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Carbohydrates of the Milk of the Platypus

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Abstract

Twelve samples of milk of the platypus, *Ornithorhynchus anatinus*, had a mean content of 3·3% hexose. Of this, almost half was L-fucose. Of the total monosaccharides present in acid hydrolysates of the water-soluble carbohydrates, L-fucose constituted 33%, D-galactose 29%, glucosamine 20%, D-glucose 11% and sialic acid 7%. Free lactose was found in only trace amounts. In all samples, the major oligosaccharide was difucosyllactose, which represented 39–52% of the total hexose. Five higher neutral oligosaccharides (from penta- to nonasaccharides) were isolated and their monosaccharide compositions determined. Each contained one or more residues of fucose, glucosamine and galactose and one residue of glucose. The presence in the milk of 4-*O*-acetyl-*N*-acetylneuraminlactose was detected by thin-layer chromatography. All milk samples examined contained protein material (probably glycoprotein), which was not precipitated by chloroform–methanol extraction. No evidence was obtained for quantitative or qualitative changes in carbohydrates during the course of the lactation season except for a small decline in total hexose towards the end of the season.

Extra keywords: monotremes.

Introduction

In almost all species of eutherian mammals, the principal carbohydrate of their milk is lactose (Jenness *et al.* 1964). Although Marston (1928) had reported lactose to be the carbohydrate of milk of the echidna, *Tachyglossus aculeatus*, more recent studies showed that free lactose is only a very minor component of the milk of both the echidna and another monotreme, the platypus, *Ornithorhynchus anatinus* (Messer and Kerry 1973; see also Griffiths 1968). In echidna milk, the major oligosaccharides were found to be sialyllactose and fucosyllactose. In platypus milk, the predominant carbohydrate was difucosyllactose.

The previous findings on platypus milk were based on one milk sample only. We now report on the carbohydrate composition of a further 12 samples of platypus milk. Preliminary results obtained with two of these were briefly reported previously by one of us (Griffiths 1978).

Materials and Methods

All the milk samples were obtained from animals captured in the upper Shoalhaven River and one of its tributary creeks. The method used for trapping has been described by Grant and Carrick (1974). The milk was taken after intramuscular injection of 2·0 i.u. of oxytocin (Syntocinon; Sandoz, Aust.) per animal. The milks were stored at −20°C for up to 3 months before analysis.

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Total carbohydrate was determined with anthrone reagent as described by Brin (1966), except that the time of heating at 100°C was reduced to 8 min from 10 min, which gave results that were 10–20% higher than those obtained with the unmodified method. This was probably because platypus milk contains fucose, which gives a lower colour yield than lactose when the heating time is extended beyond 8 min (Fig. 1). The phenol–sulfuric acid method, which has been used for the analysis of marsupial milk (Messer and Green 1979), similarly gives a lower colour yield for fucose than for lactose, and gave values for platypus milk that were 12–33% lower than those obtained with the modified anthrone method.

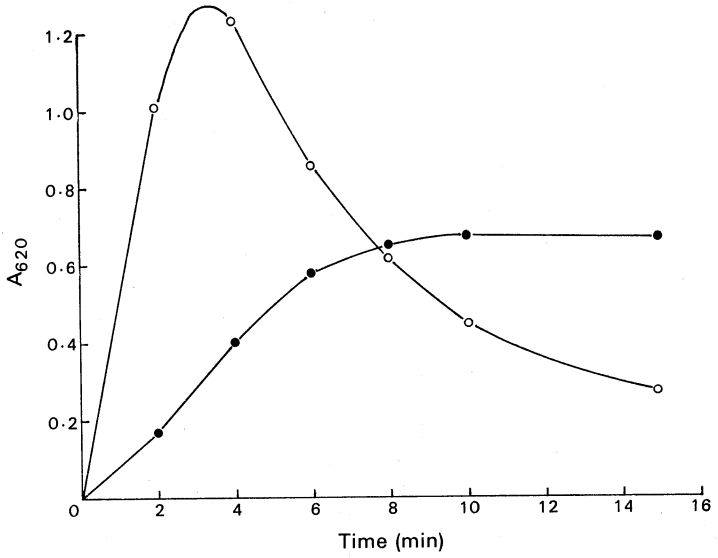

Fig. 1. Effect of heating time on colour yield in the anthrone reaction. ● 100 μg Lactose. ○ 100 μg Fucose.

Sialic acid was determined by the thiobarbituric acid method (Aminoff 1961) after liberation of free sialic acid using 0·05 M H_2SO_4 at 85°C for 45 min.

The water-soluble fractions of the milk samples were obtained by extraction with chloroform–methanol, 2 : 1 (v/v), as described previously (Messer and Mossop 1977).

To determine the monosaccharide composition of some of these fractions, approximately 4 mg of the dried material were dissolved in 0·5 ml of water. Of this solution, 0·1 ml was used for determination of sialic acid (see above); the remainder was mixed with 0·2 ml of 6 M HCl and heated at 100°C for 1 h. The hydrolysate was adjusted to pH 7 with 1 M NaOH, diluted to 5·0 ml and assayed for monosaccharides. L-Fucose and D-galactose were determined with L-fucose dehydrogenase (Sigma Chemical Co., St Louis) and galactose dehydrogenase, respectively (Finch *et al.* 1969). D-Glucose was determined with glucose oxidase and 2,2′-azino-di-(3-ethylbenzthiazoline)-6-sulfonate as chromogen (Bergmeyer and Bernt 1974), and glucosamine by the Elson–Morgan procedure of Gatt and Berman (1966).

Gel-permeation chromatography was done using two columns of Bio-Gel P-4,–400 mesh, each 150 by 1·62 cm, connected in series. Elution was done with water at the rate of 6 ml h^{-1}; fractions of 3–4 ml were collected. In some preliminary experiments, chromatography was done with Sephadex G-25, superfine, using two columns, each 150 by 1·1 cm, connected in series. Fractions were analysed for hexose by the phenol–sulfuric acid method (Messer and Green 1979), for protein by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard, and for sialic acid as described above. The contents of each major peak were pooled, isolated by freeze-drying, examined by thin layer chromatography (t.l.c.) and if necessary rechromatographed to obtain further purification.

To determine the monosaccharide composition of oligosaccharides, approximately 500 μg of purified oligosaccharide were dissolved in 0·25 ml of 2 M HCl and the solution heated at 100°C for 1 h. Of the hydrolysate, 0·02 ml were transferred to a small vial and repeatedly dried *in vacuo* over KOH to remove HCl; the residue was dissolved in 0·01 ml of water and examined for monosaccharides by t.l.c. The remainder of the hydrolysate was adjusted to pH 7 with 1 M NaOH and diluted to 2·5 ml with water. Samples of this solution were assayed for monosaccharides as described above.

T.l.c. was done on silica gel (Merck, 5553). For t.l.c. of monosaccharides, the plates had been impregnated with NaH_2PO_4 (Hansen 1975*a*). Two solvent systems were used: solvent I, 2-propanol–acetone–0·1 M lactic acid (2 : 2 : 1, v/v) (Hansen 1975*a*, 1975*b*); solvent II, ethanol–n-butanol–pyridine–water–acetic acid (100 : 10 : 10 : 30 : 3), v/v (Veh *et al.* 1981). Carbohydrates were detected with aniline–diphenylamine.

Polyacrylamide gel electrophoresis was done in sodium dodecyl sulfate (SDS), essentially as described by Fairbanks *et al.* (1971). Apparent molecular weights were determined by comparison with human red cell membrane proteins of known molecular weights.

Results

Concentration of Carbohydrate in the Milk

Table 1 gives the results of determinations of total hexose and sialic acid in 12 samples of platypus milk. One of these samples was collected in October (i.e. early during the lactation season), 10 in December (mid-lactation) and one in March (late lactation). The sample collected in March contained less hexose and sialic acid than the other samples, but none of the values appeared to be significantly different from the mean values of 3·28% (w/v) for hexose and 0·43% for sialic acid, since all were within 2 standard deviations of these means.

Table 1. Total hexose and sialic acid content of platypus milk

Date of sampling	Animal No.	Hexose[A] (g per 100 ml)	Sialic acid (g per 100 ml)
14.x.1977	—	3·51	0·41
7.xii.1977	[B]	2·59	0·37
7.xii.1981	945	3·33	0·43
8.xii.1981	166	4·38	0·61
8.xii.1981	186	3·30	0·36
8.xii.1981	948	4·18	0·46
8.xii.1981	969	2·59	0·41
9.xii.1981	973	4·05	0·59
10.xii.1981	014	2·19	0·39
10.xii.1981	921	4·56	0·62
10.xii.1981	974	2·63	0·33
16.iii.1982	918	2·00	0·23
Mean±s.d.		3·28±0·88	0·43±0·12

[A] Includes galactose, glucose and fucose (a deoxyhexose or methylpentose).
[B] This sample was pooled from three animals.

Monosaccharide Composition

Table 2 shows the monosaccharide composition of acid hydrolysates of the water-soluble carbohydrate of three milk samples. The results show that L-fucose was the predominant monosaccharide: it constituted one-third of the total carbohydrate

and 46% of the total hexose in all three samples. It was identified as the L-isomer by virtue of its susceptibility to L-fucose dehydrogenase. The other monosaccharides present were D-galactose, D-glucose, glucosamine and sialic acid. There were no obvious quantitative differences between the three samples with respect to any of these constituents. Comparison of the results for sialic acid with the data of Table 1 reveals a discrepancy, as the water-soluble carbohydrate contains less sialic acid (by a factor of about 50%) than expected from the sialic acid content of whole milk. This suggests that much of the sialic acid is bound to protein and/or lipid and is lost during the chloroform–methanol extraction.

Table 2. Monosaccharide composition of water-soluble carbohydrates in platypus milk

Values are given as percentage (w/w) of total

Date of sampling	L-Fucose	D-Galactose	D-Glucose	Glucosamine	Sialic acid
14.x.1977	35	29	11	18	7·3
10.xii.1981	33	28	11	21	7·0
16.iii.1982	34	29	11	21	5·5

Thin-layer Chromatography

When the water-soluble fractions of the milk samples were compared by t.l.c., all 12 samples gave similar patterns for carbohydrates. Each showed a prominent spot which had the same mobility as lactodifucotetraose (difucosyllactose). Only traces of fucosyllactose and lactose and no monosaccharides could be detected. All samples contained numerous higher oligosaccharides in addition to lactodifucotetraose, as well as material that remained at the origin.

Gel-permeation Chromatography

In a previous study, the carbohydrate of a sample of platypus milk separated into four main peaks during chromatography on Sephadex G-15 (Messer and Kerry 1973). In the present work, Sephadex G-25 was used in initial attempts to improve the separation, without success. However, chromatography using Bio-Gel P-4 gave better results, as the components of peaks 2 and 3 were partially separated. This is shown in Fig. 2, which illustrates the elution profile obtained with the carbohydrate of platypus milk collected in December 1977. Similar elution profiles were obtained with the other samples, i.e. in each case there were four main peaks, numbered 1 to 4 in order of elution. The contributions of each of these peaks to the total hexose of the four milk samples examined were (mean values): peak 1, 25%; peak 2, 10%; peak 3, 10%; peak 4, 46%. The remaining 9% consisted mainly of material, not further investigated, that eluted between peaks 1 and 2. In addition, there were two very minor peaks following peak 4, the contents of which were identified by t.l.c. as fucosyllactose and lactose, respectively. The contribution of each of these to the total hexose was less than 1%.

In Fig. 2, the first three peaks are subdivided into 1*a* and 1*b*, 2*a* and 2*b*, and 3*a*, 3*b* and 3*c*.

The contents of peak 1*a* reacted positively in the Lowry procedure for protein (Fig. 2). When freeze-dried, they yielded a white powder, which constituted 30% (w/w) of the material applied to the column; this corresponded to 2·3% (w/v) of the original milk sample. The material of peak 1*a* was soluble in water but not in 10% trichloroacetic acid. An amino acid analysis showed the presence of all the common amino acids, and an unusually high content of cysteine and proline, each of which constituted 12% of the total amino acids. When examined by SDS–gel electrophoresis it showed one major component with an apparent molecular weight of 33000 plus at least five minor ones with apparent molecular weights of 25000, 46000, 56000, 75000 and 88000. All these components were detected with both coomassie blue (a protein stain) and the periodate–Schiff stain for carbohydrates, and were therefore probably glycoproteins.

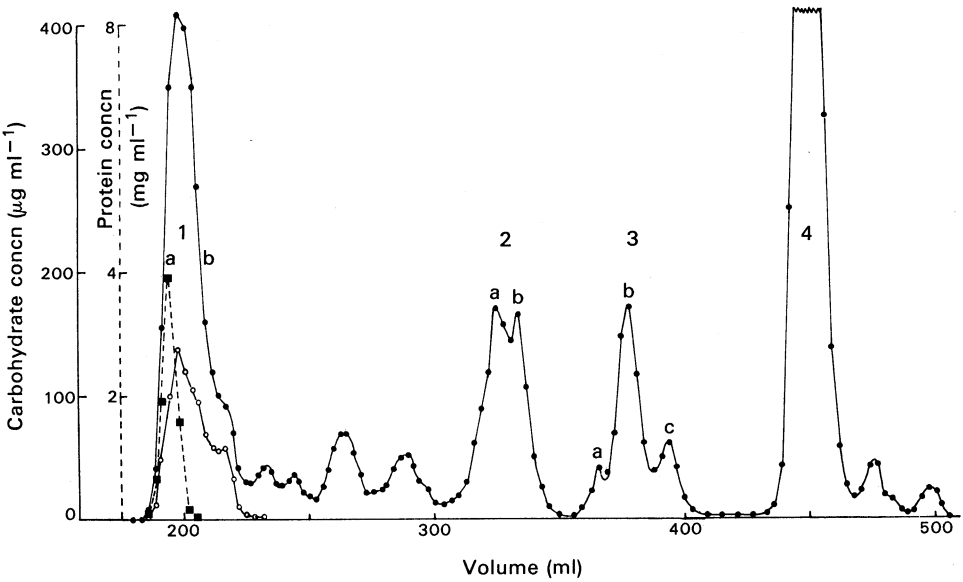

Fig. 2. Gel-permeation chromatography on Bio-Gel P-4 of platypus milk carbohydrate. The water-soluble fraction (100 mg) of a milk sample collected in December 1977 was used. ● Hexose. ○ Sialic acid. ■ Protein. The Bio-Gel column was calibrated with the following oligosaccharides (elution volumes shown in parentheses): lactose (492 ml); 3′-galactosyllactose (467 ml); digalactosyllactose (439 ml); trigalactosyllactose (415 ml); tetragalactosyllactose (393 ml); pentagalactosyllactose (374 ml). The galactosyllactoses were obtained from tammar wallaby milk as described by Messer *et al.* (1980) and Collins *et al.* (1981). For peak 4, the values for carbohydrate were 768, 1119, 963 and 625 μg ml^{-1} at elution volumes of 445, 448, 450 and 453 ml, respectively. For other details, see Materials and Methods.

The contents of peak 1*b* were very heterogeneous. T.l.c. using solvent II revealed the presence of over 12 components, of which the most rapidly migrating and most prominent had the same mobility as 4-*O*-acetyl-*N*-acetylneuraminlactose (Messer 1974), $R_F = 0{\cdot}70$ and *N*-acetylneuraminlactose, $R_F = 0{\cdot}62$.

The contents of each of peaks 2*a*, 2*b*, 3*a*, 3*b* and 3*c* appeared to be homogeneous (t.l.c.), although rechromatography of material that had been pooled from several chromatographic runs was necessary to obtain the desired purity. Each purified

oligosaccharide was subjected to monosaccharide analysis; the observed molar ratios are shown in Table 3, together with the R_F values in solvent II. The results indicate that peak 2*a* contains a nonasaccharide, 2*b* an octasaccharide, 3*a* a heptasaccharide, 3*b* a hexasaccharide and 3*c* a pentasaccharide. Each of these oligosaccharides contained either one or two residues of glucosamine, identified as such by t.l.c. The presence of glucosamine accounts for their anomalously high rates of elution (Fig. 2) since one *N*-acetylglucosamine residue in an oliogosaccharide behaves like two residues of hexose on Bio-Gel P-4 (Kobata *et al.* 1978).

Table 3. R_F values and monosaccharide compositions of contents of peaks 4, 3 and 2 of Fig. 2

Peak No.	R_F[A]	Molar ratio				Probable composition[B]
		D-Glucose	D-Galactose	Glucosamine	L-Fucose	
4	0·57	1·0	1·1	0	2·1	Glc Gal $(Fuc)_2$
3*c*	0·52	1·0	2·0	1·1	1·3	Glc $(Gal)_2$ GlcNAc Fuc
3*b*	0·49	1·0	2·0	1·0	2·2	Glc $(Gal)_2$ GlcNAc $(Fuc)_2$
3*a*	0·41	1·0	2·2	1·1	3·3	Glc $(Gal)_2$ GlcNAc $(Fuc)_3$
2*b*	0·35	1·0	3·1	2·1	2·2	Glc $(Gal)_3$ $(GlcNAc)_2$ $(Fuc)_2$
2*a*	0·33	1·0	3·1	2·0	3·2	Glc $(Gal)_3$ $(GlcNAc)_2$ $(Fuc)_3$

[A] R_F values were measured in solvent II.
[B] Glc, glucose; Gal, galactose; Fuc, fucose; GlcNAc, *N*-acetylglucosamine.

The contents of peak 4, which were homogeneous by t.l.c., had the same mobility as lactodifucotetraose of human milk (Kuhn and Gauhe 1958) during t.l.c. in solvents I and II. Monosaccharide analysis showed that they consisted of D-glucose, D-galactose and L-fucose in the molar ratio 1:1:2, respectively (Table 3). These results confirm previous data showing that the major peak observed during gel filtration contains difucosyllactose (Messer and Kerry 1973). Peak 4 contained 39–52% of the total hexose of the four samples examined.

Discussion

This work shows that the amount of carbohydrate in platypus milk is considerably greater than the previously published value of 1·7% for hexose would suggest. This value was obtained with a single sample collected in February 1972, using an unmodified anthrone method for assay of hexose (Messer and Kerry 1973). We have now shown that this method gives low results for fucose-containing milks (see Methods) and calculate that if the modified method had been used the value obtained would have been 1·9–2·0%, i.e. similar to that for the milk of the female taken in March 1982.* Both these samples were collected late during the lactation season; since they have values that are noticeably lower than the mean of 3·3% for all 12

* This platypus (No. 918) was lactating on 9 December 1981 but had very small mammary glands that yielded insufficient milk for a sample. She was caught again on 16 March 1982 and on that occasion exhibited a copious flow of milk. She was caught yet again on 18 April 1982 and was found to be no longer lactating. The fatty acid complement of her milk differed in no way from that of the platypuses milked in December (T. R. Grant and M. Griffiths, unpublished data), suggesting that her milk was normal and that she was still suckling young in mid-March. These results also support the notion (see Griffiths 1978) that lactation in the platypus lasts for *c.* 3½ months.

samples (Table 1), this suggests a fall in carbohydrate content towards the end of lactation.

The previous value of 0·05% for sialic acid (Messer and Kerry 1973) is much lower than the mean of 0·47% reported in this study. However, the latter value refers to total sialic acid including that bound to protein and lipid, whereas the previous value was obtained subsequent to removal of protein and lipid by chloroform–methanol extraction and treatment with trichloroacetic acid. As shown in this paper, about half of the sialic acid is lost during chloroform–methanol extraction, and further amounts would be lost during treatment with trichloroacetic acid. It appears that much of the sialic acid (but not hexose) of platypus milk is bound to protein and lipid.

Our results confirm that platypus milk contains a large amount of L-fucose; this constituted almost half (46%) of the total hexose (Table 2), giving a milk fucose mean content of 1·5% (cf. Table 1). The only other species whose milk has been shown to contain more than trace amounts of fucose is the echidna (Messer and Kerry 1973). In eutherian mammals, L-fucose is found as a constituent of glycoproteins, proteoglycans and glycolipids, but the amounts are small and it seems unlikely that the fucose of monotreme milk is used by the suckling young only for the synthesis of essential fucose-containing tissue constituents. One is therefore led to conclude that developing monotremes utilize L-fucose mainly as an energy source, analogous to the role of milk galactose and glucose in other mammals.

As in the previous study (Messer and Kerry 1973), a large part of the fucose was found to be present as difucosyllactose, a tetrasaccharide that has now been confirmed as the major carbohydrate of platypus milk. We have also confirmed previous findings (Messer and Kerry 1973; Hopper and McKenzie 1974) that free lactose is virtually absent from platypus milk.

All the samples examined contained, in addition to difucosyllactose, a series of higher neutral oligosaccharides, which included a penta-, hexa-, hepta-, octa- and nonasaccharide. Each of these consisted of one residue of glucose, either two or three residues of galactose, one or two residues of glucosamine and between one and three residues of fucose. It is reasonable to assume that they all have lactose at their reducing ends. The pentasaccharide and hexasaccharide have the same monosaccharide composition as human milk lacto-*N*-fucopentaoses and lacto-*N*-fucohexaoses, respectively (see Kobata 1972); the octasaccharide has the same composition as difucosyl para-lacto-*N*-hexaose or neohexaose (Yamashita *et al.* 1977). Further studies will be required to determine the exact structures of these oligosaccharides.

Previous studies showed that the major carbohydrate of echidna milk is an unusual form of sialyllactose (Messer and Kerry 1973), *viz*, 4-*O*-acetyl-*N*-acetylneuraminlactose (Messer 1974; Kamerling *et al.* 1982). This compound has not been found in the milk of other species, but we have now show that it is present in platypus milk. Yet another unusual feature of monotreme milks is the presence of protein material that is not removed by chloroform–methanol extraction. This was previously observed with echidna milk (Messer and Kerry 1973); the data of this paper suggest that platypus milk contains over 2% of this material and that it contains a protein (almost certainly a glycoprotein) with an apparent molecular weight of 33 000. It is of interest in this connection that the sialoglycoproteins of red cell membranes are found in the aqueous phase after chloroform–methanol extraction (Hamaguchi and Cleve 1972).

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