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Biochimica et Biophysica Acta 1200 (1994) 64–72

BBA Biochimica et Biophysica Acta

Chemical characterization of sialyl oligosaccharides isolated from tammar wallaby (*Macropus eugenii*) milk

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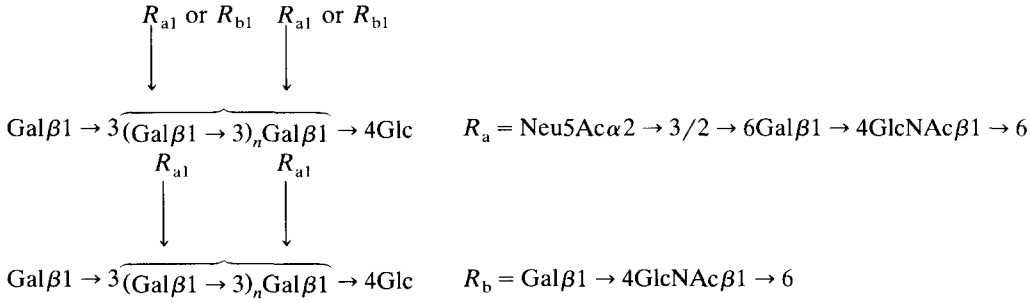
(Received 30 June 1993)
(Revised manuscript received 17 September 1993)

Abstract

Two components of the sialyl oligosaccharides were separated from milk of the tammar wallaby (*Macropus eugenii*) by gel filtration and ion exchange chromatography. Their molecular weights, estimated by gel filtration on HPLC using 5 mM triethylamine-acetate buffer (pH 5.0), were approx. 3,000. Their monosaccharide compositions, determined by GC analysis after methanolysis and by colorimetric assay, were $(Glc)_1(Gal)_9(GlcNAc)_2(Neu5Ac)_1$, and $(Glc)_1(Gal)_8(GlcNAc)_2(Neu5Ac)_2$. Their chemical structures were further elucidated by ^{1}H-NMR and methylation analysis. The results suggest that their approximate structures are:



Key words: Marsupial; Milk; Sialyl Oligosaccharide; Higher Oligosaccharide; NMR-^{1}H; (*M. eugenii*)

1. Introduction

The carbohydrates of marsupial milk are known to be unique compared with those of other mammals. Whereas the dominant carbohydrate in the milk of eutherian mammals is generally the disaccharide, lactose [1], the milk of marsupials contains a variety of oligosaccharides of which free lactose is usually only a minor component [2]. In the tammar wallaby, *Macropus eugenii*, the major oligosaccharides comprise a trisaccharide, Galß1 → 3Galß1 → 4Glc (3′-galactosyllactose) [3] and a series of homologues produced by the addition of successive ß1 → 3 galactosyl groups to the non-reducing end of lactose. Members of the series up to the heptasaccharide have been separated and their chemical structures have been determined [4].

In addition to the above, a second, branched, homologous series, the members of which contain a GlcNAc residue, has been found in milk of the tammar wallaby; their structures were Galß1 → 3[GlcNAcß1 → 6]Galß1 → 4Glc (lacto-*N*-novotetraose) [5], Galß1 → 3[Galß1 → 4GlcNAcß1 → 6]Galß1 → 4G1C (lacto-*N*-novopentaose I) and Galß1 → 3Galß1 → 3[Galß1 → 4GlcNAcß1 → 6]Galß1 → 4Glc [6].

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SSDI 0304-4165(93)E0099-5

The presence of sialyl oligosaccharides, in addition to neutral sugars, was demonstrated by gel filtration on Sephadex G25 of the carbohydrate fractions of milk from the grey kangaroo, *Macropus giganteus* [7] and the tammar wallaby [8]. However, the chemical properties of these oligosaccharides have remained unexplored. In this paper, we describe the partial chemical characterization of mono- and di-sialyl oligosaccharides from tammar wallaby milk.

2. Materials and methods

2.1. Materials

Samples of milk from animals milked at 121 to 195 days post partum, were supplied by Dr. Brian Green. 3'-Galactosyllactose, Galß1 → 6Galß1 → 4Glc (6'-galactosyllactose) and Galß1 → 4GlcNAcß1 → 3[Galß1 → 4GlcNAcß1 → 6]Galß1 → 4Glc (lacto-*N*-neohexaose) were isolated from horse colostrum [9]. Neu5Acα2 → 3Galß1 → 4Glc (3'-sialyllactose) and Neu5Acα2 → 6Galß1 → 4Glc (6'-sialyllactose) were purchased from Sigma Chemical Co., St. Louis, MO, USA. Neu5Ac was obtained from Kanto Kagaku Co, Tokyo, Japan, GlcNAc from Kishida Kagaku Co, Osaka, Japan, D-galactose from Merck Co, Darmstadt, Germany and glucosamine from Nakarai Kagaku Co, Kyoto, Japan.

2.2. Preparation of sialyl oligosaccharides from milk

Extraction of the carbohydrate from a pooled sample of milk from various animals was performed as previously described [7]. A weighed amount (usually 500 mg) of the milk carbohydrate was dissolved in 2 ml of water and the solution passed through two columns, each 3.7 × 140 cm of Sephadex G25 (Pharmacia Fine Chemicals, Uppsala, Sweden) connected in series [8]. All fractions containing saccharides which eluted prior to hexasaccharides were pooled and freeze-dried. The saccharides were then dissolved in 100 ml of distilled water and dialysed against water using dialysis tubing (Viskase Sales Corp., Chicago, IL, USA) of 43 mm width, 0.0203 mm thickness, with a molecular weight cut-off of about 12000. Dialysis was done first against 1 litre of distilled water, then against running tap water and finally against 5 l of distilled water, each for 24 h. Both the retentate and the initial 1 litre of dialysate were freeze-dried, yielding 210 mg and 190 mg, respectively, from 2 g of the milk carbohydrate.

The undialysable saccharides in the retentate were subjected to ion exchange chromatography on DEAE-Sephadex A-50 (Pharmacia Fine Chemicals, Uppsala, Sweden). For analytical scale experiments, 20 mg of the sample were dissolved in 3 ml of 50 mM Tris-(hydroxymethyl)aminomethane-hydrochloric acid buffer (pH 8.7) and the solution was applied to a column (1.5 × 25 cm) of DEAE-Sephadex A-50 equilibrated with the same buffer. The column was eluted first with 450 ml buffer and then with a linear gradient of NaCl from 0 to 1.0 M in buffer in a total volume of 500 ml. Elution was done at a flow rate of 20 ml/h and fractions of 5 ml were collected. A 0.5 ml sample of each fraction was assayed by the phenol-sulfuric acid method [10]. For preparative scale experiments, 190 mg of the sample was dissolved in 5 ml of the above-mentioned buffer and the solution applied to a column of 3.0 × 35 cm. Elution was done at a flow rate of 28 ml/h and fractions of 7 ml were collected. The fractions containing the separated saccharides were pooled and dialysed against running tap water and 5 l of distilled water, followed by freeze-drying.

2.3. Gel filtration on HPLC

HPLC analysis was performed on a Hitachi L-6200 intelligent pump using an Asahipak GS-320 column (7.6 × 500 mm, particle size 9 μm). Samples were applied in distilled water or 5 mM triethylamine-acetate buffer (pH 5.0). The column was eluted at 25°C and a flow rate of 1.0 ml/min. The peaks were detected with a UV monitor at 220 nm and with a RI detector. Molecular weights of the peak components were estimated by using a Shodex standard pullulan kit *P*-200 (mol wt. 186000), *P*-50 (mol wt. 48000), *P*-20 (mol wt. 23700), *P*-10 (mol wt. 12200) and *P*-5 (mol wt. 4800) (Showa Denko Co., Tokyo, Japan) as standards.

2.4. Methanolysis and methylation analysis

The sugar compositions of the samples were determined by GC after methanolysis and trimethylsilylation [11]. Methanolysis was performed with 2% HCl-methanol at 70°C for 16 h. HCl; 2% in methanol was prepared from 5% HCl-methanol (Nakarai Kagaku, Japan) by dilution with anhydrous methanol. The trimethylsilylation was done with a TMS-HT kit (Tokyo Kasei Co, Japan). The values for the Glc:Gal:GlcNAc ratios of the saccharides were estimated by a comparison of the peak areas in GC of their methanolysates with that of the methanolysate of lacto-*N*-neohexaose.

Reduction of the saccharides was carried out in 1 M NaBD4 solution at room temperature for 24 h in the dark. The resulting oligosaccharidiotols were permethylated by the method of Hakomori [12]. Alditol acetate derivatives of partially methylated sugars were prepared from the permethylated oligosaccharidiotols by the method of Stellner et al. [13], except that the hydrolysis of the permethylated oligosaccharidiotols was performed by heating with 90% formic acid at 100°C for 1 h and then with 2 M trifluoroacetic acid at 100°C

for 5 h. Each partially methylated alditol acetate was identified by a comparison of their GC retention times with those of the standards. Partially methylated alditol acetate standards were prepared from 3'- and 6'-galactosyllactose and lacto-*N*-neohexaose.

GC was run on a Shimadzu 6A gas liquid chromatograph equipped with a flame ionization detector and a glass column (0.2 × 300 cm) packed with 3% SE-30 on Chromosolv W. It was operated at a temperature gradient of 3°C/min from 150 to 250°C.

^{1}H-NMR

^{1}H-NMR spectra were recorded in D_2O (99.95% atom D, Merck, Germany) at 400 MHz with a JEOL-JNM-GSX-400 spectrometer operated at 300 K. Chemical shifts are expressed downfield from internal 3-(trimethylsilyl)-1-propane sulfonic acid, sodium salt (TPS), but were actually measured by reference to internal acetone ($\delta = 2.225$).

2.5. Assay methods

Neutral sugar content was assayed colorimetrically using 5 μg of sample in 100 μl of sample solution by the phenol-sulfuric acid reaction [10] using D-galactose as a standard.

Hexosamine was determined on 20 μg of sample in 100 μl by the Elson-Morgan reaction [14], after acid hydrolysis in 3 M HCl at 100°C at 4 h. Glucosamine was used as a standard.

Sialic acid was determined on 14 μg of sample in 100 μl by the periodate-resorcinol reaction [15] using *N*-acetylneuraminic acid as a standard.

All determinations were performed in triplicate per run.

3. Results

3.1. Fractionation of undialysable saccharides from tammar wallaby milk

When the undialysable saccharides from the initial gel filtration (see Section 2) were subjected to anion exchange chromatography on DEAE-Sephadex A-50 with 50 mM Tris-(hydroxymethyl)aminomethane-hydrochloric acid buffer (pH 8.7), they separated into three peaks (Fig. 1). The the first peak emerged soon after the void volume, consistent with it being a neutral saccharide. The second, SO-1, eluted later than the void volume whereas the third, SO-2, eluted only with 0.1 M NaCl in the buffer, suggesting that SO-1 and SO-2 are acidic, with SO-2 being more negatively charged than SO-1. This paper deals only with the acidic saccharides, SO-1 and SO-2.

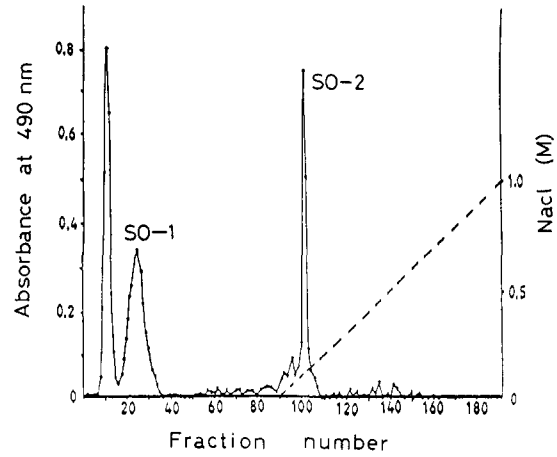


Fig. 1. An ion-exchange chromatogram of higher oligosaccharides separated from the carbohydrate fraction of milk from the tammar wallaby. A DEAE-Sephadex A-50 column (1.5×25 cm) equilibrated with 50 mM Tris-(hydroxymethyl)aminomethane-hydrochloric acid buffer (pH 8.7) was used. Elution was done first with 450 ml of the buffer and then with a linear gradient of the same buffer containing NaCl from 0 to 1.0 M. The flow rate was 20 ml/h and fractions of 5 ml were collected. The fractions were monitored by the phenol-sulfuric acid method (absorbance at 490 nm).

3.2. Gel filtration of acidic saccharides on HPLC

The compounds SO-1 and SO-2 were each analysed on HPLC, using gel permeation mode with 5 mM triethylamine-acetate buffer (pH 5.0) as eluent (Fig. 2). Their molecular weights, determined from their elution times using the pullulan standards, were estimated to be around 3000. Although each emerged as a single peak, both peaks were rather broad, consistent with their being mixtures of components with similar molecular weights.

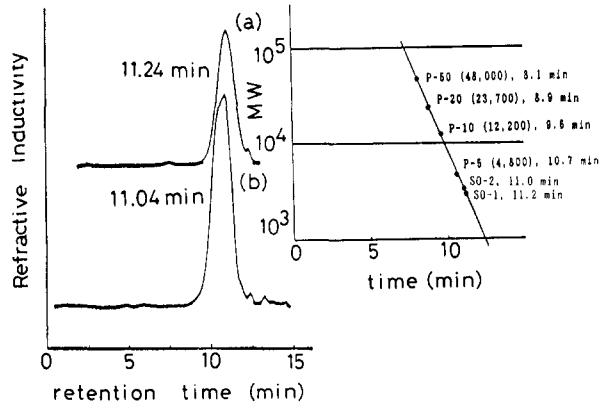


Fig. 2. HPLC of the sialyl oligosaccharides, SO-1 and SO-2, separated from tammar wallaby milk. HPLC was done on a Hitachi L-6200 intelligent pump using an Asahipak column (7.6×500 mm, particle size 9 μm, Asahi Chemical Industry Ltd., Kawasaki, Japan) with 5 mM triethylamine-acetate buffer (pH 5.0). Elution was done at a flow rate of 1.0 ml/min. The peaks were detected with a refractive index detector using a Hitachi L-3350 RI monitor. The inserted graph is a calibration curve for molecular weights of a Pullulan P kit and samples. (a) SO-1 (b) SO-2.

3.3. Chemical characterization by methanolysis, colorimetric assay and methylation analysis

GC of their trimethylsilyl ethers after methanolysis showed that both SO-1 and SO-2 contained D-glucose (Glc), D-galactose (Gal), *N*-acetylglucosamine (GlcNAc) and *N*-acetylneuraminic acid (Neu5Ac) (chromatograms not shown). The molar ratios of Glc:Gal:GlcNAc were shown by methanolysis to be 1.00:9.27:1.74 (SO-1) and 1.00:8.01:2.02 (SO-2).

Each of SO-1 and SO-2 was assayed colorimetrically for neutral saccharide, hexosamine and sialic acid. The hexosamine contents (%, w/w) of SO-1 and SO-2 were estimated as being 15.0 and 19.1, respectively, representing 1.6 and 2.1 mol/mol, respectively, when calculated from their molecular weights (see below). The sialic acid contents (%, w/w) of SO-1 and SO-2 were estimated to be 7.7 and 16.0, respectively, indicating that SO-2 contains twice as much sialic acid as SO-1. From these values, SO-2 contain 0.6 and 1.3 mol of

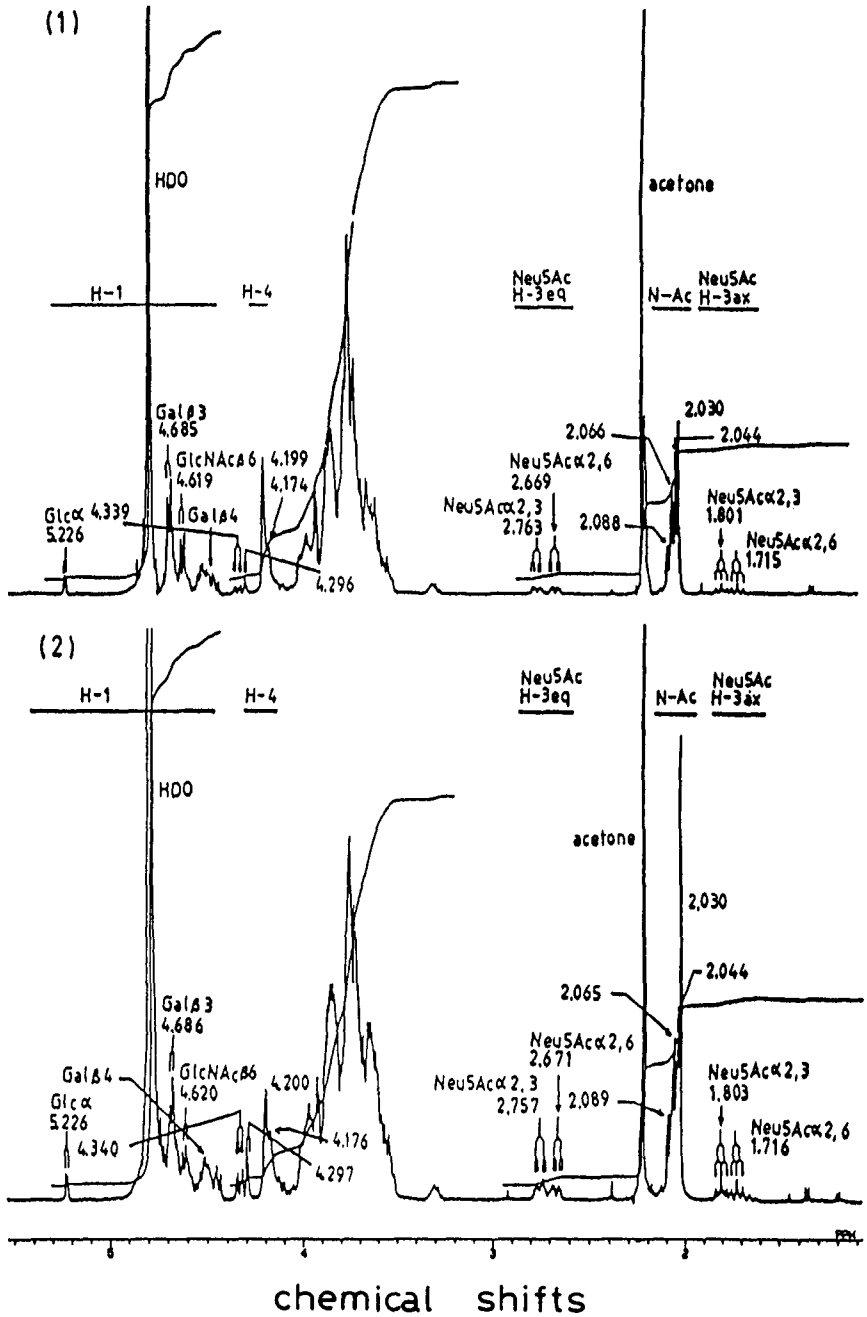


Fig. 3. 400 MHz ^{1}H-NMR spectra of the sialyl oligosaccharides (1) SO-1 and (2) SO-2 of the tammar wallaby milk. The spectra were recorded in D_2O (99.95% D). Chemical shifts (ppm) are expressed downfield from internal TPS, but were actually measured by reference to internal acetone (δ = 2.225).

Table 1
Partially methylated alditol acetates obtained from the sialyl oligosaccharides of tammar wallaby milk

Sugar derivative	Compound	
	SO-1	SO-2
1,2,3,5,6-OMe-GLc	0.6	0.8
2,3,4,6-OMe-Gal	1.4	0.8
2,4,6-OMe-Gal	4.5	2.9
2,3,4-OMe-Gal	0.3	0.6
2,4-OMe-Gal	2.0	2.0
3,6-OMe-GlcN(Me)Ac	1.7	1.7

The partially methylated alditol acetates were identified by a comparison of their retention times in GC with standard samples of partially methylated alditol acetates.The values were calculated by a comparison of the peak areas in GC with those from 3′- and 6′-galactosyllactose and lacto-*N*-neohaxaose.All values are given as the relative ratio to 2.0 moles of 2,4-OMe-Gal.

sialic acid per mol, respectively, consistent with the presence of 1 and 2 mol of sialic acid per mole of SO-1 and SO-2, respectively, if one assumes that the values obtained in the assay were somewhat low.

From the above data, the monosaccharide compositions of SO-1 and SO-2 were deduced to be, respectively; $(Glc)_1(Gal)_9(GlcNAc)_2(Neu5Ac)_1$ and $(Glc)_1(Gal)_8(GlcNAc)_2(Neu5Ac)_2$. However, these monosaccharide compositions must be regarded as average values since neither SO-1 nor SO-2 had been purified to a single compound; each of them may be heterogeneous.

From the above monosaccharides compositions, molecular weights of 2335 and 2464 were calculated for SO-1 and SO-2, respectively. These values are somewhat lower than these obtained by gel filtration on HPLC using the standard mol wt. curve obtained with the pullulan P kit. However, the values obtained by HPLC are not necessarily completely accurate, since, for example, the kinds of monosaccharide in saccharides will affect their elution volumes. The elution profiles, on HPLC, of SO-1 and SO-2 shown in Fig. 2 had broad peaks, suggesting that the saccharides might extend three-dimensionally within the column.

The GC data (Table 1) of the partially methylated alditol acetates prepared from the permethylated sacchariditols of SO-1 and SO-2 demonstrated further characteristics of their chemical structures. The presence of 4-*O*-acetyl-1,2,3,5,6-*O*-methylglucitol in the acetates from SO-1 and SO-2 showed that both have a (1 → 4) linked glucosyl residues at the reducing end. The acetates from SO-1 and SO-2 contained several mol of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol derivatized from internal *O*-3 substituted Gal residues. The presence of 1.4 and 0.8 mol of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol in the acetates from SO-1 and SO-2, respectively, indicated that these are two residues and one residue of non reducing Gal in SO-1 and SO-2, respectively. The other data for the acetates indicated that SO-1 and SO-2 contain two residues of *O*-3,6 disubstituted Gal and two residues of *O*-4 substituted GlcNAc. Therefore, SO-1 and SO-2 are (1 → 3) linked galactosylated saccharides which contain *O*-4 substituted reducing Glc, two Gal1 → 4GlcNAc1 → 6 units attached to Gal residues plus one and two non-reducing Neu5Ac residues, respectively. In addition, the finding of the small ratios of 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl galactitol in the acetates from SO-1 and SO-2 suggest that the saccharides contain Neu5Ac2 → 6Gal1 → units.

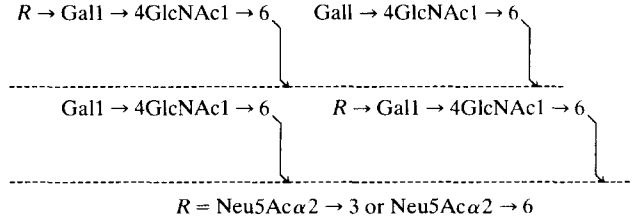
^{1}H-NMR results

Structural characterization was further done from their ^{1}H-NMR spectra (Fig. 3). The assignments shown in Table 2 were based on a comparison with the relevant chemical shifts of Galß1 → 3Galß1 → 3Galß1 → 4Glc, Galß1 → 3[GlcNAcß1 → 6]Galß1 → 4Glc, Galß1 → 3[GlcNAcß1 → 6]Galß1 → 3Galß1 → 4Glc [16], and 3′- and 6′-sialyllactose [17].

(1) SO-1

Two sets of signals of the H-3 of Neu5Ac were found at δ 2.669 and δ 2.763 as well as at δ 1.715 and δ 1.801, respectively. From a comparison with the relevant resonances of 6′ and 3′-sialyllactose, these resonances can be assigned to H-3eq and H-3ax of Neu5Ac of Neu5Acα2 → 6Gal → and Neu5Acα2 → 3Gal → units. According to Kamerling et al. [18], for α-amoners of Neu5Ac, H-3eq varies between 2.6 and 2.8; the observed chemical shifts of H-3eq therefore indicate that 1 mol configurations of Neu5Ac in SO-1 are α-anomers. Since SO-1 contains only one mole of Neu5Ac per mole, the presence of two sets of H-3eq and H-3ax signals indicates that SO-1 is heterogeneous, containing compounds which have Neu5Acα2 → 6Gal or Neu5Acα2 → 3Gal units in their structures. From a comparison of the intensity of H-3eq and H-3ax of Neu5Acα2 → 6 with that of Neu5Acα2 → 3, the presence of equimolar amounts of both Neu5Ac units can be concluded.

The *N*-acetyl (NAc) resonances observed at δ 2.044, δ 2.066 and δ 2.088 arise from GlcNAc residues. Since SO-1 contains only two mol of GlcNAc, the finding of three signals of NAc of GlcNAc residues is probably the result of SO-1 being heterogeneous. The component SO-1 has the possibility of the following four combinations of heterogeneity in its structure;



As each compound is assumed to have a different magnetic environment of NAc protons to each other,

Table 2
The assignment of ^{1}H-chemical shifts (p.p.m.) of the saccharides SO-1, SO-2 of tammar wallaby milk and of other relevant compounds

Reporter group	Residue	Chemical shift (Coupling constant)						
		*(Galβ1-3)$_2$--Galβ1-4Glc	*Galβ1-3(GlcNAcβ1-6)--Galβ1-4Glc	*Galβ1-3(GlcNAcβ1-6)--Galβ1-3Galβ1-4Glc	Neu5Acα2-3Gal--β1-4Glc	Neu5Acα2-6Gal--β1-4Glc	SO-1	SO-2
H-1	Galβ4	4.512(7.9) [1]	4.498(7.8) [1]	4.511(7.8) [1]	4.531(7.7) [1]	4.428(7.7) [1]	4.435-4.543	4.436-4.4542
	Galβ3	4.679(7.7) [1] (Gal″β3)	4.611(7.4) [1]	4.680(8.2) [1] (Gal″β3)	-	-	4.685(7.3) [1]	4.686(7.3) [1]
		4.620(7.7) [1] (Gal‴β3)		4.618(7.7) [1] (Gal‴β3)				
	GlcNacβ6.							
	α	-	4.626(8.5) [1]		-	-		
				4.586(8.4) [1]			4.619(7.7) [1]	4.620(7.3) [1]
	β	-	4.621(8.5) [1]		-	-		
H-3eq	Neu5Ac	-	-	-	2.758(4.4) [2]		2.763(4.3) [2]	2.757
						2.714(4.8) [2]	2.669(4.3) [2]	2.671(4.8) [2]
H-3ax	Neu5Ac	-	-	-	1.798(12.5 [3],-12.1 [4])		1.801(12.5 [3],-12.1 [4])	1.803(12.1 [3],-12.5 [4])
						1.744(12.1 [3],-12.5 [4])	1.715(12.5 [3],-12.1 [4])	1.716(12.5 [3],-11.7 [4])
H-4	Gal′β4	4.198	4.178(3.3) [5]	4.179(3.3) [5]	-	-	4.174	4.176
	Galβ3	4.198	-	4.166(3.3) [5]	-	-	4.174,4.199	4.176,4.200
Nac	GlcNAc	-	2.066	2.047	-	-	2.044,2.066,2.088	2.044,2.065,2.089
	Neu5Ac	-	-	-	2.030	2.029	2.030	2.030

[1],$J_{1,2}$, [2],$J_{3eq,4}$, [3],$J_{3ax,4}$, [4],$J_{3ax,3eq}$, [5],$J^{*}_{3,4}$
The data for thier chemical shifts are from Ref. 16.

the resonances of NAc of GlcNAc should resolve to three signals.

The signals at δ 4.685, δ 4.619 and δ 4.435-δ 4.543 are assigned to H-1 of Galß3, GlcNAcß6 and Galß4, respectively. As the total intensity of the shifts of NAc of GlcNAc residues was about 2-fold higher than the intensity of the chemical shifts of H-1 of Galß4 at δ 4.435-δ 4.543, H-1 of Galß4 corresponds to three protons, which are likely to be H-1 of Galß4 of two units of Galß1 → 4GlcNAc and one unit of Galß1 → 4Glc. The resonances at δ 4.199 and δ 4.174 are assigned to H-4 of ß-D-Gal residues, which are substituted at *O*-3. Because the total intensity of the H-4 resonances at δ 4.199 and δ 4.174 is 2.1-fold higher than the intensity of the H-1 resonances of Galß4, the former resonances may correspond to six protons.

The ^{1}H-NMR spectrum of SO-1 showed the characteristic chemical shift of NAc of a Neu5Ac residue at δ 2.030. By comparing its signal intensity with the total signal intensity of NAc of GlcNAc residues, the signal at δ 2.030 can be deduced to correspond to three protons of NAc of one Neu5Ac residue. The signals at δ 4.339 and δ 4.296 may be due to the H-3 and H-4 of Gal, respectively, attached by a Neu5Ac unit through a α 2 → 3 linkage, but these assignments must be regarded as very tentative at this stage. The resonance of H-1 of Glcß may overlap with the resonance at δ 4.685.

The Gal residues to which either of the GlcNAcß6 residues are linked can not readily be fixed. Our previous study showed that the ß-6-*N*-acetylglucosaminyltransferase of tammar wallaby mammary glands transfers GlcNAc to the penultimate Gal residue at the non-reducing end of Galß1 → 3Galß1 → 3Galß1 → 4Glc [16]. However, the ß 3galactosyltransferase present in these glands [20] might be able to attach Gal to the non-reducing Galβ3 of saccharides which have GlcNAc ß 6 attached to the penultimate Gal residue, to form structures such as the hexasaccharide of tammar milk described in [6]. Therefore SO-1 need not have Galß 1 → 4GlcNAcß6 attached to the penultimate Galß3; these units could be linked to any of the Gal residues of the chain except for the one at the non-reducing end.

The above considerations suggest the following approximate structure for SO-1;

$$\overset{R_{a1}\text{ or }R_{b1}\qquad R_{a1}\text{ or }R_{b1}}{\overset{\downarrow\qquad\qquad\downarrow}{\text{Gal}\beta1 \rightarrow 3\overbrace{(\text{Gal}\beta1 \rightarrow 3)_n\text{Gal}\beta1}} \rightarrow 4\text{Glc}}$$

R_b = Galβ1 → 4GlcNAcβ1 → 6

R_a = Neu5Acα2 → 3/2 → 6Galβ1 → 4GlcNAcβ1 → 6

We suggest that $n = 5$ on the basis of our estimation of the number of protons as determined by ^{1}H-NMR and the monosaccharide composition.

The ^{1}H-NMR and methylation analysis data do not negate the possibility of the presence of the following structure in SO-1;

$$\overset{R_{b1}\qquad R_{b1}}{\overset{\downarrow\qquad\downarrow}{\text{Neu5Ac}\alpha2 \rightarrow 3/2 \rightarrow 6\text{Gal}\beta1 \rightarrow 3\overbrace{(\text{Gal}\beta1 \rightarrow 3)_n\text{Gal}\beta1} \rightarrow 4\text{Glc}}}$$

However, this is unlikely because oligosaccharides with an unbranched sequence Neu5Acα2 → 3/2 → 6Galß1 → 3, as in Neu5Acα2 → 3/2 → 6Galß1 → 3Galß1 → 4Glc or Neu5Acα2 → 3/2 → 6Galß1 → 3Galß1 → 3Galß 1 → 4Glc, have not been identified in tammar wallaby milk. If the α-*N*-acetylneuraminyltransferase of tammar wallaby mammary glands had a substrate specificity directed to non-reducing Galß1 → 3 residues, oligosaccharides such as Neu5Acα2 → 3/2 → 6Galß1 → 3Galß1 → 4Glc would be present in the milk.

(2) SO-2

The pattern of the spectrum of SO-2 is quite similar to that of SO-1. The following characteristic chemical shifts are found in the spectrum; H-1 of Galß 3, GlcNAcß 6 and Galß 4 at δ 4.686, δ 4.620 and δ 4.436-δ 4.452, respectively, H-4 of ß-D-Gal residue, which are substituted at 0-3, at δ 4.200 and δ 4.176, H-3eq and H-3ax of Neu5Ac of Neu5Acα2 → 6Gal at δ 2.671 and δ 1.716, respectively, and H-3eq and H-3ax of Neu5Ac of Neu5Acα2 → 3Gal at δ 2.757 and δ 1.803, respectively. Thus, the spectrum shows that SO-2 is similar in structure to SO-1.

However, the intensity of the shift at δ 2.030 is 2-fold higher than that in SO-1, indicating that SO-2 contains 2 mol of Neu5Ac residues per mol. From a comparison of the intensity of the H-1 resonances of Galß4 with those of NAc of the GlcNAc residues at δ 2.044, δ 2.065 and δ 2.089, the presence of three protons of H-1 of Gal ß4 is deduced in SO-2. On the other hand, as the intensity of the resonances of H-4 of ß-D-Gal, which are substituted at *O*-3, is 1.6 fold as high as the intensity of the H-1 resonances of Galß4, they correspond to five protons.

From the above, the approximate structure of S0-2 is assumed to be:

$$\overset{R_{a1}\qquad R_{a1}}{\overset{\downarrow\qquad\downarrow}{\text{Gal}\beta1 \rightarrow 3\overbrace{(\text{Gal}\beta1 \rightarrow 3)_n\text{Gal}\beta1} \rightarrow 4\text{Glc}}}$$

R_a = Neu5Acα2 → 3/2 → 6Galβ1 → 4GlcNAcβ1 → 6

We suggest that $n = 4$ on the basis of our estimation of the number of protons as determined by ^{1}H-NMR and the monosaccharide composition.

4. Discussion

Since Bolliger and Pascoe's finding [19] with milk of the wallaroo (*Macropus robustus*) that its principal carbohydrate was not lactose, several studies have focused on the separation and structural elucidation of milk oligosaccharides in marsupials. Messer and Mossop [7] separated the carbohydrate fraction of milk from the eastern grey kangaroo (*Macropus giganteus*), by gel filtration on Sephadex G25, into 10 peaks of neutral saccharides plus several which contained sialyl and neutral higher oligosaccharides. Similar chromatographic patterns were obtained with milk from the tammar wallaby [8].

So far, eight neutral oligosaccharides have been purified from milk of the tammar wallaby and their chemical structures determined. The predominant oligosaccharides are 3'-galactosyllactose and its homologues, which are ß 1 → 3 linked galactosides, up to a heptasaccharide [3,4]. In addition to a series of galactosyllactose, smaller amounts of members of a second branched homologous series, lacto-*N*-novotetraose, lacto-*N*-novopentaose I and Galß1 → 3Galß1 → 3[Galß1 → 4GlcNAcß1 → 6]Galß1 → 4Glc, were isolated [5,6]. These GlcNAc-containing oliosaccharides are probably synthesized from lactose by ß3 and ß4 galactosyltransferases and a ß 6*N*-acetylglucosaminyltransferase, whose activities have been identified in tammar wallaby mammary glands [16,20].

Although milk of the eastern grey kangaroo and the tammar wallaby had been shown to contain higher neutral and sialyl oligosaccharides, the chemical properties of these oligosaccharides had not been clarified. The results obtained in this study have elucidated the main features of the chemical structures of the sialyl oligosaccharides, even though none of the components was purified into a single compound. Since these compounds have similar molecular weights of about 2500 as estimated by monosaccharide analysis and have closely related chemical structures, their complete purification may be very difficult. Our results show that the sialyl saccharides in the milk of the tammar wallaby are ß (1 → 3) linked galactosylated saccharides which contain a reducing Galß 1 → 4Glc unit, two units of Galß1 → 4GlcNAc attached ß1 → 6 to Gal residues and up to two residues of Neu5Acα2 → 3 or Neu5Acα2 → 6. This is the first demonstration that marsupial milk contains saccharides which have more than one residue of GlcNAc. It is noteworthy, however, that Messer and Mossop [7] had shown that the components in the Sephadex G-25 peaks which were larger than hexasaccharides contained up to 1.7 GlcNAc residues per mole.

Although the oligosaccharides in this paper have mol wt. of about 2500, they were not dialysable even though, normally, compounds whose molecular weights are less than about 10000 are dialysable against water. Since these saccharides have dibranched structures, it is possible that they expand three- dimensionally in water and therefore, cannot pass through the membrane of the dialysis tubing.

In the carbohydrate fraction from tammar milk, the lower oligosaccharides consist mainly of a series of unbranched ß (1 → 3) galactosyllactose; the second homologous series, whose members contain GlcNAc and are branched, are only minor components. However, our results indicate that among the sialyl oligosaccharides, branched sacchrides containing GlcNAc residues are the main components. It is likely that lacto-*N*-novotetraose, lacto-*N*-novopentaoseI and Galß1 → 3Galß1-3[Galß1 → 4GlcNAcß1 → 6]Galß1 → 4Glc are intermediates in the synthesis of these higher oligosaccharides.

In human milk, many oligosaccharides have structures in which the Gal residue of the lactosyl (Galß1 → 4Glc) core unit is substituted by Gal ß1 → 4GlcNAc or Galß 1 → 3GlcNAc unit at O-3 and O-6 [21]. These human oligosaccharides also have many heterogeneities produced by the attachment of Neu5Acα2 → 3, Neu5Acα2 → 6, Fucα1 → 2, Fucα 1 → 3 or Fucα 1 → 4 residues. Marsupial milk oligosaccharides, on the other hand, have another branching unit of Gal residue substituted by Galß 3 and GlcNAc residues at *O*-3 and *O*-6, respectively. This indicates that there are differences in the ways branching units are formed by glycosyltransferases in the milk oligosaccharides of human and marsupials. It is likely that these differences are due to varying acceptor specificities of the ß *N*-acetylglucosaminyltransferases of the mammary glands of different species. It is also noteworthy that in contrast to human milk oligosaccharides, none of the marsupial milk saccharides isolated so far contain any fucosyl residues.

High molecular weight saccharides such as those described here have not yet been found in the milk or colostrum of other mammalian species. The existence of such large, highly galactosylated, saccharides in marsupial milk may suggests a specific biological role in relation to marsupial newborn.

Acknowledgments

We thank Dr. B. Green for suppling tammar wallaby milk. This work was supported by a grant from Akiyama Life Science Commitee.

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