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Structures of the Oligosaccharides Isolated from Milk of the Platypus

JUNKO AMANO[1], MICHAEL MESSER[2] and AKIRA KOBATA[1]

[1]*Department of Biochemistry, the Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108, Japan*
[2]*Department of Biochemistry, University of Sydney, Sydney N.S.W. 2006, Australia*

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Eight major oligosaccharides were isolated from platypus milk. By sequential exoglycosidase digestion and methylation study, their structures were elucidated as shown in Fig. 9 of this paper. The characteristic feature of the platypus milk oligosaccharides is that lacto-*N*-*neo*tetraose and lacto-*N*-*neo*hexaose are the major cores in contrast to human milk oligosaccharides in which lacto-*N*-tetraose and lacto-*N*-hexaose are found as the major core.

Monotreme milk has been found to be unique in that it contains negligible amounts of free lactose but significant amount of other oligosaccharides which are composed mainly of fucose and sialic acid in addition to glucose and galactose [1]. The milk of the platypus (*Ornithorhynchus anatinus*) contains large amounts of fucosylated oligosaccharides which Messer *et al.* [2] separated into at least 14 components by gel-permeation column chromatography. Jenkins *et al.* [3], analyzed the structure of the major component using ^{13}C-NMR spectroscopy and identified it as 3,2′ -difucosyllactose (lactodifucotetraose).

This paper reports on further studies, done by sequential exoglycosidase digestion and methylation analysis, which have elucidated the structures of eight other oligosaccharides of platypus milk.

Abbreviations: All sugars mentioned in this paper have the D-configuration except for fucose which has the L-configuation. The postscript OT is used in this paper to indicate NaB^3H_4-reduced oligosaccharides. In the same way, the postscript OH is used to indicate $NaBH_4$-reduced oligosaccharides.

Materials and Methods

Materials

3-Fucosyllactose, lacto-*N*-fucopentaose II, lacto-*N*-hexaose, lacto-*N*-*neo*hexaose and LST-C (lactosialyltetrasaccharide C, NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc) were isolated from human milk as previously described [4]. Lacto-*N*-*neo*tetraose was obtained by sialidase digestion of LST-C. These oligosaccharides were labeled by reduction with NaB^3H_4 as reported previously [5]. GlcNAcβ1-3Galβ1-4GlcOT and GlcNAcβ1-6Galβ1-4 GlcOT were isolated from the partial acid hydrolysate of [^{3}H]-lacto-*N*-hexaitol by paper chromatography as reported previously [6].

β-Galactosidase and β-*N*-acetylhexosaminidase were purified from jack bean meal by the method of Li and Li [7]. Diplococcal β-galactosidase and β-*N*-acetylhexosaminidase were purified by the method of Glasgow *et al.* [8]. Diplococcal β-galactosidase cleaves the Galβ1-4GlcNAc linkage but not the Galβ1-3GlcNAc and the Galβ1-6GlcNAc linkages. In contrast, jack bean β-galactosidase cleaves all the three linkages under the conditions described in this paper. Neither enzyme can hydrolyze the β-galactosyl linkages of lactitol [9]. Three α-fucosidases with different substrate specificities were used in this study. Bovine epididymis α-fucosidase, which was purchased from Sigma Chemical Co., St. Louis, MO., USA, cleaves all fucosyl linkages so far reported in human milk oligosaccharides. *Bacillus fulminans* α-fucosidase purified by the method of Kochibe [10] cleaves only the Fucα1-2Gal linkage. Almond emulsin α-fucosidase I, which cleaves the Galβ1-4(Fucα1-3)GlcNAc, the Galβ1-3(Fucα1-4)GlcNAc and the Galβ1-4(Fucα1-3)GlcOH linkages but cannot hydrolyze other fucosyl linkages [11] was purified as reported by Imber *et al.* [12]. One unit of glycosidase activity was defined as the amount of the enzyme required to hydrolyze one μmol of the substrate/min. *Aleuria aurantia* lectin-Sepharose [13] was kindly provided by Dr. Kochibe, Gunma University. NaB^3H_4 (341 mCi/mmol) was purchased from New England Nuclear, Boston, MA, USA, and NaB^2H_4 (98%) was purchased from Merck, Darmstadt, W. Germany.

Exoglycosidase Digestion of Tritium-labeled Oligosaccharides

Sugar samples were incubated with one of the following mixtures at 37° C for 18 h: (1) bovine epididymis α-fucosidase digestion; enzyme (27 munit) in 0.2 M sodium citrate buffer, pH 6.0 (30 μl); (2) *Bacillus fulminans* α-fucosidase digestion; enzyme (17.5 μg) in 0.05 M sodium phosphate buffer, pH 6.6 (20 μl); (3) almond α-fucosidase I digestion; enzyme (0.09 munit) in 0.05 M sodium acetate buffer, pH 5.0 (20 μl); (4) jack bean β-galactosidase digestion; enzyme (0.1 unit) in 0.05 M sodium citrate buffer, pH 4.0 (40 μl); (5) jack bean β-*N*-acetylhexosaminidase digestion; enzyme (0.2 unit) in 0.05 M sodium citrate buffer, pH 4.0 (40 μl); (6) Diplococcal β-galactosidase and diplococcal β-*N*-acetylhexosaminidase digestion; a mixture of β-galactosidase (2 munit) and β-*N*-acetylhexosaminidase (10 munit) in 0.05 M sodium citrate buffer, pH 6.0 (50 μl). One drop of toluene was added to all reaction mixtures to inhibit bacterial growth during incubation. The reaction was terminated by heating the reaction mixture in a boiling water bath for 3 min.

Affinity Chromatography of Oligosaccharides on an Aleuria aurantia Lectin Column

Tritium-labeled oligosaccharides (3×10^4 cpm, 1-2 nmol) were dissolved in 100 μl of phosphate-buffered saline (6.7 mM KH_2PO_4, 0.015 M NaCl, pH 7.4) and applied to *Aleuria aurantia* lectin-Sepharose columns (1.0 ml) previously equilibrated with the buffered saline and allowed to stand at room temperature (20-25°C) for 30 min. The columns were then eluted with 15 ml of the same buffer, followed by the buffer containing 0.5 mM L-fucose. Fractions (1.0 ml) were collected at 15 ml/h and the radioactivity in each fraction was determined.

Analytical Methods

Radioactivity was measured with an Aloka liquid scintillation spectrometer (LSC-700). Radiochromatoscanning was performed with a Packard radiochromatogram scanner model 7201. Methylation analysis of oligosaccharides was done as described previously [14]. For the separation of 2,4,6-tri- and 3,4,6-tri-*O*-methyl galactitols, a glass column (3 mm × 2.0 m) of 3% ECNSS-M on Gas-chrom Q (100-200 mesh) was used for gas chromatography. Bio-Gel P-4 column chromatography of radioactive oligosaccharides was performed by the method of Yamashita *et al.* [15]. Descending paper chromatography of radioactive oligosaccharides was done by using either ethyl acetate/pyridine/acetic acid/water, 5/5/1/3 by vol (solvent I) or ethyl acetate/pyridine/water, 12/5/4 by vol (solvent II).

Results

Isolation of Oligosaccharides

The oligosaccharides obtained from platypus milk were obtained by gel permeation chromatography on Bio-Gel P-4 as reported previously [2]. Fractions 2a, 2b, 3a, 3b and 3c, thus obtained, were each re-purified on Bio-Gel P-4 and aliquots of each were then converted to the corresponding alcohols by reduction with NaB^3H_4; the remaining oligosaccharides were reduced with NaB^2H_4 to obtain samples suitable for methylation analysis. After reduction, all oligosaccharide samples were purified by paper chromatography using solvent I. Fraction 3b was further separated into three oligosaccharides by affinity chromatography on an *Aleuria aurantia* lectin-Sepharose column (Fig. 1); the other four fractions behaved as single components on this column. The three oligosaccharides of fraction 3b were named 3b-I, 3b-II and 3b-III (Fig. 1).

Structural Study of Oligosaccharide 2a

Upon Bio-Gel P-4 column chromatography, the radioactive oligosaccharide was eluted at 10.6 glucose units (solid line in Fig. 2A). No degradation occurred when it was incubated with either jack bean β-galactosidase or jack bean β-*N*-acetylhexosaminidase (data not shown), but it was completely converted to a radioactive oligosaccharide with mobility of 8.6 glucose units by bovine epididymis α-fucosidase, indicating that two or three

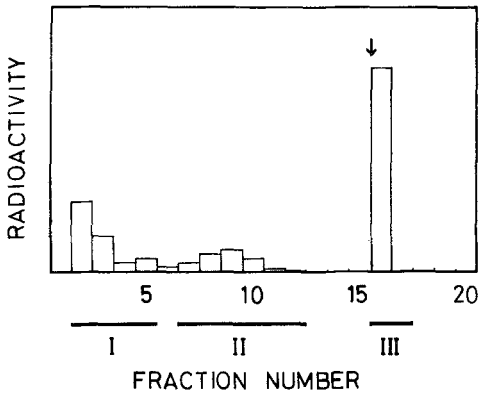


Figure 1. *Aleuria aurantia* lectin-Sepharose column chromatography of oligosaccharide 3b. Conditions of chromatography are described in Materials and Methods. Fractions 1-15 contained material eluted with starting buffer, fractions 16-20 contained material eluted with 0.5 mM L-fucose as indicated by an arrow.

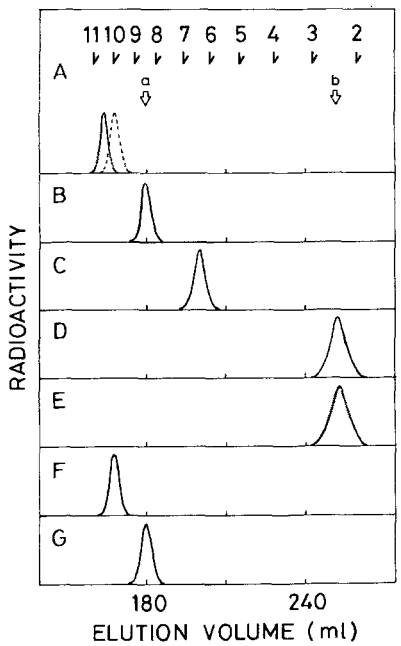
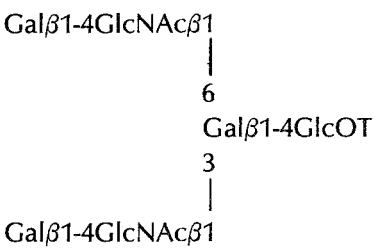


Figure 2. Sequential exoglycosidase digestion of radioactive oligosaccharides 2a and 2b. The reaction mixtures were subjected to Bio-Gel P-4 column chromatography and the radioactivity in each tube (2.0 ml) was determined on an aliquot of sample by liquid scintillation spectrometry. The arrows indicate the elution positions of glucose oligomers (numbers indicate the glucose units). The elution positions of standard oligosaccharides, lacto-*N*-*neo*hexaitol,(a); and lactitol,(b); are also given. A, intact 2a [^{3}H] and 2b [^{14}C]; B, the radioactive peak (2a or 2b) in (A) incubated with bovine epididymis α-fucosidase; C, the radioactive peak in (B) incubated with jack bean β-galactosidase; D, the radioactive peak in (C) incubated with jack bean β-*N*-acetylhexosaminidase; E, the radioactive peak in (B) incubated with diplococcal β-galactosidase and β-*N*-acetylhexosaminidase; F, 2a incubated with *B. fulminans* α-fucosidase; G, the radioactive peak in (F) and 2b incubated with almond α-fucosidase I.

fucose residues were removed by the enzymatic digestion (Fig. 2B). Upon sequential digestion with jack bean β-galactosidase and jack bean β-*N*-acetylhexosaminidase, the defucosylated radioactive oligosaccharide was converted to a radioactive compound eluting at the same position as lactitol, releasing 2 mol each of galactose and *N*-acetylglucosamine (Fig. 2C and D, respectively). The radioactive oligosaccharide in Fig. 2B was also converted to radioactive lactitol by incubation with a mixture of diplococcal β-galactosidase and diplococcal β-*N*-acetylhexosaminidase (Fig. 2E). Since diplococcal β-galactosidase cleaves the Galβ1-4GlcNAc linkage but neither the Galβ1-3GlcNAc nor the Galβ1-6GlcNAc linkage [16], the results indicated that the radioactive oligosaccharide in Fig. 2B has the following structure:

(Galβ1-4GlcNAcβ1-)$_2$-Galβ1-4GlcOT

Oligosaccharide 2a should be a di- or tri-α-fucosyl derivative of the hexasaccharide. In order to determine its complete structure, it was subjected to methylation analysis. Only 1,2,3,5,6-penta-*O*-methyl sorbitol was detected as partially *O*-methylated sorbitol. Therefore, the glucose at the reducing terminal of oligosaccharide 2a occurs as Galβ1-4Glc and should not be substituted by other sugars. Accordingly, the molar ratios of partially *O*-methylated monosaccharides were calculated by assigning the penta-*O*-methyl sorbitol a value of 1.0 (Table 1). This evidence also indicated that the two Galβ1-4GlcNAcβ1- groups should be linked to the galactose residue of the lactitol moiety. Since one mol of 2,4-di-*O*-methyl galactitol was detected as the only di-*O*-methyl galactitol, the structure of the fucose-free hexaitol, corresponding to the radioactive peak in Fig. 2B, should be:



Detection of one mol each of 3,4,6-tri- and 2,3,4,6-tetra-*O*-methyl galactitol indicated that one terminal galactose residue of the hexaitol is substituted by an α-fucosyl residue at its C-2 position. Although the recovery of 2-*N*-methylacetamido-2-deoxyglucitol was not quantitative, detection of only the 6-mono-*O*-methyl derivative indicated that each of the two *N*-acetylglucosamine residues of the hexaitol is substituted by an α-fucosyl residue at its C-3 position.

In order to determine which Galβ1-4GlcNAc outer chain is monofucosylated, radioactive fraction 2a was subjected to another series of sequential exoglycosidase digestions as follows. First, the Fucα1-3GlcNAc linkages were cleaved by incubation with almond α-fucosidase I. The radioactive product in Fig. 3A was then incubated with a mixture of jack bean β-galactosidase and jack bean β-*N*-acetylhexosaminidase to remove the

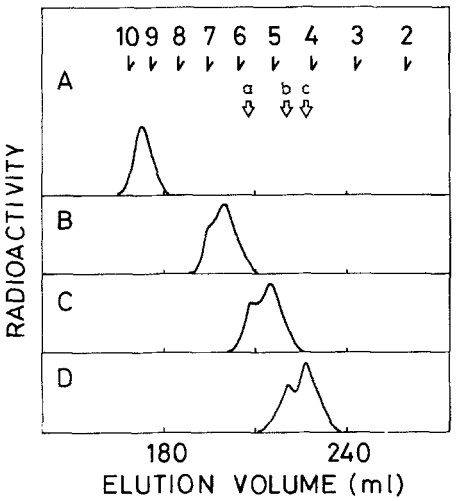


Figure 3. Assignment of the monofucosylated outer chain in compound 2a. Analytical conditions and arrows are the same as in Fig. 2. The elution positions of standard oligosaccharides lacto-*N*-*neo*tetraitol, (a); GlcNAcβ1-3Galβ1-4GlcOT, (b); and GlcNAcβ1-6Galβ1-4GlcOT, (c) are shown. A, oligosaccharide 2a incubated with almond α-fucosidase; B, the radioactive peak in (A) incubated with jack bean β-galactosidase and β-*N*-acetylhexosaminidase; C, the radioactive peak in (B) incubated with *B. fulminans* α-fucosidase; D, the radioactive peak in (C) incubated with jack bean β-galactosidase.

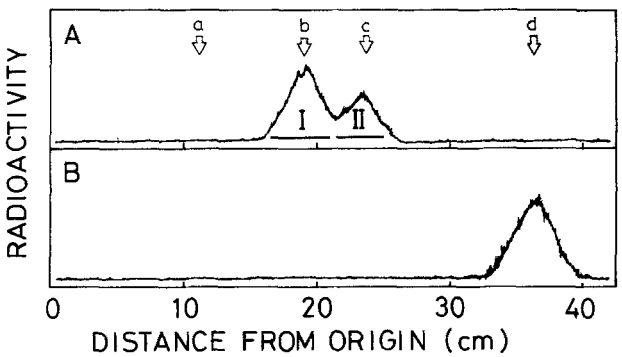


Figure 4. Paper chromatography of the radioactive products corresponding to that in Fig. 3D using solvent II. Arrows indicate the migration positions of standard oligosaccharides: a, lacto-*N*-*neo*tetraitol; b, GlcNAcβ1-6Galβ1-4GlcOT; c, GlcNAcβ1-3Galβ1-4GlcOT; d, lactitol. A and B, the radioactive products in Fig. 3D before and after jack bean β-*N*-acetylhexosaminidase digestion.

Table 1. Methylation analysis of the oligosaccharide fractions.

Methylated sugars	Molar ratio[a]			
	2a	2b	3b	3c
Sorbitol				
1,2,3,5,6-Penta-*O*-methyl (4-mono-*O*-acetyl)	*1.0*	*1.0*	0.2	0.6
1,2,5,6-Tetra-*O*-methyl (3,4-di-*O*-acetyl)	0	0	0.5	0
Fucitol				
2,3,4-Tri-*O*-methyl (1,5-di-*O*-acetyl)	3.2	1.9	1.6	1.0
Galactitol				
2,3,4,6-Tetra-*O*-methyl (1,5-di-*O*-acetyl)	1.0	2.0	0.5	*1.0*
3,4,6-Tri-*O*-methyl (1,2,5-tri-*O*-acetyl)	1.1	0	0.4	0
2,4,6-Tri-*O*-methyl (1,3,5-tri-*O*-acetyl)	0	0	*1.0*	1.0
2,4-Di-*O*-methyl (1,3,5,6-tetra-*O*-acetyl)	0.9	1.0	0	0
2-*N*-Methylacetamido-2-deoxyglucitol				
3,6-Di-*O*-methyl (1,4,5-tri-acetyl)	0	0	0	0
4,6-Di-*O*-methyl (1,3,5-tri-*O*-acetyl)	0	0	0	0
6-Mono-*O*-methyl (1,3,4,5-tetra-*O*-acetyl)	0.5	0.8	0.8	0.2

[a] Numbers in the table were calculated by taking the italicised values as 1.0.

Galβ1-4GlcNAc outer chain (Fig. 3B). The remaining one α-fucosyl residue in the radioactive product in Fig. 3B was removed by incubation with *B. fulminans* α-fucosidase. The product was separated into two components by Bio-Gel P-4 column chromatography; the elution position of the first component eluted was the same as that of authentic lacto-*N*-*neo*tetraitol (Fig. 3C). Upon removal of the β-galactosyl residues, the two components were converted to compounds with the same elution volumes as GlcNAcβ1-3 Galβ1-4GlcOT and GlcNAcβ1-6Galβ1-4GlcOT, respectively (Fig. 3D). That the two radioactive trisaccharides are GlcNAcβ1-3Galβ1-4GlcOT and GlcNAcβ1-6Galβ1-4GlcOT was confirmed by paper chromatography before and after jack bean β-*N*-acetylhexosaminidase digestion (Fig. 4). These results indicated that fraction 2a is a mixture of the two isomeric oligosaccharides shown in Fig. 9.

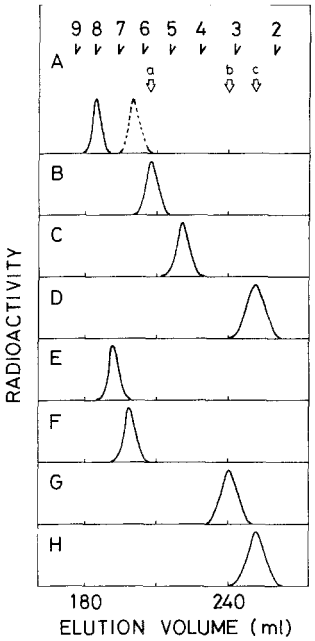


Figure 5. Sequential exoglycosidase digestion of radioactive oligosaccharides 3a and 3c. Analytical conditions and black arrows are the same as in Fig. 2. The elution positions of standard oligosaccharides lacto-*N*-*neo*-tetraitol, (a); 3-fucosyllactitol, (b); and lactitol, (c), are shown. A, 3a (solid line) and 3c (dotted line); B, 3a or 3c incubated with bovine epididymis α-fucosidase; C, the radioactive peak in (B) digested with jack bean β-galactosidase; D, the radioactive peak in (C) incubated with jack bean β-*N*-acetylhexosaminidase; E, 3a incubated with *B. fulminans* α-fucosidase; F, the radioactive peak in (E) incubated with almond α-fucosidase I; G, the radioactive peak in (F) incubated with a mixture of diplococcal β-galactosidase and β-*N*-acetylhexosaminidase; H, the radioactive peak in (G) incubated with almond α-fucosidase I.

Structural Study of Oligosaccharide 2b

Oligosaccharide 2b eluted at ten glucose units on a Bio-Gel P-4 column (dotted line in Fig. 2A). This oligosaccharide, like 2a, was resistant to jack bean β-galactosidase and jack bean β-*N*-acetylhexosaminidase digestion (data not shown). The radioactive oligosaccharide was converted to an oligosaccharide with a mobility of 8.6 glucose units by bovine epididymis α-fucosidase digestion, indicating the removal of two fucose residues (Fig. 2B). The defucosylated radioactive oligosaccharide gave the same results as the hexaitol obtained from oligosaccharide 2a upon sequential exoglycosidase digestion, indicating that it also has the following structure:

(Galβ1-4GlcNAcβ1-)$_2$-Galβ1-4GlcOT

Methylation analysis of oligosaccharide 2b (Table 1) indicated that the two Galβ1-4GlcNAcβ1- groups are linked at the C-3 and C-6 positions of the galactose residue of the lactitol moiety, and that the two non-reducing terminal fucoses are linked at the C-3 positions of the two *N*-acetylglucosamine residues. Therefore, the structure of oligosaccharide 2b should be as shown in Fig. 9.

Structural Study of Oligosaccharides 3a and 3c

Oligosaccharides 3a and 3c eluted at 8.0 and 6.5 glucose units, respectively, on the Bio-Gel P-4 column, (Fig. 5A). Both radioactive oligosaccharides were converted to another radioactive oligosaccharide, with a mobility of 6.0 glucose units, by incubation with bovine epididymis α-fucosidase (Fig. 5B), indicating that three and one fucosyl residues were removed from 3a and 3c, respectively, by the fucosidase digestion. That the resulting defucosylated oligosaccharides obtained are both Galβ1-4GlcNAcβ1-Galβ1-4GlcOT was confirmed by the following experiments. Since the same results were obtained from both tetraitols, only the data of the tetraitol obtained from oligosaccharide 3c is documented. By sequential digestion with jack bean β-galactosidase (Fig. 5C) and jack bean β-*N*-acetylhexosaminidase (Fig. 5D), the radioactive tetraitol was converted to [^{3}H]-lactitol, releasing one mol each of galactose and *N*-acetylglucosamine. The tetraitol was also converted to lactitol by digestion with a mixture of diplococcal β-galactosidase and diplococcal β-*N*-acetylhexosaminidase (data not shown), indicating that the non-reducing terminal galactose is linked at the C-4 position of the *N*-acetylglucosamine residue. Oligosaccharide 3c should be a monofucosyl derivative of this tetraitol. Methylation analysis of oligosaccharide 3c indicated (Table 1) that it is lacto-*N*-fucopentaose III (Fig. 9). Oligosaccharide 3a is a trifucosyl derivative of the tetraitol. Although methylation analysis could not be performed on this oligosaccharide because of the limited amount of sample available, the three fucose residues were assigned as shown in Fig. 9, based on the following evidence. When the radioactive oligosaccharide 3a was incubated with *B. fulminans* α-fucosidase, one fucose was removed (Fig. 5E). Since this enzyme cleaves Fucα1-2Gal linkages only, this probably indicated that an α-fucosyl residue in oligosaccharide 3a is linked at the C-2 position of the terminal β-galactosyl residue of the tetraitol. The radioactive oligosaccharide in Fig. 5E released another fucose residue upon incubation with almond α-fucosidase I (Fig. 5F). Almond α-fucosidase I cleaves only the α-fucosyl residue located closer to the non-reducing terminal of the two octasaccharides: Galβ1-3 and 4(Fucα1-4 and 3)GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ 1-4Glc [14]. The enzyme also cannot cleave the α-fucosyl linkage of lacto-*N*-fucopentaitol V (Yamashita, K. *et al.*, unpublished data). The resistant α-fucosyl linkage, however, can be cleaved by almond α-fucosidase I after removal of the outer Galβ-GlcNAc residues by incubation with β-galactosidase and β-*N*-acetylhexosaminidase. Therefore, one α-fucosyl residue in oligosaccharide 3a should occur as a Fucα1-3GlcNAc group. The radioactive oligosaccharide in Fig. 5F was then converted to a radioactive oligosaccharide with the same mobility as authentic 3-fucosyllactitol by incubation with a mixture of diplococcal β-galactosidase and β-*N*-acetylhexosaminidase (Fig. 5G). The radioactive trisaccharide was then converted to radioactive lactitol by incubation with almond α-fucosidase I (Fig. 5H), indicating that the radioactive trisaccharide is most probably 3-fucosyllactitol. Neither diplococcal β-galactosidase nor jack bean β-galactosidase can cleave the β-galactosyl linkages in the Galβ1-3 and 4(Fucα1-4 and 3)GlcNAc groups [9]. Therefore, oligosaccharide 3a should have the structure as shown in Fig. 9.

Structural Studies of Oligosaccharides 3b-I, 3b-II and 3b-III

Oligosaccharides 3b-I and 3b-II eluted at 7.0 glucose units in a Bio-Gel P-4 column (Fig. 6A and 7A, respectively), whereas oligosaccharide 3b-III eluted at 7.4 glucose units

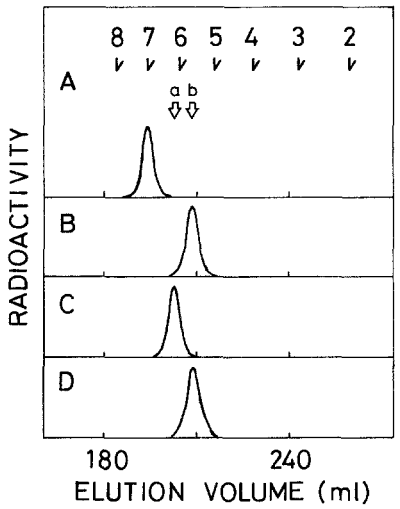


Figure 6. Sequential exoglycosidase digestion of radioactive 3b-I. Analytical conditions and black arrows are as given in Fig. 2. The elution positions of standard oligosaccharides lacto-*N*-fucopentaitol II, (a); and lacto-*N*-tetraitol, (b) are shown. A, intact 3b-I; B, 3b-I incubated with bovine epididymis α-fucosidase; C, the radioactive peak in (A) incubated with *B. fulminans* α-fucosidase; D, the radioactive peak in (C) incubated with almond α-fucosidase I.

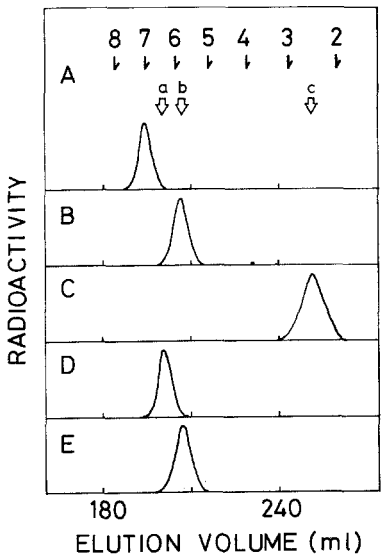


Figure 7. Sequential exoglycosidase digestion of radioactive 3b-II. Analytical conditions and black arrows are the same as shown in Fig. 2. The elution positions of standard oligosaccharides lacto-*N*-fucopentaitol III, (a); lacto-*N*-*neo*tetraitol, (b); and lactitol, (c) are given. A, intact 3b-II; B, 3b-II incubated with bovine epididymis α-fucosidase; C, the radioactive peak in (B) incubated with a mixture of diplococcal β-galactosidase and β-*N*-acetylhexosaminidase; D, the radioactive peak in (A) incubated with *B. fulminans* α-fucosidase; E, the radioactive peak in (D) incubated with almond α-fucosidase I.

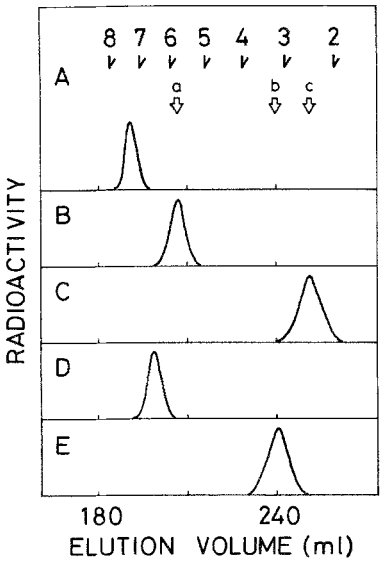


Figure 8. Sequential exoglycosidase digestion of radioactive 3b-III. Analytical conditions and black arrows are the same as shown in Fig. 2. The elution positions of standard oligosaccharides lacto-*N*-*neo*tetraitol, (a); 3-fucosyllactitol, (b); and lactitol, (c) are shown. A, intact 3b-III; B, 3b-III incubated with bovine epididymis α-fucosidase; C, the radioactive peak in (B) incubated with a mixture of diplococcal β-galactosidase and β-*N*-acetylhexosaminidase; D, the radioactive peak in (A) incubated with almond α-fucosidase; E, the radioactive peak in (D) incubated with a mixture of diplococcal β-galactosidase and β-*N*-acetylhexosaminidase.

(Fig. 8A). All three oligosaccharides were resistant to jack bean β-galactosidase or jack bean β-*N*-acetylhexosaminidase digestion (data not shown). Radioactive oligosaccharide 3b-I was converted to a radioactive oligosaccharide with a mobility of 5.5 glucose units by incubation with bovine epididymis α-fucosidase (Fig. 6B). Radioactive oligosaccharides 3b-II and 3b-III were converted to a radioactive oligosaccharide with a mobility of 5.7 glucose units by the same enzymatic digestion (Fig. 7B and 8B, respectively). All these radioactive oligosaccharides were converted to [^{3}H]-lactitol by sequential digestion with jack bean β-galactosidase and jack bean β-*N*-acetylhexosaminidase, releasing one mol each of galactose and *N*-acetylglucosamine (data not shown), indicating that they all have the following structure:

Galβ1-?GlcNAcβ1-?Galβ1-4GlcOT

When incubated with a mixture of diplococcal β-galactosidase and β-*N*-acetylhexosaminidase, the radioactive tetraitols from oligosaccharides 3b-II (Fig. 7B) and 3b-III (Fig. 8B) were each converted to [^{3}H]-lactitol (Fig. 7C and 8C, respectively). Therefore, these tetraitols should have the following structure:

Galβ1-4GlcNAcβ1-?Galβ1-4GlcOT

However, the tetraitol from oligosaccharide 3b-I (Fig. 6B) remained intact after the mixed glycosidase digestion (data not shown). Methylation analysis of a mixture of the unfractionated oligosaccharides 3b (Table 1) gave only 6-mono-*O*-methyl-2-*N*-methylacetamido-2-deoxyglucitol as the glucosamine derivative. Therefore, all *N*-acetylglucosamine residues in the oligosaccharides of this fraction occur as 3,4-disubstituted GlcNAcβ1-. Furthermore, the data indicated that all oligosaccharides in this fraction have two non-reducing terminal fucoses although the number of 2,3,4-tri-*O*-methyl fucitols was somewhat less than 2.0. One of these fucoses should occur as either the Galβ1-4(Fucα1-3)GlcNAc or the Galβ1-3(Fucα1-4)GlcNAc group. Therefore, oligosaccharide 3b-I should have the Galβ1-3(Fucα1-4)GlcNAc group because it could not be hydrolyzed by diplococcal β-galactosidase after complete defucosylation. In contrast, oligosaccharides 3b-II and 3b-III should have the Galβ1-4(Fucα1-3)GlcNAc group. Both radioactive oligosaccharides 3b-I and 3b-II released one fucose residue upon *B. fulminans* α-fucosidase digestion (Fig. 6C and 7D, respectively) indicating that both of them have the Fucα1-2Gal group. Since the sum of oligosaccharides 3b-I and 3b-II was 46% of the total oligosaccharides in fraction 3b, all the 3,4,6-tri-*O*-methyl galactitol in Table 1 should be derived from the Fucα1-2Gal group. Therefore, the GlcNAcβ1-Gal group in the three oligosaccharides of fraction 3b should exclusively occur as GlcNAcβ1-3Gal because 1.0 mol of 2,4,6-tri-*O*-methyl galactitol was detected. That the radioactive oligosaccharides in Fig. 6C, Fig. 7D and Fig. 8D all released one fucosyl residue upon almond α-fucosidase I digestion (Figs. 6D, 7E and 8D, respectively) further confirmed that all three oligosaccharides contain one Fucα1-3GlcNAc or Fucα1-4GlcNAc residue. The radioactive product in Fig. 8D was converted to 3-fucosyllactitol by incubation with a mixture of diplococcal β-galactosidase and β-*N*-acetylhexosaminidase (Fig. 8E), indicating that oligosaccharide 3b-III contains the Fucα1-3GlcOT residue. That the radioactive trisaccharide in Fig. 8E is 3-fucosyllactitol was further confirmed by the evidence that it is converted to lactitol by almond α-fucosidase I digestion (data not shown). The presence of 1,2,5,6-tetra-*O*-methyl sorbitol in the methylation data for 3b (Table 1) also supports the presence of a Fucα1-3GlcOT residue. The series of results described indicates that the structures of the three oligosaccharides in fraction 3b should be as shown in Fig. 9.

Discussion

In a previous paper [2] it was suggested, on the basis of their monosaccharide compositions, that these platypus milk oligosaccharides may be related in structure to some of the human milk oligosaccharides. Examination of the structures shown in Fig. 9 demonstrates that this is the case. Thus oligosaccharide 3c has the same structure as human milk lacto-*N*-fucopentaose III [17], and 3b-I is identical with lacto-*N*-difucohexaose I [18]. The other oligosaccharides have not so far been found in human milk [4, 19, 20], but 3a and 3b-III were reported as human urinary oligosaccharides [21, 22].

With the exception of 3b-I (and possibly 3a) these oligosaccharides appear to be derived from 3c. Thus 3b-II and 3b-III can each be assumed to have been formed by the addition of one fucosyl residue to 3c. Oligosaccharide 2b, which is a difucosyl derivative of lacto-*N*-*neo*hexaose [23] appears to be derived from 3c *via* the addition of one residue each of *N*-acetylglucosamine, galactose and fucose, and to give rise to 2a (trifucosyl derivatives of lacto-*N*-*neo*hexaose) by the addition of a fucosyl residue at either one or the

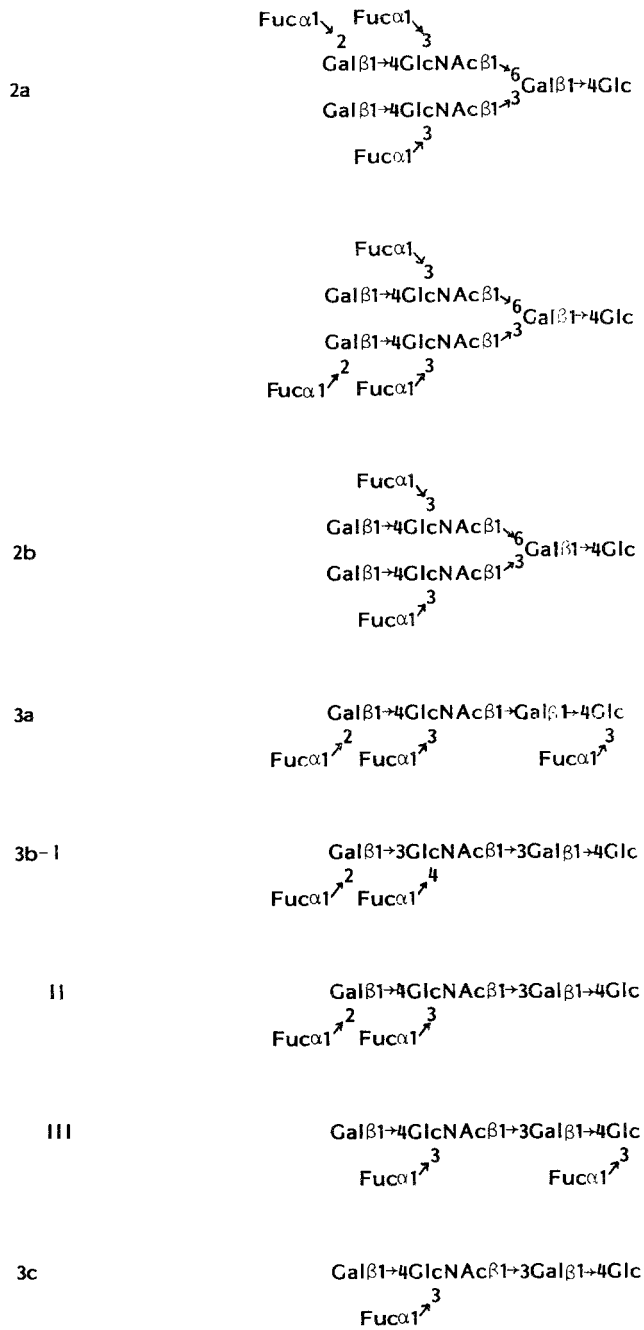


Figure 9. Structures of the oligosaccharides isolated from the milk of the platypus. The linkage between the *N*-acetylgalactosamine and the galactose in oligosaccharide 3a could not be determined.

other of its terminal galactoses. Oligosaccharide 3b-I is a difucosyl derivative of lacto-*N*-tetraose. Oligosaccharide 3a is probably derived from either 3b-II or 3b-III. It is noteworthy that all of these oligosaccharides contain either lacto-*N*-*neo*tetraose or lacto-*N*-tetraose as a part of their structures. This suggests that these tetrasaccharides (especially the former) are intermediates in the synthesis of the higher oligosaccharides within the platypus mammary gland, even though their presence has not been detected in platypus milk [2].

Although, quantitatively, the major component of platypus milk is 3,2'-difucosyllactose [2], none of the higher oligosaccharides is derived from it. This suggests that once fucose has become attached to the galactose residue of lactose, this prevents the further attachment of any other monosaccharide to that residue. A similar situation applies in the case of the human milk oligosaccharides. It is, in fact, remarkable how closely related the platypus milk oligosaccharides are to the human ones, bearing in mind that platypuses and humans belong to two different sub-classes (Prototheria and Eutheria, respectively) of the Class Mammalia. It is noteworthy, in this connection, that the milk oligosaccharides of marsupials (sub-class Metatheria) are very different from the human ones; none of them contain fucose [24] and they are all based on 3'-galactosyllactose [25], a trisaccharide which is not found in human milk.

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