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THE SEPARATION BY LIQUID CHROMATOGRAPHY (UNDER ELEVATED PRESSURE) OF PHENYL, BENZYL, AND *o*-NITROPHENYL GLYCOSIDES OF OLIGOSACCHARIDES. ANALYSIS OF SUBSTRATES AND PRODUCTS FOR FOUR *N*-ACETYL-D-GLUCOSAMINYLTRANSFERASES INVOLVED IN MUCIN SYNTHESIS*,†

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ABSTRACT

Liquid chromatography under elevated pressure (h.p.l.c.) has been applied to the separation of the phenyl, benzyl, and *O*-nitrophenyl glycosides of 2-acetamido-2-deoxy-D-galactopyranose and of various mucin-type, di-, tri-, and tetra-saccharides. The separations were carried out with a Whatman Partisil PXS 5/25 PAC column and various proportions of acetonitrile and water in the mobile phase. These methods were subsequently used to separate the substrates and products of the following *N*-acetylglucosaminyltransferase reactions: UDP-GlcNAc + β-Gal-(1→3)-GalNAc-R → β-Gal-(1→3)-[β-GlcNAc-(1→6)]-GalNAc-R + UDP (*1*); UDP-GlcNAc + β-Gal-(1→3)-[β-GlcNAc-(1→6)]-GalNAc-R → β-GlcNAc-(1→3)-β-Gal-(1→3)-[β-GlcNAc-(1→6)]-GalNAc-R + UDP (*2*); UDP-GlcNAc + GalNAc-R′ → β-GlcNAc-(1→3)-GalNAc-R′ + UDP (*3*); and UDP-GlcNAc + β-GlcNAc-(1→3)-GalNAc-R′ → β-GlcNAc-(1→6)-[β-GlcNAc-(1→3)]-GalNAc-R′ + UDP (*4*), where R = benzyl or *o*-nitrophenyl, and R′ = benzyl or phenyl α-D-glycoside. Reaction *1* is catalyzed by a transferase in canine submaxillary glands and porcine gastric mucosa, and reaction *2* by an enzyme in porcine gastric mucosa. Enzyme activities catalyzing reactions *3* and *4* have recently been demonstrated in rat colonic mucosa. Liquid chromatography can be used at the preparative level for the purification and identification of the transferase products, and at the analytical level in the assay of glycosyltransferases.

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INTRODUCTION

Mucins are high-molecular-weight glycoproteins that contain oligosaccharides bound to a polypeptide backbone with an *O*-glycosyl linkage between a 2-acetamido-2-deoxy-D-galactose and either a serine or threonine residue. These oligosaccharides vary greatly in size and complexity. In a recent review, Schachter and Williams[2] pointed out that mucin oligosaccharides can be classified into simple oligosaccharides containing only a single sugar residue* (GalNAc) or one of the following three disaccharides, α-Sia-(2→6)-GalNAc, β-Gal-(1→3)-GalNAc, or β-GlcNAc-(1→3)-GalNAc, and into larger oligosaccharides having at least four classes of core, *i.e.*, β-Gal-(1→3)-GalNAc (core *I*), β-Gal-(1→3)-[β-GlcNAc-(1→6)]-GalNAc (core *II*), β-GlcNAc-(1→3)-GalNAc (core *III*), and β-GlcNAc-(1→3)-[β-GlcNAc-(1→6)]-GalNAc (core *IV*). All four classes are usually elongated by the addition of oligosaccharide sequences containing β-(1→3)- and β-(1→4)-linked Gal residues; and β-(1→3)-, β-(1→4)-, and β-(1→6)-linked GlcNAc residues[2]. In this laboratory, four novel *in vitro* *N*-acetylglucosaminyltransferase (GlcNAc-transferase) activities involved in mucin oligosaccharide synthesis have been demonstrated: (a) the addition of GlcNAc in β-D-(1→6) linkage to the GalNAc residue of β-Gal-(1→3)-GalNAc-mucin to form the class *II* core[3-5], (b) the elongation[1,6,7] of core II class, and (c) the synthesis of the class *III* and *IV* core structures[7]. The reactions catalyzed are as follows:

UDP-GlcNAc + β-Gal-(1→3)-GalNAc-R → β-Gal-(1→3)-[β-GlcNAc-(1→6)]-GalNAc-R + UDP (*1*)

UDP-GlcNAc + β-Gal-(1→3)-[β-GlcNAc-(1→6)]-GalNAc-R → βGlcNAc-(1→3)-β-Gal-(1→3)-[β-GlcNAc-(1→6)]-GalNAc-R + UDP (*2*)

UDP-GlcNAc + GalNAc-R′ → β-GlcNAc-(1→3)-GalNAc-R′ + UDP (*3*)

and UDP-GlcNAc + β-GlcNAc-(1→3)-GalNAc-R′ → β-GlcNAc-(1→6)-[β-GlcNAc-(1→3)]-GalNAc-R′ + UDP (*4*)

Reaction *1* is catalyzed by UDP-GlcNAc:β-Gal-(1→3)-GalNAc-R (GlcNAc-to-GalNAc) 6-β-GlcNAc-transferase (core 6-β-GlcNAc-transferase A) present in canine submaxillary glands and porcine gastric mucosa. Reaction *2* is catalyzed by UDP-GlcNAc:β-Gal-(1→3)-[-β-GlcNAc-(1→6)]-GalNAc-R (GlcNAc-to-Gal) 3-β-GlcNAc-transferase (elongation 3-β-GlcNAc-transferase) present in porcine gastric mucosa. Enzyme activities catalyzing reactions *3* and *4* have recently been demonstrated in rat colonic mucosa and are named, respectively, UDP-GlcNAc:GalNAc-R 3-β-GlcNAc-transferase (core 3-β-GlcNAc-transferase) and UDP-GlcNAc:β-GlcNAc-(1→3)-GalNAc-R (GlcNAc-to-GalNAc) 6-β-GlcNAc-transferase (core 6-β-GlcNAc-transferase B). The four enzymes are readily as-

*Unless otherwise stated, all monosaccharides are in the D configuration and the pyranose form

sayed by use as GlcNAc acceptors, of synthetic oligosaccharide α-D-glycosides [R = phenyl (Ph), benzyl (Bn), or *o*-nitrophenyl (ONP)].

The assay of enzymes in crude membrane preparations may result in the formation of more than one radioactive product. This may occur because more than one enzyme acts on a particular substrate, or because degradation of substrate by lysosomal glycosidases leads to substrates for other enzymes. We have, therefore, developed methods of liquid chromatography under elevated pressure (h.p.l.c.) for separating the oligosaccharide glycosides that are either substrates for or products from the four aforementioned reactions. We have also examined the chromatographic properties of some synthetic isomers of these oligosaccharides. Most previous studies on glycosyltransferase products have involved separation of reduced oligosaccharides or oligosaccharide glycosides by gel filtration, paper chromatography, thin-layer chromatography, and paper electrophoresis. Recent reports have described the use of liquid chromatography under elevated pressure to separate reduced oligosaccharides formed by sialyl- and galactosyl-transferases[8–11], but this is the first report on the use of h.p.l.c. in the separation of GlcNAc-transferase products, and of the phenyl, benzyl, and *o*-nitrophenyl glycosides of oligosaccharides. Liquid chromatography under elevated pressure has also been used to separate oligosaccharides and oligosaccharide derivatives obtained from glycoproteins, lipid-linked oligosaccharides, and other sources[12–18].

EXPERIMENTAL

Materials. — The following materials were purchased from commercial sources: UDP-2-acetamido-2-deoxy-D-[U-^{14}C]glucose, 213 mCi/mmol, and ACS (Aqueous Counting Scintillant) scintillation fluid (Amersham); UDP-GlcNAc and Triton X-100 (Sigma); *p*-nitrophenyl β-D-galactopyranoside (P-L Biochemicals); phenyl 2-acetamido-2-deoxy-α-D-galactopyranoside (Koch-Light); sodium 2-(*N*-morpholino)ethanesulfonate (MES) (Calbiochem-Behring); Bio-Gel P-2 (200–400 mesh), Bio-Gel P-4 (−400 mesh), Chelex-100 (Na^+, 100–200 mesh), and AG 1 X8 (100–200 mesh) (Bio-Rad); and acetonitrile (u.v. grade, Caledon Laboratories).

The following compounds were synthesized by methods that are described elsewhere: Ph α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-α-D-Gal*p*NAcide[19]; Bn β-D-Glc*p*NAc-(1→3)-α-D-Gal*p*NAcide and β-D-Glc*p*NAc-(1→6)-α-D-Gal*p*NAcide[20]; Bn β-D-Glc*p*NAc-(1→6)-[β-D-Glc*p*NAc-(1→3)]-α-D-Gal*p*NAcide[21]; Bn α-D-Gal*p*NAcide; ONP β-D-Glc*p*NAc-(1→6)-α-D-Gal*p*NAcide; Bn β-D-Fuc*p*-(1→3)-[β-D-Glc*p*NAc-(1→6)]-α-D-Gal*p*NAcide; Bn β-D-Glc*p*NAc-(1→6)-β-D-Gal-(1→3)-[β-D-Glc*p*NAc-(1→6)]-α-D-Gal*p*NAcide; Bn and ONP β-D-Gal*p*-(1→3)-α-D-Gal*p*NAcide; and Bn, Ph, and ONP β-D-Gal*p*-(1→3)-[β-D-Glc*p*NAc-(1→6)]-α-D-Gal*p*NAcide [22].

Liquid chromatography under elevated pressure (h.p.l.c.). — Separations were performed with a Beckman Model 324 Gradient Liquid Chromatograph equipped with a Model 421 System Controller, a Model 100A solvent metering sys-

tem, and a Partisil PXS 5/25 PAC column (Whatman) protected with a CSK guard column packed with ODS (Whatman) under 6.4–8.6 MPa of pressure. Samples (2–25 μL) were injected with a Beckman Model 210 sample-injection valve. The radioactive samples contained ~2000–6000 d.p.m. per injection. Standard compounds were determined each experiment day. Mixtures of acetonitrile (u.v. grade) and de-ionized water were used as the mobile phase at room temperature. The flow rate was 1 mL/min. Chromatographic separations were monitored by u.v. absorption at 195 nm with a Hitachi Model 100-40 spectrophotometer. The elution of radioactive compounds was determined by collecting fractions and liquid-scintillation counting. The reproducibility of retention times from experiment to experiment was routinely >90%.

Enzyme preparations. — *(a) From pig gastric mucosa.* Stomachs from freshly-slaughtered pigs were washed and the mucosal lining was cleared of overlying mucus with a glass slide. The cardiac zone of the stomach was discarded. The mucosa of the remaining stomach was scraped off with a scalpel blade and homogenized with a Polytron homogenizer[3] in ice-cold 0.25M sucrose-0.2M NaCl (300 mL). All subsequent steps were at 4°. The homogenate was centrifuged for 15 min at 10 000 r.p.m. with a JA17 rotor in a Beckman J21 centrifuge, and the supernatant was centrifuged at 140 000*g* for 20 min to produce a microsome pellet. Pellets were homogenized in minimal volumes of 0.25M sucrose and stored at −70°.

(b) From rat colon. Adult rats were starved for 24–48 h. Colons were removed and washed with 0.9% NaCl at 4°. The overlying mucus was removed by gentle scraping with a glass slide, and the mucosa was scraped off with a glass slide, hand homogenized in minimal volumes of 0.25M sucrose, and stored at −70°. The protein concentration was 14 mg/mL.

Preparation of radioactive enzyme products. — All incubations were carried out at 37°.

(a) With o-nitrophenyl β-Gal-(1→3)-α-GalNAcide as acceptor. The incubation mixture contained the following components in a final volume of 1.0 mL: mM acceptor, 10mM $MnCl_2$, 0.1M MES (pH 7.0), 2.9mM UDP-[^{14}C]GlcNAc (434–520 d.p.m./nmol), 0.25% (v/v) Triton X-100, and porcine-gastric mucosa (4.9 mg of protein). After 3 h of incubation, the reaction was stopped by freezing and the incubation mixture subjected to high-voltage paper electrophoresis in 1% sodium tetraborate, followed by descending paper chromatography for 9 h with 80% ethanol. The product was eluted from the paper with 50% methanol, and borate ions were removed by repeated flash-evaporation from a methanol solution containing 0.5% acetic acid. Gel filtration on Bio-Gel P-2 removed salt and sucrose, and resolved two radioactive fractions. The product eluted first from the P-2 column was identified as ONP β-[^{14}C]GlcNAc-(1→3)-β-Gal-(1→3)-{β-[^{14}C]GlcNAc-(1→6)}-α-GalNAcide and the retarded fraction as ONP β-Gal-(1→3)-{β-[^{14}C]GlcNAc-(1→6)}-α-GalNAcide[1,7].

(b) With Bn β-Gal-(1→3)-[β-GlcNAc-(1→6)]-α-GalNAcide as acceptor. The incubation mixture contained the following components in a final volume of 1.0

mL: 2mM acceptor, 10mM $MnCl_2$, 0.1M MES, (pH 7.0), 3.3mM UDP-[^{14}C]GlcNAc (434–520 d.p.m./nmol), 0.1% (v/v) Triton X-100, and porcine gastric mucosa (7.4 mg of protein). After 1 h, further enzyme (3.7 mg) was added. The incubation was carried out for a total of 22 h, and the product was isolated as described under *(a)*. A single radioactive fraction was obtained and was subjected to ^{1}H-n.m.r. spectroscopy. The compound was identified as Bn β-[^{14}C]GlcNAc-(1→3)-β-Gal-(1→3)-[β-GlcNAc-(1→6)]-α-GalNAcide[1,7].

*(c) With phenyl 2-acetamido-2-deoxy-α-*D*-galactopyranoside as acceptor.* The product was prepared by incubating the following in a total volume of 2.5 mL: 4mM acceptor, 10mM $MnCl_2$, 0.1M MES (pH 6.5), 2.9mM UDP-[^{14}C]GlcNAc (180–550 d.p.m./nmol), 0.2% (v/v) Triton X-100, and rat colon mucosa (14 mg of protein). After 2 h, the product was prepared as described under *(a)*, except for the omission of the 80%-ethanol chromatographic step. Gel filtration on Bio-Gel P-2 resolved two radioactive fractions, the larger of which was analyzed by ^{1}H-n.m.r. spectroscopy.

*(d) With benzyl 2-acetamido-2-deoxy-α-*D*-galactopyranoside as acceptor.* The following were incubated in a total volume of 3.75 mL: 4mM acceptor, 10mM $MnCl_2$, 0.1M MES (pH 6.5), 2.9mM UDP-[^{14}C]GlcNAc (180–550 d.p.m./nmol), 0.2% (v/v) Triton X-100, and rat colon mucosa (21 mg of protein). After 2 h, the product was prepared as described under *(c)*. Gel filtration on Bio-Gel P-2 column (twice, 118 × 0.7 cm) and P-4 column (200 × 1.5 cm) in water resolved two radioactive fractions. The major peak (85% of total radioactivity) was retarded relative to the minor peak.

In order to test the usefulness of liquid chromatography as a routine assay for GlcNAc-transferase activity, the aforementioned incubation was scaled down to a total volume of 50 μL. The reaction was stopped after 2 h by the addition of 20mM sodium tetraborate–mM EDTA (0.4 mL), and the incubation mixture was passed through a 1-mL column (Pasteur pipette) of AG 1 X8 (Cl^-, 100–200 mesh) resin equilibrated with water. The product was obtained by washing the column with water (3.0 mL), and either subjected to liquid scintillation counting, or dried by flash evaporation or lyophilization and redissolved in a minimal amount of water for analysis by h.p.l.c.

Protein determination. — The protein content was determined by the procedure of Lowry *et al.*[23] using bovine serum albumin as standard.

^{1}H-N.m.r. spectroscopy. — Heavy metals were removed by passage of the sample through Chelex 100 columns (0.7 × 8 cm) extensively washed with distilled water. The samples were flash-evaporated three times from a solution in 99.8% D_2O, dried from 99.96% D_2O (Aldrich) in a vacuum desiccator in the presence of P_2O_5, and dissolved in a dry box in 99.96% D_2O. Spectra were recorded with a Nicolet 360-MHz spectrometer as previously described[24,25].

RESULTS AND DISCUSSION

Synthetic oligosaccharide glycosides. — Table I summarizes the separations achieved when various synthetic phenyl, benzyl, and *o*-nitrophenyl oligosaccharide α-D-glycosides were subjected to h.p.l.c. on a Partisil PAC column. Several investigations[8-14] have reported good resolution of unsubstituted or reduced oligosaccharides on h.p.l.c. with amino columns. PAC columns were used in this work since the presence of both nitrile and amino functional groups make the PAC-column, bonded phase less polar than that of an amino column, and the PAC column should, therefore, be preferable for resolving the hydrophobic glycosides shown in Table I. However, a detailed comparative study of PAC and amino columns was not carried out. Also shown in Table I are the retention times of sucrose,

TABLE I

RETENTION TIMES OF SYNTHETIC OLIGOSACCHARIDE GLYCOSIDES[a]

Compound[b]	*Retention time (min)*		
	Solvent A	*Solvent* B	*Solvent* C
Ph αGalNAc	4.5	5.5	
Ph βGal→3(βGlcNAc→6)αGalNAc	20	49	
Ph αL-Fuc→2βGal→3αGalNAc	11		
Bn αGalNAc	4.5	5.5	6
Bn βGal→3αGalNAc	8.5	13	22
Bn βGal→3(βGlcNAc→6)αGalNAc	20	47	
Bn βGlcNAc→6βGal→3(βGlcNAc→6)αGalNAc	47		
Bn βD-Fuc→3(βGlcNAc→6)αGalNAc	14		
Bn βGlcNAc→3αGalNAc	10.5	15.5	31
Bn βGlcNAc→6αGalNAc	9.5	13.5	25
Bn βGlcNAc→3(βGlcNAc→6)αGalNAc	25	61	
Bn β[^{14}C]GlcNAc→3βGal→3 (βGlcNAc→6)αGalNAc	42		
ONP βGal→3αGalNAc	8	11.5	17
ONP βGlcNAc→6αGalNAc	8.5	13	21
ONP βGal→3(βGlcNAc→6)αGalNAc	17	38	
ONP β[^{14}C]GlcNAc→3βGal→3 (β[^{14}C]GlcNAc→6)αGalNAc	40		
PNP βGal		5	5
GlcNAc	8.5	12	15
Sucrose	14	19	28
Triton X-100	4		4

[a]Compounds were analyzed by l.c. as described in the Experimental section. Three solvent systems were used as the mobile phase (all v/v) (*A*) 83:17 acetonitrile–water, (*B*) 87.13 acetonitrile–water, and (*C*) 9:1 acetonitrile–water. [b]Abbreviations: Ph, phenyl; Bn, benzyl; ONP, *o*-nitrophenyl, and PNP, *p*-nitrophenyl.

Triton X-100, and free 2-acetamido-2-deoxy-D-glucose. Sucrose and Triton X-100 are present in the incubation mixtures, and 2-acetamido-2-deoxy-D-glucose may be formed from the breakdown of UDP-GlcNAc. Three different isocratic acetonitrile–water solvent systems were tested.

In general, resolution was enhanced and retention times were increased by decreasing the water content of the mobile phase. Two of the three phenyl glycosides available to us showed retention times identical to those of the corresponding benzyl glycosides in both solvent systems *A* and *B*. However, the three *o*-nitrophenyl oligosaccharides tested were eluted earlier than the corresponding benzyl derivatives in all three solvent systems. This indicates that the *o*-nitrophenyl glycosides are more hydrophobic than the benzyl derivatives, possibly owing to hydrogen-bond formation between hydroxyl and nitro groups.

Monosaccharide glycosides were eluted first, within 4–6 min depending on the solvent system. Disaccharide glycosides were eluted next (8–11 in solvent *A*, 11–16 in solvent *B*, and 17–31 min in solvent *C*), followed by trisaccharide glycosides containing fucose (11–14 min in solvent *A*), trisaccharide glycosides lacking fucose (17–25 in solvent *A*, and 38–61 min in solvent *B*) and, finally, tetrasaccharide glycosides (47 min in solvent *A*).

The isomers Bn β-GlcNAc-(1→3)- and -(1→6)-α-GalNAcide separated well in all three solvent systems, the (1→6) always being eluted before the (1→3) isomer. This separation suggests that the two isomers differ in their three-dimensional orientations and in the availability of hydroxyl groups for binding to the PAC column.

R β-Gal-(1→3)-α-GalNAcide → R β-Gal-(1→3)-[β-GlcNAc-(1→6)]-α-GalNAcide → R β-GlcNAc-(1→3)-β-Gal-(1→3)-[β-GlcNAc-(1→6)]-α-GalNAcide

R = Bn or ONP

Scheme 1

Products formed by core 6-β-GlcNAc-transferase A and elongation 3-β-GlcNAc-transferase. — The two enzymes carrying out the sequence of reactions described in Scheme 1 have been studied *in vitro*[1–7]. The oligosaccharides produced all occur in mucins, and both enzymes have been shown to act on mucin substrates as well as on the benzyl and *o*-nitrophenyl glycosides. H.p.l.c. was used to verify the product identifications previously carried out by other means. When ONP β-Gal-(1→3)-α-GalNAc was incubated with UDP-[^{14}C]GlcNAc and porcine, gastric-mucosal extract under the conditions described in the Experimental section, the product previously identified as ONP β-[^{14}C]GlcNAc-(1→3)-β-Gal-(1→3)-{β-[^{14}C]GlcNAc-(1→6)}-α-GalNAcide was eluted as a single radioactive peak with a retention time of about 40 min in solvent *A* (Peak B, Fig. 1c), and the product pre-

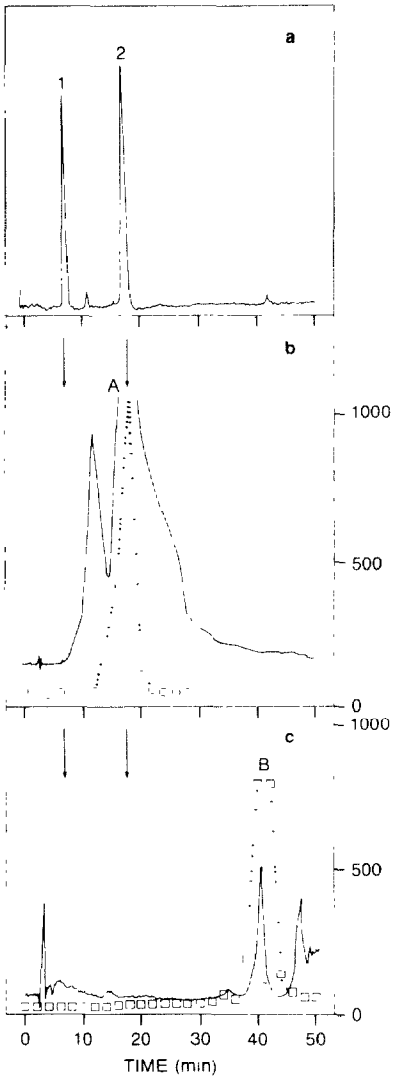


Fig. 1 Liquid chromatograms under elevated pressure of *o*-nitrophenyl glycosides with solvent system *A* (83:17, v/v acetonitrile–water) as described in the Experimental section: (a) Standard synthetic compounds: (1) ONP β-Gal-(1→3)-α-GalNAcide, and (2) ONP β-Gal-(1→3)-[β-GlcNAc-(1→6)]-α-GalNAcide. The radioactive products obtained by the action of porcine-gastric mucosal extract on ONP β-Gal-(1→3)-α-GalNAcide were analyzed on this system (b) Lower-molecular-weight fraction from the Bio-Gel P-2 column. A is ONP β-Gal-(1→3)-{β-[^{14}C]GlcNAc-(1→6)}-α-GalNAcide (c) Higher-molecular-weight-fraction from the Bio-Gel P-2 column: B is ONP β-[^{14}C]GlcNAc-(1→3)-β-Gal-(1→3)-{β-[^{14}C]GlcNAc-(1→6)}-α-GalNAcide Absorbance at 195 nm, ——, and d.p.m., ····.

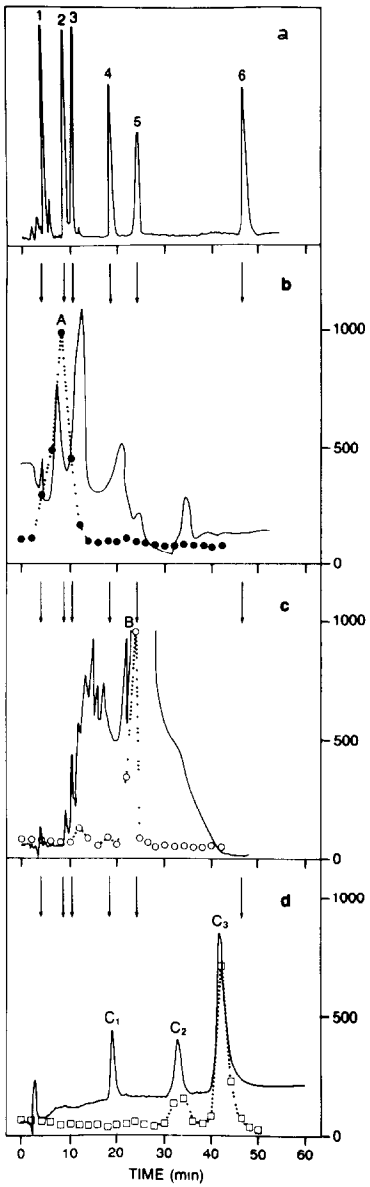


Fig. 2. Liquid chromatograms under elevated pressure of benzyl glycosides with solvent system *A*(83:17, v/v acetonitrile–water) as described in the Experimental section. (a) Standard synthetic compounds: (1) Bn α-GalNAcide; (2) Bn β-GlcNAc-(1→6)-α-GalNAcide; (3) Bn β-GlcNAc-(1→3)-α-GalNAcide; (4) Bn β-Gal-(1→3)-[β-GlcNAc-(1→6)]-α-GalNAcide; (5) Bn β-GlcNAc-(1→3)-[β-GlcNAc-(1→6)]-α-GalNAcide; and (6) Bn β-GlcNAc-(1→6)-β-Gal-(1→3)-[β-GlcNAc-(1→6)]-α-GalNAcide. The radioactive products obtained by the action of rat-colon-mucosal extract on Bn α-GalNAcide were analyzed on this system. (b) Lower-molecular-weight-fraction from the Bio-Gel P-4 column: A is [^{14}C]GlcNAc. (c) Higher-molecular-weight fraction from the Bio-Gel P-4 column: B is Bn β-[^{14}C]GlcNAc-(1→3)-{β-[^{14}C]GlcNAc-(1→6)}-α-GalNAcide. (d) The product obtained by the action of porcine-gastric-mucosal extract on Bn β-Gal-(1→3)-[β-GlcNAc-(1→6)]-α-GalNAcide: (C_1) Bn β-Gal-(1→3)-[β-GlcNAc-(1→6)]-α-GalNAcide; (C_2) non identified; and (C_3) Bn β-[^{14}C]GlcNAc-(1→3)-β-Gal-(1→3)-[β-GlcNAc-(1→6)]-α-GalNAcide. Absorbance at 195 nm, ——; and d.p.m., ·····.

viously identified as ONP β-Gal-(1→3)-{β-[^{14}C]GlcNAc-(1→6)}-α-GalNAcide was eluted with a retention time of about 17 min in solvent *A* (Peak A, Fig. 1b). The latter retention time is identical to that of the synthetic compound (Peak 2, Fig. 1a). A synthetic ONP α-tetrasaccharide standard was not available for testing, but a retention time of 40 min is consistent with what would be expected for a tetrasaccharide. We did not have synthetic ONP β-GlcNAc-(1→3)-β-Gal-(1→3)-α-GalNAcide in order to determine whether this compound would be resolved from ONP β-Gal-(1→3)-[β-GlcNAc-(1→6)]-α-GalNAcide. However, the presence of a single, sharp radioactive peak in the region of ONP β-Gal-(1→3)-[β-GlcNAc-(1→6)]-α-GalNAcide on h.p.l.c. in both solvents *A* (Fig. 1b) and *B* (not shown) supports our previous conclusion that the 3-β-GlcNAc-transferase does not act on ONP β-Gal-(1→3)-α-GalNAcide to form ONP β-[^{14}C]GlcNAc-(1→3)-β-Gal-(1→3)-α-GalNAcide[1,7].

The product of 3-β-GlcNAc-transferase action on Bn β-Gal-(1→3)-[β-GlcNAc-(1→6)]-α-GalNAcide (see Experimental section) has previously been identified as Bn β-[^{14}C]GlcNAc-(1→3)-β-Gal-(1→3)-[β-GlcNAc-(1→6)]-α-GalNAcide[1,7]. On h.p.l.c. analysis of this product with solvent system A (Fig. 2d), three u.v.-absorbing peaks were obtained. The first peak (C_1) was nonradioactive and was eluted with the same retention time as Bn β-Gal-(1→3)-[β-GlcNAc-(1→6)]-α-GalNAcide (Peak 4, Fig. 2a); therefore, it represents the substrate that had not been removed from the product. The second peak (C_2, Fig. 2d), eluted at 33 min, was radioactive (24% of total radioactivity) and remains to be identified. The third peak (C_3, Fig. 2d) represented the main radioactive product and was eluted just before Bn β-GlcNAc-(1→6)-β-Gal-(1→3)-[β-GlcNAc-(1→6)]-α-GalNAcide (Peak 6, Fig. 2a) with a retention time of about 42 min. This retention time is consistent with the radioactive compound being a tetrasaccharide. It is interesting that the (1→3) isomer of the tetrasaccharide (Peak C_3, Fig. 2d) was eluted prior to the (1→6) isomer (Peak 6, Fig. 2a), whereas the (1→6) isomer of Bn GlcNAc→α-GalNAcide was eluted before the (1→3) isomer (Peaks 2 and 3, Fig. 2a).

In our previous work on the 3-β-GlcNAc-transferase[1], apparent enzyme activity was found when *p*-nitrophenyl β-D-galactopyranoside was used as acceptor. Analysis of the radioactive compound by h.p.l.c. using solvent systems *B* and *C* indicated the material to be free, radioactive 2-acetamido-2-deoxy-D-glucose, presumably formed by the breakdown of UDP-[^{14}C]GlcNAc (data not shown).

R α-GalNAcide → R β-GlcNAc-(1→3)-α-GalNAcide →
R β-GlcNAc-(1→3)-[β-GlcNAc-(1→6)]-α-GalNAcide

R = Bn or Ph

Scheme 2

The enzymatic products of core 3-β-GlcNAc-transferase and core 6-β-GlcNAc-transferase B. — A recent preliminary report[7] has suggested the presence, in rat colon mucosa, of two GlcNAc-transferases involved in the synthesis of mucin oligosaccharide cores of classes *3* and *4*, as shown in Scheme 2. The ^{1}H-n.m.r. spectrum of the higher-molecular-weight of the two products formed by use of phenyl 2-acetamido-2-deoxy-α-D-galactopyranoside as acceptor showed doublet signals in the anomeric hydrogen region at δ 5.570 ($J_{1,2}$ 3.5 Hz) due to a 2-acetamido-2-deoxy-α-D-galactopyranosyl residue, 4.701 ($J_{1,2}$ 8.6 Hz) due to a (1→3)-linked 2-acetamido-2-deoxy-β-D-glucopyranosyl residue, and 4.490 ($J_{1,2}$ 8.8 Hz) due to a (1→6)-linked 2-acetamido-2-deoxy-β-D-glucopyranosyl residue. The spectrum therefore indicates incorporation of 2-acetamido-2-deoxy-β-D-glucopyranosyl groups at both O-3 and O-6 of the 2-acetamido-2-deoxy-α-D-galactopyranosyl residue.

The product formed with benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside as substrate was also resolved into two radioactive fractions by gel filtration. Both fractions were analyzed by h.p.l.c. The low-molecular-weight fraction (Fig. 2b) contained sucrose, [^{14}C]GlcNAc (Peak A, Fig. 2b), and unidentified components. The higher-molecular-weight radioactive fraction was eluted at the same position (Peak B, Fig. 2c) as Bn β-GlcNAc-(1→3)-[β-GlcNAc-(1→6)]-α-GalNAcide (Peak 5, Fig. 2a). Further characterization of this product by ^{1}H-n.m.r. spectroscopy and methylation analysis is under way.

We are interested in using h.p.l.c. not only as a means of product purification but also as a relatively rapid method for assaying transferases in crude membrane preparations. Small-scale incubations containing rat-colon mucosal extracts, benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside, and UDP-[^{14}C]GlcNAc were therefore analyzed on h.p.l.c. directly, without prior product purification by gel filtration (see Experimental section). Passage of the sample through AG 1 X8 resin removed UDP-[^{14}C]GlcNAc and [^{14}C]GlcNAc phosphate, but [^{14}C]GlcNAc, sucrose, Triton X-100, and salts remained. Triton X-100 was nonradioactive and was eluted well before the oligosaccharide glycosides with either solvent system *A* or *C* (Table I). Sucrose was also nonradioactive and eluted between the critical di- and tri-saccharide regions in solvent system *A*, and between the two disaccharides in solvent system *C* (Table I). [^{14}C]GlcNAc, however, was eluted in the disaccharide region with solvent system *A*, and proper identification of disaccharide products is difficult with this solvent. For this reason, we have developed a sequential solvent system in which solvent *C* is used first to separate disaccharides and GlcNAc, followed by solvent *A* to elute higher-molecular-weight oligosaccharides (Fig. 3).

The products of rat-colon-mucosa enzyme action on benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside were resolved into three radioactive peaks with this system (Fig. 3b): [^{14}C]GlcNAc (Peak A, 75% of total radioactivity), Bn β-[^{14}C]GlcNAc-(1→3)-α-GalNAcide (Peak B, 5% of total radioactivity), and Bn β-[^{14}C]GlcNAc-(1→3)-{β-[^{14}C]GlcNAc-(1→6)}-α-GalNAcide (Peak C, 20% of total radioactivity). These radioactive peaks were eluted like the corresponding synthet-

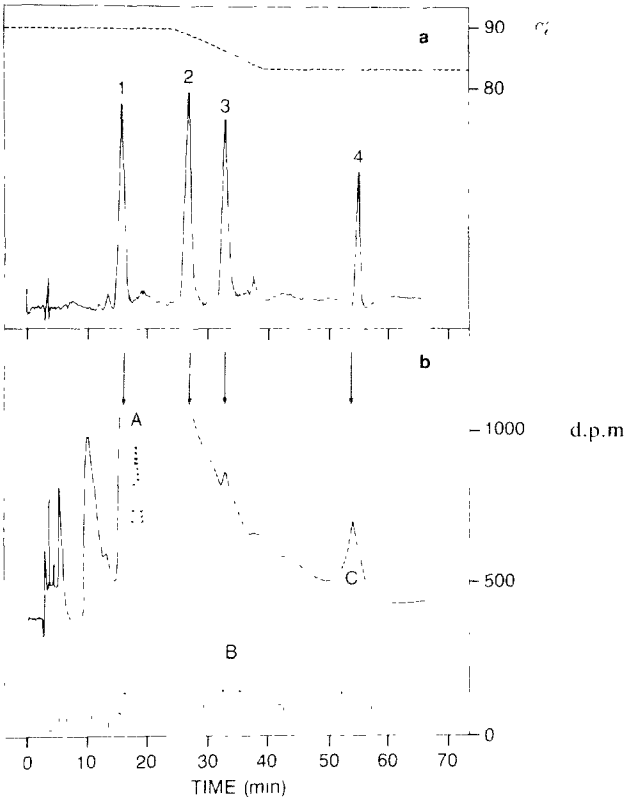


Fig. 3. Liquid chromatograms under elevated pressure of benzyl glycosides. Initial elution was isocratic with solvent system *C* (9:1, v/v acetonitrile–water) for 24 min. At this time, the water content of the mobile phase was increased linearly for 15 min to a final concentration of 83.17 (v/v) acetonitrile–water (solvent *A*). Elution was continued isocratically with solvent *A*. (a) Standard synthetic compounds: (1) GlcNAc, (2) Bn β-GlcNAc-(1→6)-α-GalNAcide, (3) Bn β-GlcNAc-(1→3)-α-GalNAcide, (4) Bn β-GlcNAc-(1→3)-[β-GlcNAc-(1→6)]-α-GalNAcide. (b) A small-scale incubation (50 μl) of rat-colon mucosal extract, UDP-[^{14}C]GlcNAc, and Bn α-GalNAcide was analyzed on this system after passage through a column of AG 1 X8 (Cl^-) resin. (A) [^{14}C]GlcNAc; (B) Bn β-[^{14}C]GlcNAc-(1→3)-α-GalNAcide, and (C) Bn β-[^{14}C]GlcNAc-(1→3)-{β-[^{14}C]GlcNAc-(1→6)}-α-GalNAcide. Absorbance at 195 nm, —— d.p.m., · · · ·, and acetonitrile (%) in the mobile phase, -------

ic compounds (Peaks 1, 3, and 4, respectively, Fig. 3a). Further, there was no formation of Bn β-[^{14}C]GlcNAc-(1→6)-α-GalNAcide (standard compound is Peak 2, Fig. 3a), indicating that the 3-β-GlcNAc-transferase must act before the 6-β-GlcNAc-transferase, and that the latter enzyme cannot act on the 2-acetamido-2-deoxy-α-D-galactopyranosides.

Table II summarizes the incorporation of [^{14}C]GlcNAc catalyzed by rat-colon-mucosal extracts in the presence of UDP-[^{14}C]GlcNAc. It is interesting that Bn β-GlcNAc-(1→3)-α-GalNAcide is an appreciably more effective acceptor than benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside. H.p.l.c. analysis of the product formed with the disaccharide acceptor (data not shown) revealed [^{14}C]GlcNAc

TABLE II

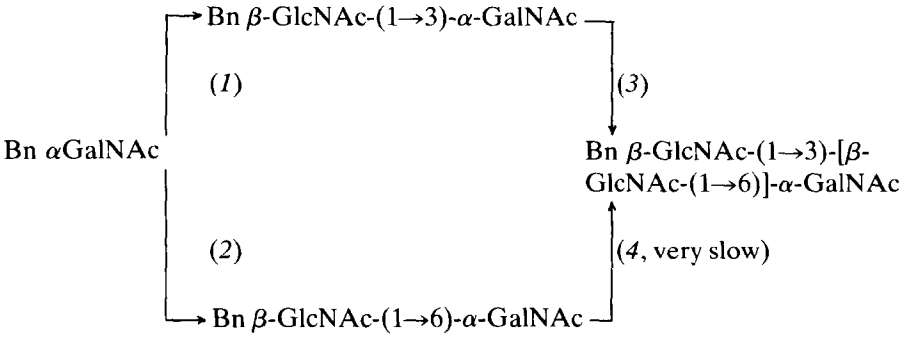
INCORPORATION OF GlcNAc CATALYZED BY RAT-COLON-MUCOSAL EXTRACTS[a]

Substrate	*Product formed (nmol/h)*	
	Bn βGlcNAc→3αGalNAc	*Bn βGlcNAc→3(βGlcNAc→6)αGalNAc*
Bn αGalNAc	0.7	1.5
Bn βGlcNAc→3αGalNAc		22.6
Bn βGlcNAc→6αGalNAc		0.5

[a]The following components were incubated at 37° for 2 h in a total volume of 50 μL: 4mM substrate, 10mM $MnCl_2$, 0.1M MES buffer (pH 6.5), 0.2% Triton X-100, 2.9mM UDP-[^{14}C]GlcNAc (480 d.p m./nmol), and rat-colon-mucosal extract (0.28 mg of protein). Radioactive product was analyzed as described for small-scale incubations in the Experimental section [(*d*), preparation of radioactive enzyme products]

and Bn β-GlcNAc-(1→3)-{β-[^{14}C]GlcNAc-(1→6)}-α-GalNAcide (60% of total radioactivity) as the only radioactive peaks. Table II shows that Bn β-GlcNAc-(1→6)-α-GalNAcide is a relatively poor substrate. H.p.l.c. analysis of the product formed with the latter acceptor (data not shown) revealed primarily [^{14}C]GlcNAc and a very small radioactive peak in the trisaccharide region.

The aforementioned data suggest Scheme 3 for the synthesis of oligosaccharides of core classes *3* and *4*. Reaction (*2*) does not appear to take place. This is perhaps to be expected since the disaccharide β-GlcNAc-(1→6)-GalNAc has not as yet been isolated from a mucin. Even if this disaccharide were to be formed, it is a poor substrate for 3-β-GlcNAc-transferase (Reaction *4*). The physiological pathway therefore appears to be *via* Reactions (*1*) and (*3*).



Scheme 3

Although rat colon mucosa has a highly active 6-β-GlcNAc-transferase, it is interesting that rat colonic mucin[26] has oligosaccharides with core class *3* but not core class *4*. It is possible that further work may yet reveal core-class *4* oligosac-

charides in this mucin. Alternatively, substrate-specificity studies with low-molecular-weight oligosaccharides may not apply in all cases to mucin substrates. Another possibility is that β-GlcNAc-(1→3)-GalNAc may be acted on more effectively by other glycosyltransferases [*e.g.*, a sialyltransferase incorporating an α-sialyl group at O-6 of a GalNAc residue] than by 6-β-GlcNAc-transferase. H.p.l.c. is obviously a very powerful tool at the analytical level in the study of glycosyltransferase–substrate specificities.

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