A antigen, each of these structures is thought to be rather uncommon on *N*-linked oligosaccharides.

In the preceding papers (22, 23), we described an approach to studying the "biosynthetic capability" of the intact Golgi apparatus. When Golgi-enriched fractions were incubated with UDP-[^{3}H]GlcNAc, UDP-[^{3}H]Gal, CMP-[^{3}H]Neu5Ac,[1] or [*acetyl*-^{3}H]Ac-CoA label was transferred to endogenous acceptors that were protected from proteases. The majority of the label in each case was releasable by PNGase F and most of the structural intermediates predicted by the established model of *N*-linked oligosaccharide biosynthesis (1–5) were detected, while some novel or unexpected structures were found. Little, if any, of the UDP-[^{3}H]GlcNAc was epimerized to UDP-[^{3}H]GalNAc prior to transfer. Based on this

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[1] The abbreviations used are: Neu5Ac, *N*-acetylneuraminic acid; PAGE, polyacrylamide gel electrophoresis; ConA, concanavalin A; PNGase F, peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase; PAPS, adenosine 3′phosphate,5′-phosphosulfate; HPLC, high performance liquid chromatography.

result and the lack of reports of GalNAc-containing *N*-linked oligosaccharides in rat liver, we predicted that if similar incubation were done with UDP-[^{3}H]GalNAc, no labeled *N*-linked oligosaccharides would be found. Alternatively, if *N*-linked oligosaccharides were labeled, they might represent previously undescribed oligosaccharide structures made by the rat liver. In fact, we found that incubations with UDP-[^{3}H]GalNAc gave several distinct labeled glycoproteins, a few of which were sensitive to PNGase F. This paper describes the structural characterization of these novel [^{3}H]GalNAc-containing *N*-linked oligosaccharides, and also of the PNGase F-resistant (*O*-linked) oligosaccharides synthesized during the same labeling.

EXPERIMENTAL PROCEDURES

Many of the materials and methods used in this study are exactly as described in the preceding papers (22, 23), with the following additions and changes.

Materials—UDP-[6-^{3}H]GalNAc (6 Ci/mmol) was synthesized as previously described (24). Tritium-labeled dermatan sulfate oligomers were kindly provided by Dr. D. Tollefson, Washington University School of Medicine, St. Louis. *Patella vulgata* α-*N*-acetylgalactosaminidase (0.1 units/25 μl) was from V-Labs; chicken liver α-fucosidase (2 units/ml) from Oxford Glycosystems; and EN^{3}HANCE fluorographic solution for PAGE gels from Du Pont-New England Nuclear. All other reagents were obtained from commercial sources and were of the highest quality available.

Incorporation of [^{3}H]GalNAc into Endogenous Macromolecules—To a Golgi-enriched subcellular fraction from rat liver (3.5 ml), $MnCl_2$ (final 1 mM) and ~0.45 mCi of UDP-[^{3}H]GalNAc (21 μM final) were added. The labeling was allowed to proceed for 20 min at room temperature. The sample was then diluted to 30 ml with 50 mM sodium maleate, 5 mM $MgCl_2$, 0.25 M sucrose, pH 6.5. The membranes were pelleted by centrifugation at 100,000 × *g* for 30 min. The pellet was resuspended in ~1 ml of 2% (w/v) SDS containing 40 mM 2-mercaptoethanol and solubilized with sonication and heating to 90 °C for 10 min. Similar labelings were also chased with either 20 μM UDP-GalNAc alone or with 20 μM each of UDP-GalNAc, UDP-GlcNAc, UDP-Gal, CMP-Neu5Ac, and PAPS. The chase was allowed to proceed for an additional 12 min at room temperature and then diluted and centrifuged as above. In some cases, more SDS was needed to fully solubilize the pellets.

Isolation of [^{3}H]GalNAc-labeled Oligosaccharides—The [^{3}H]GalNAc-labeled *N*-linked oligosaccharides were released and purified as described in the preceding paper (22), except for some differences in the exact amount of SDS used to dissolve the macromolecules and the amount of PNGase F used to release the *N*-linked oligosaccharides. Also, unlike the case with the [^{3}H]GlcNAc-labeled oligosaccharides (22), recovery of the released [^{3}H]GalNAc-labeled oligosaccharides from the Bio-Beads SM-2 column was only ~25% indicating that a significant fraction of these oligosaccharides were hydrophobic. These hydrophobic molecules were not investigated further in this study. To confirm the completeness of release, the labeled macromolecules remaining in the void volume of the Sephacryl S-200 column after the first PNGase F treatment were pooled, acetone precipitated, dissolved in SDS, aliquots retreated or sham-treated with PNGase F, and re-examined on the same S-200 column.

Base Release of O-Linked Oligosaccharides—The *O*-linked oligosaccharides were released by β-elimination (25) from the PNGase F-resistant labeled glycoproteins that eluted in the void volume of the S-200 column. For analytical purposes, 100 μl of the macromolecules were mixed 1 ml of 100 mM NaOH, 1 M $NaBH_4$, and incubated at 37 °C for 18 h. Acetone was added to consume the $NaBH_4$, the mixture was acidified with HOAc, and analyzed on the same S-200 column. For preparative purposes, the HEPES buffer was omitted from the column buffer to facilitate subsequent purification. The fractions from the S-200 column that contained the released *O*-linked oligosaccharides were pooled and dodecyl sulfate was precipitated by adding saturated potassium acetate (final 2% (v/v)) and placing the mixture at 4 °C overnight. After removing the potassium dodecyl sulfate by centrifugation, the supernatant liquid was applied to a 2-ml column of Dowex 50 (H^+ form) that was prewashed with at least 10 column volumes of water. The flow-through and a 10-ml water wash were collected into a single tube and lyophilized. The oligosaccharides were resuspended in 5–10% (v/v) HOAc in methanol. The mixtures were dried and the procedure was repeated 2–3 times to remove traces of residual borates.

SDS-PAGE and Fluorography—Discontinuous SDS-PAGE was performed with a 10% (w/v) acrylamide separating gel and a 3.3% (w/v) acrylamide stacking gel. The samples were dissolved in 65 mM Tris, 2% (w/v) SDS, 100 mM 2-mercaptoethanol, pH 6.8, with boiling and loaded onto the gel. Following electrophoresis, the gels were soaked in EN^{3}HANCE fluorographic solution and washed with water according to the manufacturers instructions. The gels were dried under vacuum at 60 °C and exposed to film at −80 °C.

Protection of Radiolabeled Endogenous Acceptors from Proteases—A 1.1-ml aliquot of a Golgi-enriched fraction (22) was incubated with ~16 μCi of UDP-[^{3}H]GalNAc at room temperature for 12 min. The reaction was chased by adding UDP-GalNAc 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 150 μl. To two were added 20 μl of 1% (v/v) Triton X-100 in 50 mM maleate, 5 mM $MgCl_2$, 0.25 M sucrose, pH 6.5; two others received 20 μl of 0.1% (v/v) Triton X-100 in the same buffer, and the remaining two aliquots received buffer alone. One of each pair of aliquots was treated with Proteinase K (2 mg/ml final), the other was incubated with bovine serum albumin (1 mg/ml final). Duplicate aliquots of 25 μl were removed from each of these samples after 0, 0.5, and 16 h of incubation at room temperature. Macromolecules were precipitated from each aliquot with 8% (v/v) perchloric acid on ice, and the radioactivity determined as previously described (22).

Exoglycosidase Digestions—All exoglycosidase digestions were performed in 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 some of the reactions are listed in the appropriate legends.

For release of labeled monosaccharides, anionic *N*-linked oligosaccharides or *O*-linked oligosaccharides were treated overnight at 37 °C with 0.5–1.0 units of Jack bean β-hexosaminidase, 8 milliunits of α-*N*-acetylgalactosaminidase, or no enzyme in 50–60 mM sodium citrate, 0.02% (w/v) NaN_3, pH 4.6. In some cases, the oligosaccharides were heated to 80 °C in 2 M HOAc for 3 h, lyophilized, and resuspended in water. In some cases, release of monosaccharide was monitored by QAE-Sephadex, where released monosaccharides passed though the column unretained. In other cases, the reactions were spotted and chromatographed on Whatman No. 1 paper as described below. Less than 5% of the radioactivity migrated with monosaccharide in untreated controls.

Time Course of Mild Acid Hydrolysis of Anionic N-Linked Oligosaccharides—A 2 M acetic acid solution was preheated to 80 °C. The anionic oligosaccharides were added and the mixture returned to 80 °C. Aliquots were removed after 5, 10, 20, 30, and 180 min of incubation and added to an equal volume of 2 M NH_4OH. These were immediately frozen in a dry ice/ethanol bath and lyophilized. The oligosaccharides were resuspended in water, analyzed by paper chromatography as described below, and 1-cm strips were cut out and counted. The radioactivity migrating with an authentic GalNAc standard and that which remained at the origin (oligosaccharides) was determined. The zero time point was determined by first mixing the HOAc and NH_4OH, then adding the oligosaccharide and lyophilizing.

Radiochemical Composition—The radiolabeled neutral and anionic oligosaccharides were hydrolyzed with 1 M HCl at 105 °C for 4 h and lyophilized. The hydrolysate was dissolved in water and analyzed by HPLC on a Dionex CarboPac PA1 column using 18 mM NaOH as the eluant (26). Nonradioactive $GlcNH_2$ and $GalNH_2$ were included with each sample as internal standards and identified on-line with a pulsed amperometric detector. Fractions (0.33 min) were neutralized with HOAc and the radioactivity determined. Alternatively, the acid hydrolysates were lyophilized, and re-*N*-acetylated with a freshly made solution of 2% (v/v) acetic anhydride, 0.5 M sodium bicarbonate at room temperature for 30 min. The mixtures were acidified with HOAc, lyophilized, resuspended in water, and desalted by ion exchange chromatography on sequential columns of Dowex 50 (H^+ form) and Dowex 3-X4a (free base) as described (22). The samples were resuspended in water and analyzed by paper chromatography on borate-impregnated papers as described below.

Lectin Affinity Chromatography—Lectin affinity chromatography on ConA-Sepharose was performed essentially as described (22, 27).

Paper Chromatography—Paper chromatography was performed in 5:5:1:3 solvent as described in the preceeding paper (22). Borate-impregnated papers were used to separate GlcNAc and GalNAc as

previously described (28) with 1-butanol:pyridine:water (6:4:3) as the solvent.

High Pressure Liquid Chromatography (HPLC)—Neutral oligosaccharides were analyzed using a bonded-amine, MicroPak AX-5 HPLC column developed with a gradient of decreasing acetonitrile in water (22, 29). 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. After washing with water for 5 min the column was eluted with a gradient of increasing NaCl concentration was begun. For the analysis of [^{3}H]GalNAc-labeled *N*-linked oligosaccharides the gradient was 0–300 mM NaCl over 85 min. For the analysis of *O*-linked oligosaccharides the gradient was 0–500 mM NaCl over 85 min. The flow rate was 0.6 ml/min and fractions were collected every 1 min. The *O*-linked oligosaccharides were also analyzed by DEAE HPLC at pH 5.5 or 2.45. In these cases, KP_i buffer was added to the eluant.

GalNAcitol and GlcNAcitol were separated (30) on the AX-5 column equilibrated and run in acetonitrile, water, 15 mM KPO_4, pH 5.2 (85:5:10). The flow rate was 2 ml/min and fractions were collected every 0.33 min.

RESULTS

In the preceding paper (22), we showed that little, if any, UDP-[^{3}H]GlcNAc was epimerized to UDP-[^{3}H]GalNAc prior to transfer of the monosaccharide to endogenous acceptors in intact Golgi apparatus-enriched fractions. Since *N*-linked oligosaccharides containing GalNAc residues have not been described in rat liver, similar incubations with UDP-[^{3}H]GalNAc were not expected to yield radioactive *N*-linked chains. However, as shown below, a novel family of *N*-linked chains was found to be labeled.

Transfer of [^{3}]GalNAc to Endogenous Acceptors and Release of the N-Linked and O-Linked Oligosaccharides—A Golgi-enriched fraction from rat liver was incubated with UDP-[^{3}H]GalNAc as described under "Experimental Procedures" in the presence of 1 mM $MnCl_2$. After labeling, the membranes were collected, solubilized with SDS, and the radiolabeled macromolecules isolated from the void volume of an Sephacryl S-200HR column. As shown in Fig. 1, digestion with PNGase F (31) released oligosaccharides that migrate in the included volume of the same S-200HR column, separated from PNGase F-resistant material (32). About 8% of the radioactivity was released from macromolecules with PNGase F, and <1% of the radioactivity runs in the same region after a control incubation, indicating that the release was specific (the amount specifically released is reproducible between similar preparations). This release was ~10 times lower than that obtained from similar incubations with [^{3}H]GlcNAc-, [^{3}H]Gal-, [^{3}H]Neu5Ac-, or [^{3}H]acetate-labeled macromolecules (22, 23). This is not surprising since GalNAc is most commonly found in *O*-linked oligosaccharides and proteoglycans.

Redigestion of an aliquot of the PNGase F-resistant macromolecules gave no additional release of radioactivity (see Fig. 2). In contrast, the PNGase F-resistant radioactivity was almost quantitatively released by the alkaline sodium borohydride treatment, a β-elimination procedure that releases *O*-linked oligosaccharides (see Fig. 2). These data indicate that while a significant portion of the macromolecular radioactivity is specifically associated with *N*-linked oligosaccharides, most of the remainder occurs in *O*-linked oligosaccharides.

SDS-PAGE Analysis of the Radiolabeled Glycoproteins—The radiolabeled glycoprotein acceptors labeled by UDP-[^{3}H]GalNAc and UDP-[^{3}H]GlcNAc were compared by SDS-PAGE and fluorography. As shown in Fig. 3, very few of the most prominent radiolabeled bands are common to both labelings, indicating that different endogenous acceptors are labeled with UDP-[^{3}H]GalNAc and UDP-[^{3}H]GlcNAc. Treatment with PNGase F also gave different results. As expected from data in the preceding paper (22), almost all of the [^{3}H]

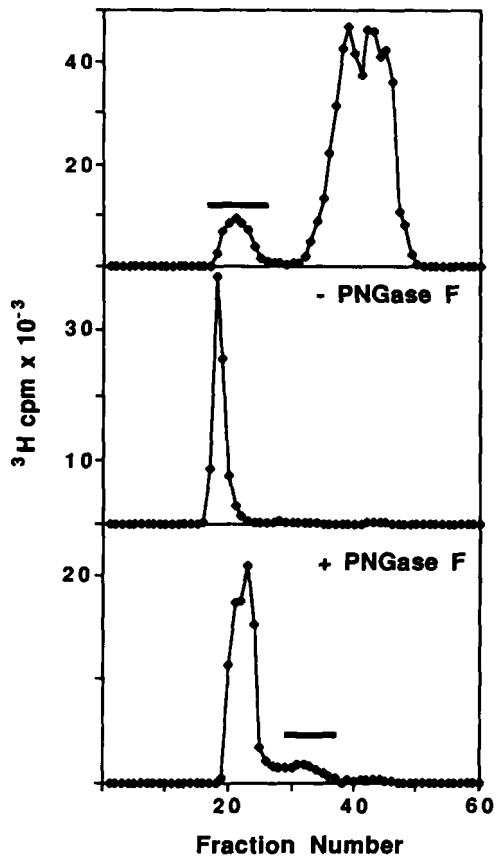

FIG. 1. **Isolation of [^{3}H]GalNAc-labeled macromolecules and release of *N*-linked oligosaccharides.** A Golgi-enriched subcellular fraction was labeled with UDP-[^{3}H]GalNAc as described under "Experimental Procedures." The pelleted membranes were solubilized with SDS and the radiolabeled macromolecules (fractions *under bar*) were isolated by Sephacryl S-200HR chromatography run in SDS (*top panel*). The radiolabeled macromolecules were treated without and with PNGase F and rechromatographed on the same column. The radioactivity released by PNGase F was pooled (fractions *under bar, lower panel*).

GlcNAc-labeled bands were eliminated by PNGase F (compare *lanes A* and *B*). In contrast, only a few specific [^{3}H]GalNAc-labeled bands were eliminated or diminished by the same treatment (compare *lanes C* and *D*). These bands do not appear to be in common with the [^{3}H]GlcNAc-labeled bands, indicating that specific glycoprotein acceptors carried [^{3}H]GalNAc-labeled *N*-linked oligosaccharides. These data also suggest that enzymatic epimerization of UDP-[^{3}H]GalNAc to UDP-[^{3}H]GlcNAc is not occurring during the incubation. This was directly proven by monosaccharide analysis (see below).

Protection of the Radiolabeled Acceptors from Proteases—If the Golgi compartments being labeled are intact and have correct topological orientation, the products of the transfer reaction should be resistant to exogenously added proteases. To address this, a Golgi-enriched fraction labeled with UDP-[^{3}H]GalNAc and chased with excess nonradioactive UDP-GalNAc was treated with different combinations of detergent and protease for different periods of time. The proteolytic degradation of the [^{3}H]GalNAc-labeled proteins was followed by acid precipitation, and the 100% control value defined as the radioactivity that remained precipitable after a 30-min incubation in the absence of protease or detergent. The results are very similar to those reported for UDP-[^{3}H]GlcNAc in the preceding paper (22). Addition of Proteinase K alone gave a small decrease in acid-precipitable radioactivity, which did

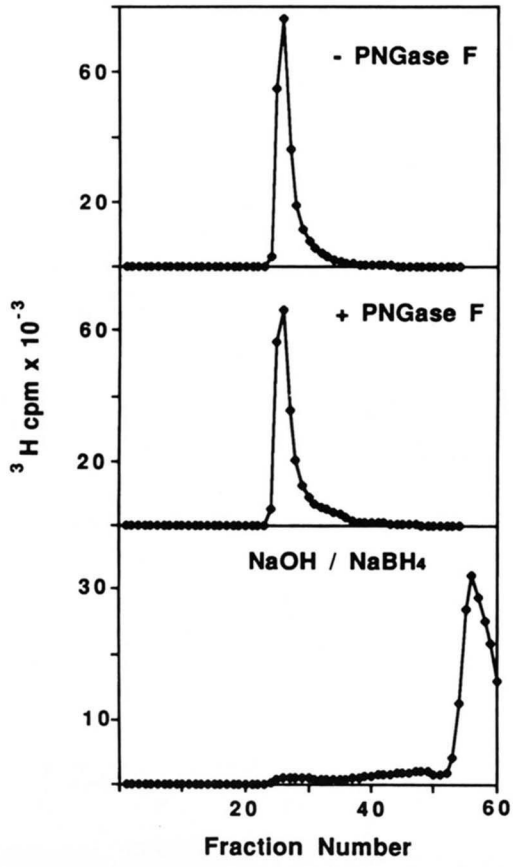

FIG. 2. **Retreatment of the PNGase F-resistant macromolecules with PNGase F or with alkaline sodium borohydride.** The PNGase F-resistant macromolecules described in the legend to Fig. 1 were pooled, and an aliquot was retreated with PNGase F and rechromatographed on the same Sephacryl S-200HR column used to isolate the radiolabeled macromolecules. Another aliquot of the PNGase F-resistant macromolecules was treated with alkaline borohydride, neutralized, and analyzed on the same column.

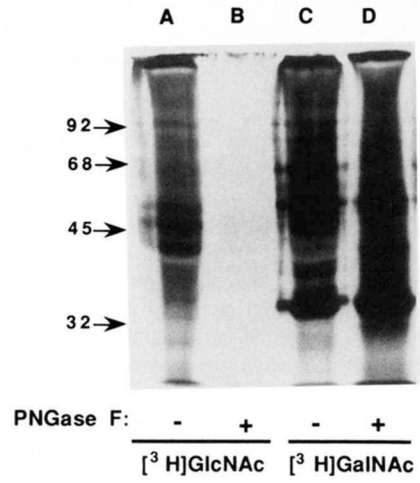

FIG. 3. **Fluorogram of [^{3}H]GalNAc- and [^{3}H]GlcNAc-labeled glycoproteins.** A Golgi-enriched fraction was radiolabeled with either UDP-[^{3}H]GalNAc or UDP-[^{3}H]GlcNAc, pelleted, solubilized, treated with or without PNGase F, and separated by SDS-PAGE on a 10% (w/v) acrylamide gel. The gels were subjected to fluorography as described under "Experimental Procedures." The samples and the PNGase F treatment status are indicated on the figure. The migration of molecular mass standards (in kDa) is indicated.

not change appreciably with time (increasing from 10% at t = 0 to 15% at t = 16 h). In contrast, if Triton X-100 was added to a sufficiently high concentration (0.10%, v/v), there was a marked protease-dependent decrease in the acid precipitability of macromolecules. With prolonged incubation times, the acceptors remained intact in the absence of detergent, whereas a combination of detergent and protease lowered acid-precipitable radioactivity to 6% of control after 16 h of incubation (data not shown). These results indicate that the great majority of the acceptors labeled by UDP-[^{3}H]GalNAc are in sealed compartments of the appropriate topological orientation.

Fractionation of N-Linked Oligosaccharides on QAE-Sephadex—The released *N*-linked oligosaccharides were freed of detergents, desalted, and fractionated into neutral and anionic species on QAE-Sephadex as described under "Experimental Procedures." Of the radioactivity recovered, 55% passed through the QAE column (neutral *N*-linked oligosaccharides) and 45% was eluted with 1 M NaCl (anionic *N*-linked oligosaccharides).

Radiochemical Purity of [^{3}H]GalNAc—The UDP-GlcNAc 4′-epimerase that can interconvert UDP-GlcNAc and UDP-GalNAc is a soluble cytosolic NAD^+-requiring enzyme. To be certain that no epimerization took place during the labeling, the neutral and anionic oligosaccharides were hydrolyzed with HCl and the resulting ^{3}H-labeled monosaccharides analyzed by Dionex HPLC as described under "Experimental Procedures." Of the radioactivity that co-migrated with either $GlcNH_2$ or $GalNH_2$, the great majority was associated with $GalNH_2$ (85% from the neutral oligosaccharides, and 96% from the anionic fraction). These results were confirmed by paper chromatography of the labeled hydrolysates following re-*N*-acetylation as described under "Experimental Procedures" (81% GalNAc was found in neutral oligosaccharides and 95% in anionic oligosaccharides). These data indicate that although a small amount of contaminating [^{3}H]GlcNAc is present (primarily in the neutral fraction), the great majority of the radiolabel transferred to *N*-linked oligosaccharides is in [^{3}H]GalNAc.

Analysis of the Neutral [^{3}H]GalNAc-labeled N-Linked Oligosaccharides—These oligosaccharides were first analyzed on concanavalin A (ConA)-Sepharose as described under "Experimental Procedures." The specificity of ConA is well known (Ref. 27, and references therein). 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 or biantennary chains with a single Gal on the α1,3 arm elute with 100 mM α-methylmannopyranoside (ConA-III). Of the radioactivity recovered in oligosaccharides, 84% was in ConA-I, with 8% in each of the ConA-II and ConA-III fractions. Since ConA recognizes the trimannosyl core rather than the terminal sugars, these data indicate that the majority of [^{3}H]GalNAc is likely to be associated with triantennary, tetra-antennary, or bisected oligosaccharides. However, the exact percentages are difficult to determine because of the small amount of contaminating [^{3}H]GlcNAc described above. Because such a large proportion of the radioactivity was unretained by ConA, further analysis was done without preparative ConA fractionation.

The mixture of neutral oligosaccharides was analyzed by HPLC with an AX-5 chromatography column as described under "Experimental Procedures." This column separates neutral oligosaccharides on the basis of size, with smaller ones eluting first (29). As shown in Fig. 4, several poorly resolved

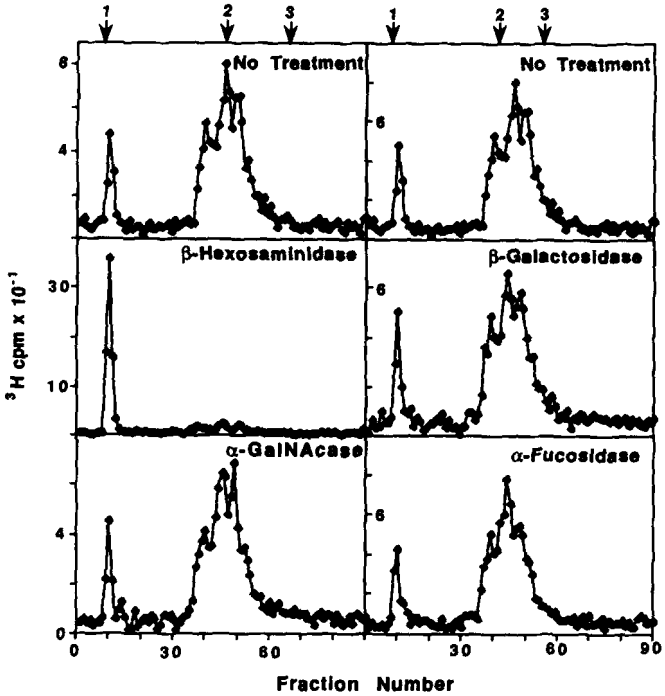

FIG. 4. **HPLC size analysis of the neutral *N*-linked oligosaccharides.** The oligosaccharides were treated overnight at 37 °C with 0.5 unit of Jack bean β-hexosaminidase, 12 milliunits of *P. vulgata* α-*N*-acetylgalactosaminidase (*α-GalNAcase*), 14 milliunits of bovine testicular β-galactosidase, 10 milliunits of chicken liver α-fucosidase, or no enzyme in 20 μl of 50 mM sodium cacodylate, pH 5.8, 0.02% (w/v) NaN_3. The samples were boiled, adjusted to 450 μl with 70% acetonitrile in water, and analyzed by AX-5 HPLC as described under "Experimental Procedures." Fractions (0.75 min) were monitored for radioactivity. The standards *1–3* are GlcNAc, $Man_5GlcNAc_2$itol, and $Man_9GlcNAc_2$itol, respectively.

peaks ranging in approximate size from the standards $Man_4GlcNAc_2$itol to $Man_8GlcNAc_2$itol are found. A peak that accounted for 10% of the radioactivity co-migrates with the free monosaccharide standard. In light of the original elution position of the labeled oligosaccharides in the S-200 gel filtration, this free monosaccharide is likely to have arisen from breakdown of a labile linkage such as a phosphodiester, during the preparation and purification of the oligosaccharides (see below). The neutral oligosaccharides were also treated with several glycosidases and re-analyzed by HPLC. The broad spectrum β-hexosaminidase from Jack bean released 68% of the radiolabel, indicating that the majority of the [^{3}H]GalNAc associated with neutral oligosaccharides is terminal and β-linked. Treatment with α-*N*-acetylgalactosaminidase did not release any monosaccharide, indicating that label was not associated with blood group A structures. Bovine testicular β-galactosidase (releases β1,3- and β1,4-linked Gal), and chicken liver α-*fucosidase* (releases α1,2-, α1,4-, and α1,6-linked Fuc), had no affect on the elution profiles. Because of the resistance to exoglycosidases other than β-hexosaminidase, and the terminal location of most of the label, further structural analysis was not pursued. However, taken together, these data indicate that [^{3}H]GalNAc was transferred to a variety of small neutral *N*-linked oligosaccharides previously undescribed in rat liver.

Analysis of the Anionic [^{3}H]GalNAc-labeled N-Linked Oligosaccharides—As described above, 45% of the [^{3}H]GalNAc-labeled *N*-linked oligosaccharides is anionic. Of this material, 97% passed through a ConA-Sepharose column and the remaining 3% was eluted with 10 mM α-methylglucopyranoside. This indicates that the [^{3}H]GalNAc in anionic oligosaccharides was essentially all transferred to complex-type oligosaccharides with three or more antennae. GalNAc residues have been described on anionic *N*-linked oligosaccharides, either α-linked on blood group A antigens (7–10) or β-linked as on pituitary hormone oligosaccharides (6) or the Cad, or Sda blood group antigen (21). GalNAc has also been reported to be phosphodiester-linked on *O*-linked oligosaccharides of the adhesion molecule, *N*-cadherin (33, 34). The latter linkage should be susceptible to mild acid cleavage in a manner analogous to the phosphodiester-linked GlcNAc precursor of the Man-6-phosphate lysosomal enzyme recognition marker (35).

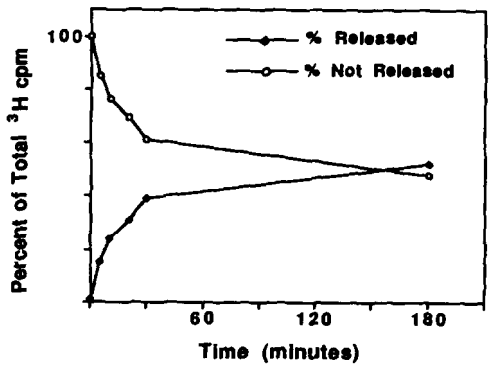

FIG. 5. **Time course of release of [^{3}H]GalNAc from anionic *N*-linked oligosaccharides by mild acid.** These oligosaccharides were treated with 2 M HOAc at 80 °C for various times and analyzed by paper chromatography as described under "Experimental Procedures." The maximum release (52%) was obtained at 3 h.

Only 24% of the [^{3}H]GalNAc on the anionic oligosaccharides is released with β-hexosaminidase, whereas 52% is released with α-*N*-acetylgalactosaminidase. A significant fraction (~25%) of the label is not released by either of these treatments suggesting that these [^{3}H]GalNAc residues are either covered by other monosaccharides, or modified or linked in a fashion that is resistant to these enzymes. Interestingly, 52% of the label was also released by mild acid treatment, indicating that a significant portion of the [^{3}H]GalNAc on these anionic *N*-linked oligosaccharides might be phosphodiester-linked. These data also suggest that the putative phosphodiester-linked GalNAc is α-linked, similar to the phosphodiester-linked GalNAc on *N*-cadherin (33) and the phosphodiester linked GlcNAc precursor of Man-6-phosphate (35).

The anionic oligosaccharides were treated with mild acid on a preparative scale and the label fractionated into released monosaccharide and intact oligosaccharides by Sephadex G-10 chromatography. As predicted from the compositional analysis above, the mild acid-released monosaccharide co-migrated with GalNAc on paper chromatography, and there was no detectable radioactivity co-migrating with GlcNAc (data not shown). The kinetics of the release of [^{3}H]GalNAc by mild acid was determined to further demonstrate that the acid-labile [^{3}H]GalNAc was phosphodiester-linked. As shown in Fig. 5, a maximum of 52% of the total [^{3}H]GalNAc was released from the anionic oligosaccharides after a 3-h incubation in 2 M HOAc at 80 °C. Of this mild-acid releasable monosaccharide, 46% was released following a 10-min incubation. These kinetics are very similar to those that demonstrated the phophodiester linked GlcNAc of lysosomal enzymes (the pH of HOAc was 2.3, compared with a pH of 2.1 used to demonstrate the phophodiester-linked GlcNAc) (35). Since such HOAc treatment does not release terminal glycosidically linked monosaccharides such as β-GlcNAc or β-Gal,[2]

[2] B. K. Hayes and A. Varki, unpublished observations.

glycosidically linked [^{3}H]GalNAc is probably not being released under these conditions. Taken together, these data demonstrate that a significant fraction of the [^{3}H]GalNAc transferred to *N*-linked oligosaccharides is phosphodiester-linked.

DEAE HPLC was used to determine the number and nature of the negative charges on the anionic oligosaccharides. As shown in Fig. 6, a range of anionic molecules was separated, with the most prominent species having 3–4 negative charges. Treatment with a broad spectrum sialidase (*Arthrobacter ureafaciens* sialidase) gave neutral species that were apparently derived from those with 2–4 negative charges (note the much flatter baseline in this region of the chromatogram following the treatment, and a shift in elution position of some of the smaller peaks). Mild acid treatment, which should release both sialic acid residues (36), and the phosphodiester-linked [^{3}H]GalNAc described above, neutralized essentially all of the anionic species. These results indicate that a variety of anionic oligosaccharides are labeled with [^{3}H]GalNAc. The major anionic substituents are sialic acids and phosphodiester-linked [^{3}H]GalNAc, which in some cases may be present on the same oligosaccharide chain.

Analysis of the [^{3}H]GalNAc-labeled O-Linked Oligosaccharides—As described earlier, alkaline sodium borohydride treatment released 95% of the PNGase F-resistant radiolabel from the macromolecular acceptors. The base-released molecules were freed of SDS, desalted, and characterized further. Ninety-seven percent of the radioactivity passed through a QAE-Sephadex column, indicating a lack of anionic substituents. When analyzed by paper chromatography, essentially all of the radioactivity migrated ahead of the disaccharide Galβ1,4GlcNAc, in the position expected for a monosaccharide (data not shown). Since borohydride was present during the base release, released monosaccharides should have been reduced to alditols. Since the majority of the radiolabel transferred to *N*-linked oligosaccharides was [^{3}H]GalNAc (see above), the majority of the released radiolabel from the *O*-linked saccharides is likely to be [^{3}H]GalNAcitol. However, if any epimerization to UDP-[^{3}H]GlcNAc had occurred prior to transfer, a portion of the released monosaccharide could be in [^{3}H]GlcNAcitol, arising from GlcNAc *O*-linked to Ser/Thr residues (37). In fact, HPLC analysis of the released material showed only [^{3}H]GalNAcitol (data not shown), indicating that the original *O*-linked saccharide was exclusively [^{3}H]GalNAc-*O*-Ser/Thr. This saccharide accounts for the great majority of the [^{3}H]GalNAc transferred to endogenous acceptors, and explains the major differences in the fluorograms of the glycoproteins labeled by UDP-[^{3}H]GlcNAc and UDP-[^{3}H]GalNAc (Fig. 3).

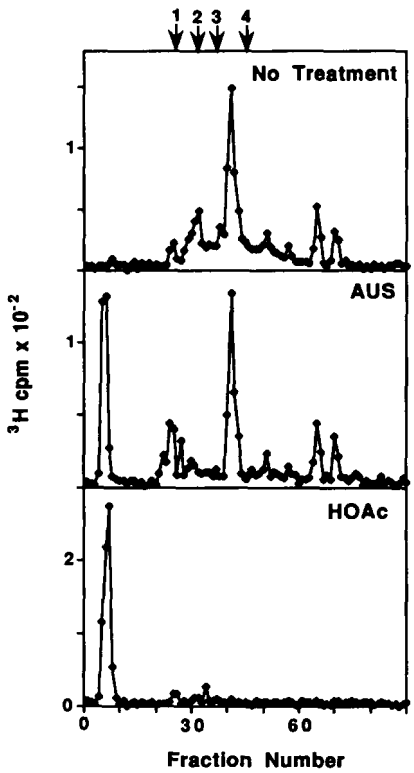

FIG. 6. **DEAE HPLC analysis of the anionic *N*-linked oligosaccharides.** These oligosaccharides were treated for 2 h at 37 °C with or without *Arthrobacter ureafaciens* sialidase (*AUS*) (4 milliunits) in 50 mM sodium cacodylate, pH 5.8. The samples were heated to 90 °C, diluted with 500 μl of water, and analyzed by DEAE HPLC as described under "Experimental Procedures." Another aliquot was treated with 2 M HOAc at 80 °C for 3 h, lyophilized, resuspended in water, and similarly analyzed. 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.

It has been suggested that other glycosyltransferases that can extend *O*-linked saccharides may not be co-localized with the first enzyme, UDP-GalNAc:peptide-α-*N*-acetylgalactosaminyltransferase (38). To address this possibility, two separate samples were incubated with UDP-[^{3}H]GalNAc in the presence of 1 mM $MnCl_2$; one was chased with nonradioactive UDP-GalNAc (20 μM), and the other with 20 μM each of UDP-GalNAc, UDP-GlcNAc, UDP-Gal, CMP-Neu5Ac, and PAPS (the latter combination is referred to as ALL for convenience). The radiolabeled macromolecules were obtained, the *N*-linked oligosaccharides released, and the PNGase-F-resistant macromolecules treated with alkaline borohydride as described above. Over 90% of the PNGase F-resistant radioactivity was released with alkaline borohydride in each case. As expected, >95% of the radioactivity of the base-released saccharides from the sample chased with UDP-GalNAc alone was neutral on a QAE-Sephadex column, confirming the earlier observations. In contrast, 70% of the material from the sample chased with ALL bound to the QAE-Sephadex column. Since overall incorporation of radioactivity was not significantly altered by the addition of ALL, the [^{3}H]GalNAc must have been further modified during the chase reaction. The percent of radioactivity that bound to QAE-Sephadex was variable between otherwise similar preparations, ranging from 8 to 70% in different experiments. This may either represent differences in the metabolic state of the animal at the time of sacrifice and/or differences in the efficiency of the various Golgi-enriched preparations to add multiple monosaccharides to a single GalNAc-Ser/Thr residue. Detailed analyses is presented on the preparation in which 70% of the label was associated with anionic molecules.

The labeled *O*-linked saccharides from the two samples were next analyzed on paper chromatography to determine their approximate size. As shown in Fig. 7, almost all of the radioactivity from the sample chased with UDP-GalNAc only co-migrated with the monosaccharide standard, compared with only 25% of that from the sample chased with ALL. The remaining 75% migrated as a broad series of peaks that represent either differences in the number of monosaccharides or the number of charges on these saccharides, or both. Since none of the radioactivity was retained at the origin, these saccharides must be relatively small. The percentage of radioactivity migrating with monosaccharides on paper is very similar to the percent that passed through a QAE-Sephadex column. This was further confirmed by preparative QAE-

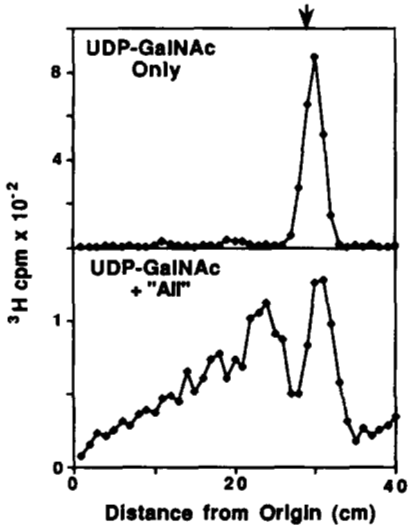

FIG. 7. **Paper chromatography of the *O*-linked saccharides.** *O*-Linked saccharides released from samples labeled with UDP-[^{3}H]GalNAc and chased with either nonradioactive UDP-GalNAc or ALL as described in the text, were analyzed on Whatman No. 1 paper as described under "Experimental Procedures." The *arrow* indicates the position of authentic GalNAc.

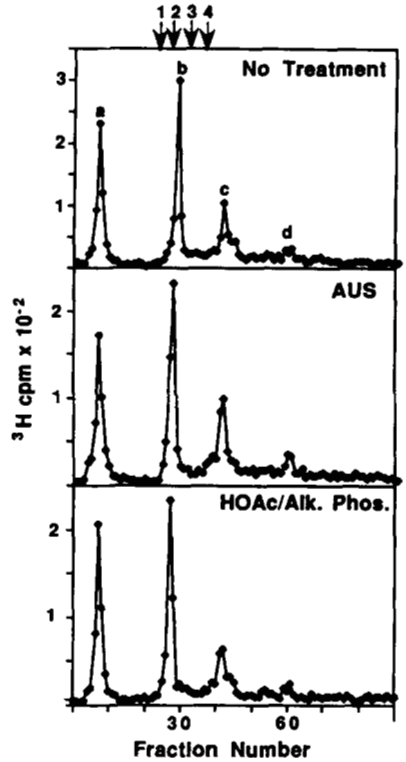

FIG. 8. **DEAE HPLC analysis of the *O*-linked saccharides.** The saccharides released by alkaline borohydride from the sample chased with ALL were treated with or without *A. ureafaciens* sialidase (*AUS*) (20 milliunits) in 30 μl of 35 mM sodium cacodylate, pH 5.8, or calf intestinal phosphatase (2 units) in 37 mM glycine, 190 mM NaCl, 18 mM Na_2CO_3, pH 10.6, for 6 h at 37 °C. Other aliquots were incubated in 2 M HOAc at 80 °C for 3 h, lyophilized, and treated with and without alkaline phosphatase. The samples were 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. Treatments with HOAc alone or alkaline phosphatase alone gave no change in the profile (not shown, see data in Table I).

Sephadex fractionation and reanalysis. The neutral species quantitatively co-migrated with the monosaccharide standard on paper chromatography, and with [^{3}H]GalNAcitol in AX-5 HPLC analysis (data not shown). These data indicate that if additional sugars (*e.g.* Gal and/or GlcNAc) are added to the [^{3}H]GalNAc-Ser/Thr residue, the resulting chains are almost completely converted to anionic species. The relatively small size of the anionic oligosaccharides was confirmed by gel permeation chromatography on Sephadex G-10, in which it was not possible to completely separate the saccharides from the salt peak (data not shown).

Exoglycosidase Digestion of the O-Linked Saccharides—GalNAc can occur at the nonreducing termini of *O*-linked oligosaccharides, *e.g.* GalNAcβ1,4Gal as on the Cad or Sda antigen (39, 40), GalNAcα1,3GalNAc-Ser/Thr (Core 6), or in blood A group antigens (41). However, treatment of the anionic molecules with Jack bean β-hexosaminidase or α-*N*-acetylgalactosaminidase gave no significant increase (<2%) in radioactivity passing through QAE-Sephadex (data not shown). This indicates that either the [^{3}H]GalNAc is not at the nonreducing termini, or that it is resistant to the action of these glycosidases because of ionic substitutions. However, since there was no increase in incorporation of total label when ALL was added to the chase, it is very likely that all of the transferred label is in the linkage region, and not at nonreducing termini. Thus, [^{3}H]GalNAc is transferred from UDP-[^{3}H]GalNAc to Ser/Thr residues of the polypeptide acceptors, and in the absence of added nucleotide donors, these residues are not extended. If these nucleotide donors are added, the [^{3}H]GalNAc-Ser/Thr is not only covered by other monosaccharides, but these are efficiently converted into anionic species.

DEAE HPLC of the O-Linked Saccharides—The total *O*-linked saccharides from the sample chased with ALL was analyzed by DEAE HPLC in conjunction with various treatments to determine the number and nature of the negative charges. As shown in Fig. 8, the expected fraction (~30%) passed through the column and there were two major and one minor anionic species in the retained material (see Table I for percentages). Treatment with *A. ureafaciens* sialidase, alkaline phosphatase, or a combination of mild acid followed by alkaline phosphatase did not substantially affect the elution profile (mild acid treatment alone caused a very slight shift toward fewer negative charges). Thus, the great majority of the negative charge on the [^{3}H]GalNAc-labeled *O*-linked saccharides is not due to sialic acid residues, phosphomonoesters, or phophodiesters. While sulfate esters are the most likely explanation, the lack of availability of a broad spectrum sulfatase makes it difficult to address this possibility directly. Besides sulfate, the only other possible explanations for the negative charge on these saccharides is the presence of uronic

TABLE I

Distribution of radioactivity in O-linked oligosaccharides

The radioactivity associated with peaks *a–d* (see Fig. 8, for examples) was determined and the percent in each peak relative to the sum of the peaks was calculated.

Treatment	HPLC peak			
	a	*b*	*c*	*d*
	% of total			
None	36	36	23	5
AUS[a]	32	36	26	6
Alkaline phosphatase	34	38	23	5
HOAc	40	37	20	4
HOAc/alkaline phosphatase	38	38	21	4

[a] *A. ureafaciens* sialidase.

acids or portions of peptide. The arguments against the [^{3}H]GalNAc being associated with peptide are 2-fold. First, the radiolabel did not bind to Dowex 50 during the purification. Second, the radioactivity migrated as relatively small species on paper chromatography in a system where peptides markedly retard migration. Uronic acids are typically associated with proteoglycans, and have not been previously reported on mammalian *O*-linked saccharides. However, their presence cannot be ruled out based on the data presented so far.

To obtain alternate evidence for sulfate esters and to rule out uronic acids, we performed DEAE HPLC fractionation at two different pH values. At pH 5.5, all possible anionic substituents of oligosaccharides should be deprotonated, whereas at pH 2.45 sialic acids, uronic acids, and phosphates should be at least partially protonated, while sulfate groups should remain fully negatively charged. The samples were therefore analyzed at the two different pH values, in comparison with dermatan sulfate fragments (consisting of alternating iduronic acid and GalNAc-4-sulfate residues). As shown in Fig. 9, the anionic [^{3}H]GalNAc-labeled *O*-linked oligosaccharides eluted from the DEAE column in two major peaks. At pH 2.45 (where ~80% of the iduronic acid should be protonated), the dermatan sulfate standards elute from the DEAE column at a lower NaCl concentration than at pH 5.5. At pH 2.45, the anionic [^{3}H]GalNAc-labeled *O*-linked oligosaccharides eluted from the DEAE column at a position similar to the dermatan sulfate fragments, suggesting the presence of one and two sulfate groups. The first anionic species split into a doublet, suggesting either differences in the position of the presumed sulfate groups, or differences in the charge:mass ratio of these saccharides. Interestingly, the [^{3}H]GalNAc-labeled *O*-linked oligosaccharides actually eluted with a slightly higher concentration of NaCl at pH 2.45 than at pH 5.5. This cannot be accounted for by the decrease in pH because the saccharides should be either unchanged or more protonated at the lower pH. The most likely explanation is that since the ionic strength of the potassium phosphate used to buffer the column is somewhat lower at pH 2.45 than at pH 5.5, and slightly more NaCl was needed to elute the bound molecules. A similar effect may not be as evident with the dermatan sulfate oligomers because the charge on these molecules is also being partially reduced by protonation of the uronic acid groups. Taken together, these data indicate that the negative charge on the anionic [^{3}H]GalNAc-labeled *O*-linked saccharides is most likely due to sulfate esters.

DISCUSSION

When rat liver Golgi preparations are incubated with UDP-[^{3}H]GalNAc, label is transferred to *N*- and *O*-linked oligosaccharides on glycoproteins that are within the lumen of intact compartments. These are protected from degradation by added protease unless Triton X-100 is added to a sufficiently high concentration (greater than the critical micellar concentration), indicating that they are located in the lumen of intact vesicles that have the appropriate topological orientation, and that are therefore similar to the compartments of the Golgi apparatus in the intact cell. Several glycoproteins are radiolabeled, and most are distinct from those radiolabeled with UDP-[^{3}H]GlcNAc. A few specific [^{3}H]GalNAc-labeled proteins are susceptible to PNGase F, indicating that a portion of the [^{3}H]GalNAc had been transferred to *N*-linked oligosaccharides. The radiolabeled *N*-linked oligosaccharides were isolated and their structures characterized. Several neutral oligosaccharides were labeled with [^{3}H]GalNAc, and the majority of these were not retained by ConA-Sepharose, indicating that they were tri- or tetra-antennary in nature. Their small size indicates that they may be biosynthetic intermediates. A variety of anionic oligosaccharides were also detected. The anionic oligosaccharides quantitatively passed through the ConA column, indicating that they are also tri- or tetra-antennary in nature. The negative charge on these molecules is due to sialic acid and phosphate. A novel acid labile GalNAc was detected that is released with kinetics consistent with it being linked through a phosphodiester bond. In contrast to the GlcNAc-P-Man phosphodiesters of lysosomal enzymes which are found on high mannose- and hybrid-type *N*-linked oligosaccharides (35, 42), these GalNAc phosphodiesters were found exclusively on complex-type chains. These data show the existence of a whole new class of GalNAc-containing *N*-linked oligosaccharides, and demonstrate that this *in vitro* approach to studying *N*-linked oligosaccharide biosynthesis can detect previously undescribed structures.

This approach is also extended here to studying *O*-linked oligosaccharide biosynthesis. Sucrose density gradient centrifugation has previously been used to study the possible colocalization of UDP-GalNAc:peptide-α-*N*-acetylgalactosaminyltransferase and UDP-Gal:GalNAcβ1,3-galactosyltransferase, the first enzyme to elongate the commonest type of *O*-linked oligosaccharide core (38, 41). The two activities fractionated into regions of different density, the galactosyltrans-

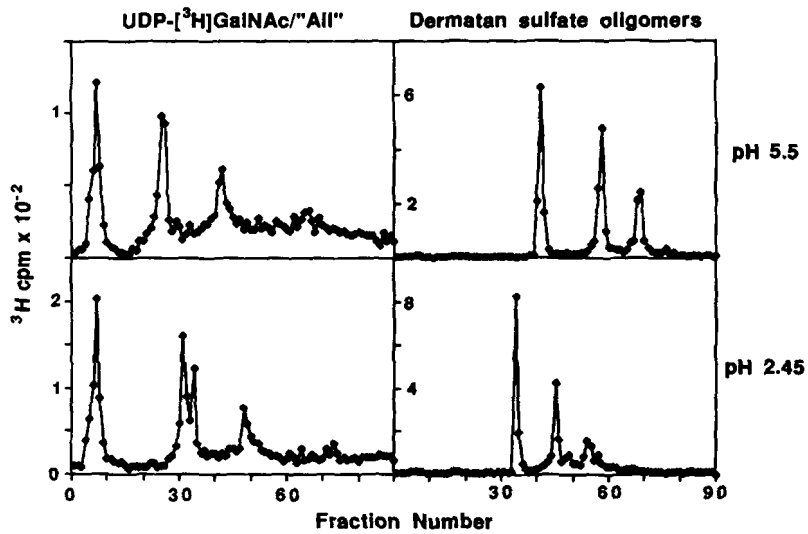

FIG. 9. **DEAE HPLC analysis of the anionic *O*-linked saccharides at different pH values.** The saccharides released by alkaline borohydride from the sample chased with ALL were analyzed by DEAE HPLC as described in Fig. 8 except that the column was buffered with 10 mM potassium phosphate at pH 5.5 or 2.45. Oligomers of dermatan sulfate are presented for comparison. The structures of these are (IdAβ1,3(4)GalNAc-4-sulfate)$_n$, where n = 1, 2, and 3, and the last monosaccharide is reduced.

ferase co-migrating with UDP-Gal:GlcNAcβ1,4-galactosyltransferase and the GalNAc transferase migrating in a region of higher density, presumably representing a location in earlier regions of the Golgi apparatus, or the endoplasmic reticulum. However, as with many such studies, there was a region of the density gradient that contained both activities. This region of overlap may represent incomplete separation of two distinct compartments carrying the two enzymes, or may reflect a compartment that contains both enzyme activities. The data presented in this paper provide alternate evidence for such compartmental overlap.

The underlying structure of the presumed sulfated *O*-linked saccharides described here remains unclear, and the lack of a broad-spectrum sulfatase limits the analysis. While it is possible that the [^{3}H]GalNAc residue is itself sulfated, this structure has not been described so far. It is more likely that the [^{3}H]GalNAc has had other monosaccharides attached to it which are themselves sulfated. *O*-Linked oligosaccharides with class 1 and class 2 core structures have been described in the rat liver (Ref. 43, and references therein). These saccharides have the structures Galβ1,3GalNAc-*O*-Ser/Thr and Galβ1,3(GlcNAcβ1,6)GalNAc-*O*-Ser/Thr, respectively (41). There are several reports of such *O*-linked oligosaccharides with sulfate linked to the 6-position of GlcNAc, or the 3- or 6-position of a Gal residue (44–48), in other tissues. While several of these fit the criterion of being small, anionic, and having sulfate as the only anionic substituent, it is unclear which, if any, of these is being synthesized in the rat liver. The minor fractions with several negative charges (peaks *c* and *d* in Fig. 8) cannot be accounted for by any previously reported structures and could represent novel multiply sulfated *O*-linked saccharides in the rat liver. One common aspect of all the oligosaccharides is that they have 2 or more monosaccharide residues, and most have a β1,3-linked galactose. Thus, one or more glycosyltransferases, such as the UDP-Gal:GalNAcβ1,3-galactosyltransferase (an enzyme known to be in the rat liver (43), must be functionally co-localized with UDP-GalNAc:peptide-*N*-acetylgalactosaminyltransferase. The complete lack of sialylation of these molecules may indicate that the sialyltransferases which recognize *O*-linked saccharides are located in more distal compartments of the Golgi apparatus, or that the CMP-sialic acid donor is unavailable in the compartment where the [^{3}H]GalNAc is being added.

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