*Aust. J. Biol. Sci.*, 1977, **30**, 379–88

# Milk Carbohydrates of Marsupials
# I. Partial Separation and Characterization of Neutral Milk Oligosaccharides of the Eastern Grey Kangaroo

*M. Messer and G. S. Mossop*

Department of Biochemistry, University of Sydney, Sydney, N.S.W. 2006.

*Abstract*

The carbohydrate fractions of two samples of milk from the eastern grey kangaroo, *Macropus giganteus*, were each separated by gel filtration on Sephadex G25 into 10 peaks of neutral saccharides, plus between two and four peaks which contained sialyl saccharides and neutral polysaccharides.

Of the neutral saccharides, a small monosaccharide peak contained mainly galactose and glucose, a disaccharide peak (which constituted less than 5% of the total hexose) contained lactose, and eight other peaks had the elution volumes expected for tri- to decasaccharides; these contained oligosaccharides which, upon acid hydrolysis, yielded varying proportions of galactose, glucosamine, and glucose.

In both milk samples the principal carbohydrates were neutral tri-, tetra-, penta-, and hexasaccharides, in each of which galactose was the predominant monosaccharide.

The results are discussed in relation to carbohydrates previously found in the milk of marsupials, monotremes, and eutherians.

## Introduction

The carbohydrate composition of the milk of marsupials is known to be very different from that of other mammals. Whereas the principal carbohydrate of the milk of almost all eutherians is lactose (Jenness *et al.* 1964) and that of monotremes is either sialyllactose (echidna) or difucosyllactose [platypus (Messer and Kerry 1973; Messer 1974)], the milk of at least five species of marsupials has been shown to contain, in addition to monosaccharides and a small amount of lactose, one or more oligosaccharides which are composed mainly of galactose (Bolliger and Pascoe 1953; Gross and Bolliger 1958, 1959; Jenness *et al.* 1964; Bergman and Housley 1968).

The exact nature of these marsupial milk oligosaccharides—their number, variety and chemical structure—has remained unexplored, and there are few data on differences in milk carbohydrates between various species or at different times of lactation. Furthermore the mechanism by which marsupial milk oligosaccharides are synthesized in the mammary gland, or digested and absorbed in the intestinal tract of the pouch young, is unknown.

In this paper we describe the partial separation and monosaccharide composition of the neutral oligosaccharides of two milk samples from eastern grey kangaroo, *Macropus giganteus*.

## Materials and Methods

*Milk Samples*

Sample A (30 ml), supplied by Professor G. B. Sharman, Macquarie University, North Ryde, N.S.W., was pooled from three separate milkings of one animal (Code No. G-4) about 280 days

after birth of the pouch young. Prior to extraction of the carbohydrate fraction, sample A was stored at −20°C in the presence of a few drops of toluene (as a bacteriostatic agent) for about 18 months.

Sample B (9·5 ml), supplied by Mr W. E. Poole, Division of Wildlife Research, CSIRO, Canberra, was obtained from an animal (Code No. G-469) whose pouch young was 250 days of age. It was stored at −20°C for a few days only.

In both cases milking was done following an intramuscular injection of oxytocin (Griffiths *et al.* 1972).

*Extraction of Milk Carbohydrate*

The milk was thawed and then extracted with 4 volumes of chloroform–methanol, 2 : 1 (v/v), as described by Öhman and Hygstedt (1968). The emulsion was centrifuged at 4°C and 3500 *g* for 30 min, the lower chloroform layer and the denatured protein were discarded, the methanol was removed from the upper layer by evaporation *in vacuo* at 25°C, and the solution that remained was freeze-dried. The resulting white powder was called the 'carbohydrate fraction'; it was stored dessicated at −20°C. The yield of carbohydrate fraction was 74 mg per gram of milk (sample A) and 75 mg/g (sample B).

*Gel Filtration*

A weighed amount (usually 100 mg) of the carbohydrate fraction was dissolved in 2 ml of water and the solution passed through either two or three columns of Sephadex G15, G25, or G50 (Pharmacia Fine Chemicals, Uppsala, Sweden) or Bio-Gel P4, 400-mesh (Bio-Rad Laboratories, Richmond, California, U.S.A.), each 100 cm long and 2·5 cm in diameter, connected in series. Elution was done with water (saturated with chloroform) at a flow rate of 20–25 ml/h, and fractions of 6–7 ml were collected. Samples (0·2–0·3 ml) of each fraction were analysed for hexose and for sialic acid, as described below. Void volumes were determined with Blue Dextran 2000 (Pharmacia). The columns were calibrated with the following saccharides (5 mg of each): galactose (monosaccharide), lactose (disaccharide), raffinose or maltotriose (trisaccharides) and stachyose or maltotetraose (tetrasaccharides).

For removal of acidic (sialyl) saccharides prior to gel filtration, 100 mg of the carbohydrate fraction dissolved in 2 ml of water were passed through a column of DEAE-Sephadex A25 (Pharmacia), acetate form, 22 cm long and 1·5 cm in diameter. The neutral saccharides were eluted with water; fractions containing hexose were pooled, concentrated, and freeze-dried.

*Monosaccharide Composition*

For qualitative and quantitative determination of the monosaccharide composition of elution peaks from gel filtration, fractions constituting peaks were pooled, concentrated to about 3 ml on a rotating evaporator *in vacuo* at 50–70°C, and freeze-dried. An amount of freeze-dried material calculated to correspond to about 2 μmol (i.e. 0·7–3·3 mg) was weighed into a culture tube (Corning Glass Works, New York, U.S.A., type 9826, 13 by 100 mm) and dissolved in 3·0 ml of 2 M HCl. The tube was flushed with nitrogen gas, sealed with a Teflon-lined screw cap, and placed in a boiling water bath for 1 h. Of the hydrolysate, 0·30 ml was transferred to a small vial and dried *in vacuo* over KOH; the dried material was dissolved in 0·1 ml of water, and the monosaccharide composition of the solution examined by thin-layer chromatography. The remainder of the hydrolysate was adjusted to pH 7 with 1 M NaOH solution and diluted to 10 ml with water. Samples of this diluted hydrolysate were then analysed for glucose, galactose, and glucosamine as described below.

*Assay Methods*

*Total hexose* of fractions from gel filtration was determined by the anthrone method as described by Roe (1955), except that the time of heating was 10 min rather than 15 min. Lactose was used as the standard.

*Sialic acid* was determined by the thiobarbituric acid method of Aminoff (1961), following hydrolysis with 0·05 M $H_2SO_4$ at 85°C for 45 min. *N*-Acetylneuraminic acid was used as the standard.

*Glucose* was determined by the following modification of the glucose oxidase method of Huggett and Nixon (1957). The test solution (1·0 ml), containing up to 0·1 μmol of glucose, was mixed with 1·0 ml of 0·2 M sodium phosphate–potassium phosphate buffer, pH 7·0, containing 0·01%

*o*-dianisidine and 0·001% peroxidase (Sigma Chemical Co., St. Louis, U.S.A., Type I), followed by 0·2 ml of 0·2% highly purified glucose oxidase (Nagase and Co., Ohama, Japan). The mixture was incubated at 37°C for 30 min, 1·0 ml of 50% (v/v) $H_2SO_4$ was then added, and the absorbancy of the solution measured at 530 nm (Messer and Dahlqvist 1966).

*Galactose* was estimated with galactose dehydrogenase (Boehringer, Mannheim, Germany) as described by Finch *et al.* (1969).

*Glucosamine* was determined with an amino acid analyser (JEOL Co. Ltd, Tokyo, Japan, type JLC-6AH) using the long column only. Samples were applied in 0·1 M sodium citrate buffer, pH 2·2. The column was eluted at 53°C, first with 0·2 M sodium citrate buffer, pH 3·33, and then with 0·2 M sodium citrate buffer, pH 4·46; the peak for glucosamine emerged about 40 min after the buffer change, well ahead of that for galactosamine which emerged after about 65 min. Standard solutions of D-glucosamine (0·05 and 0·10 mM) were run with each set of determinations.

*Chromatography*

*Paper chromatography* was done by the descending method on Whatman papers No. 1 or No. 2 (42 cm long) with ethyl acetate–pyridine–water, 12 : 5 : 4 (v/v), as solvent (Kobata 1972) for 24–48 h. Saccharides were located with alkaline $AgNO_3$ reagent (Trevelyan *et al.* 1950).

*Thin-layer chromatography* was done on precoated silica gel 60 plates (E. Merck, Darmstadt, Germany, Art. 5553, 20 by 20 cm) with isopropanol–acetone–0·1 M lactic acid, 4 : 4 : 2 (v/v), as solvent (Hansen 1975); saccharides were located with aniline–diphenylamine reagent.

## Results

*Gel Filtration*

Since Sephadex G15 had previously been used with success in the separation of milk carbohydrates of monotremes (Messer and Kerry 1973), the same gel was used in an initial attempt to separate those of grey kangaroo. Although the lower saccharides separated into five peaks, the major part of the carbohydrate remained unresolved. Even less separation was obtained with Sephadex G50, but better results were achieved with Bio-Gel P4 or Sephadex G25. Of the materials tried, the best was Sephadex G25, superfine grade; either two or three 100 by 2·5 cm columns of this gel, connected in series, separated the carbohydrate fraction into 10 peaks of neutral saccharides (numbered 1–10 in reverse order of elution) which emerged from the column following two to four indistinct peaks of sialic acid-containing material (Fig. 1). The two milk samples gave similar elution profiles, but there were differences in the shapes and sizes of the peaks.

Removal of the acidic (sialyl) saccharides with DEAE–Sephadex before gel filtration resulted in each case in a considerable reduction in the amount of carbohydrate which eluted prior to peak 10; however, some neutral saccharides, which did not resolve into peaks even on Sephadex G50, remained (Fig. 2).

*Molecular Size of the Neutral Saccharides*

Peaks 1, 2, 3, and 4 eluted at volumes corresponding to those of standard mono-, di-, tri-, and tetrasaccharides, respectively, with both Sephadex G15 and G25. No standard oligosaccharides larger than tetrasaccharides were available, but it is seen in Fig. 3 that the relative elution volumes of peaks 3–10, when plotted against the logarithms of the molecular weights of tri- to decasaccharides, fell on a straight line; therefore peaks 5–10 eluted at the volumes expected for penta- to decasaccharides, respectively* (Andrews 1964). Confirmatory evidence pertaining to the molecular

* The anomalous elution volumes of peaks 1 and 2 on Sephadex G25, and of peak 1 on Sephadex G15, suggest that mono- and disaccharides have molecular weights below the optimum fractionation range of Sephadex G25, and monosaccharides below that of Sephadex G15.

size of the neutral oligosaccharides was obtained from quantitative determination of their monosaccharide composition (see below).

Since oligosaccharides are conventionally considered to be carbohydrates containing no more than 10 monosaccharide residues, the neutral carbohydrates which emerged prior to the decasaccharide peak (Fig. 2) would appear to be polysaccharides rather than large oligosaccharides. No definite conclusions could be drawn, however, about the molecular size of the sialic acid-containing carbohydrates; these may have emerged soon after the void volume, not because of their large size, but because negatively charged substances are excluded from the gel phase at low ionic strength (Kobata *et al.* 1969).

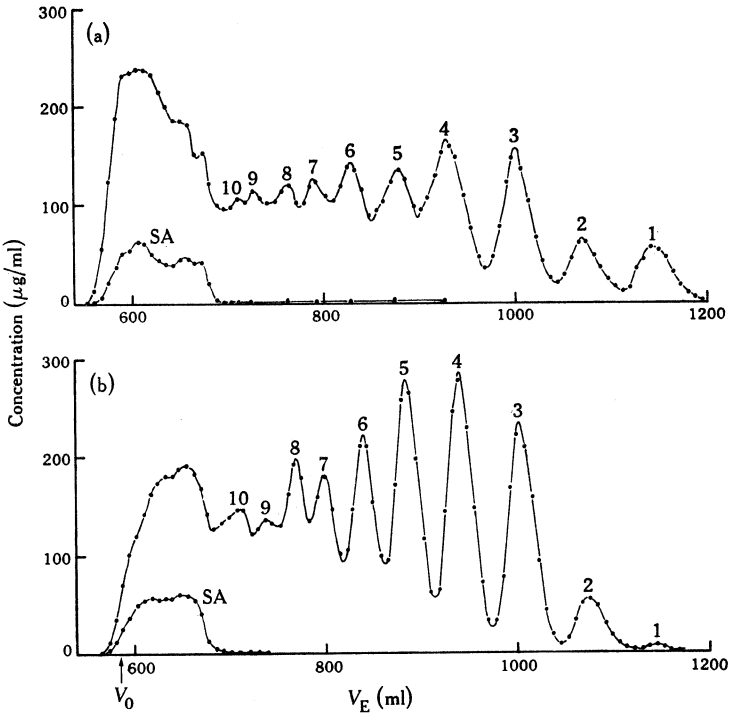


**Fig. 1.** Gel filtration of the carbohydrate fraction of (*a*) milk sample A and (*b*) milk sample B of eastern grey kangaroo. In each case 100 mg of the carbohydrate fraction, containing 59 and 71 mg of hexose, respectively, dissolved in 2 ml of water, were passed through Sephadex G25, superfine grade, using three columns, each 100 by 2·5 cm, connected in series. Elution was done with water. ● Hexose (lactose equivalents, anthrone method). ○ (Curve SA) Sialic acid (*N*-acetylneuraminic acid equivalents). $V_0$, void volume (593 ml). $V_E$, elution volume.

Table 1 presents the composition of the carbohydrate of both milk samples in terms of the amount of hexose in each size class. It is seen that the principal carbohydrates were neutral tri- to hexasaccharides, which together comprised 39% (sample A) and 48% (sample B) of the total hexose. The main differences between the two samples were that sample A contained more monosaccharide and more sialyl saccharides than sample B.

*Thin-layer and Paper Chromatography of the Neutral Saccharides*

Although gel filtration separated the neutral milk saccharides into different size classes, each of the latter was not necessarily homogeneous. To obtain information

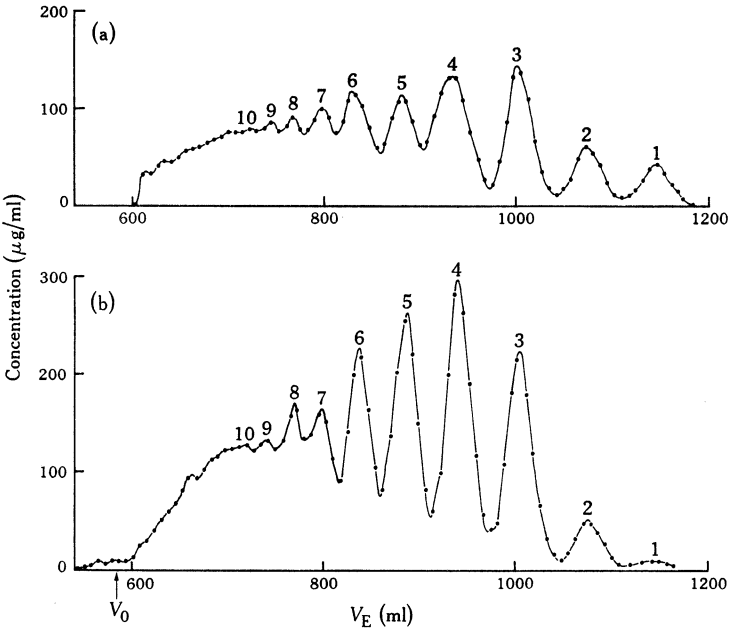


**Fig. 2.** Gel filtration of the neutral carbohydrates of (*a*) milk sample A and (*b*) milk sample B. The neutral carbohydrates were obtained by removal of the acidic (sialyl) saccharides from 100 mg of the carbohydrate fraction, using DEAE-Sephadex as described in Materials and Methods. For other details, see Fig. 1.

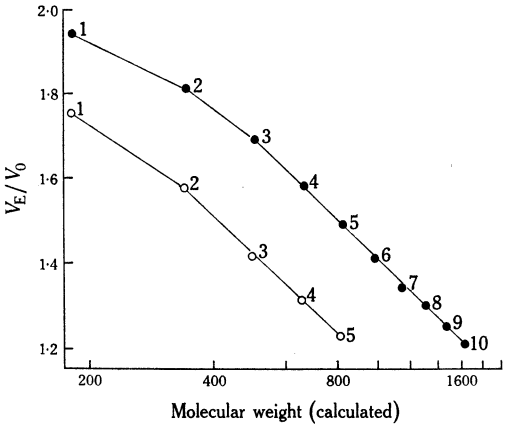


**Fig. 3.** Plots of relative elution volumes, $V_E/V_0$, of the neutral milk saccharides on Sephadex G25 (●) and G15 (○) against the log (molecular weight) of mono- to decasaccharides. The relative elution volumes are mean values from a number of experiments. The numbers refer to the peaks of Fig. 1. A molecular weight of 162 was assumed for the monosaccharide residues of oligosaccharides.

on the degree of heterogeneity, the fractions constituting elution peaks were pooled, concentrated, freeze-dried and then examined by both thin-layer chromatography (for monosaccharides and lower oligosaccharides) and paper chromatography.

Chromatography of the monosaccharide peak of milk sample A showed that this consisted mainly of galactose; in addition, small amounts of glucose, inositol,

*N*-acetylgalactosamine and fucose were detected. The monosaccharide peak of milk sample B consisted mainly of galactose and glucose in about equal amounts, plus small amounts of inositol.

**Table 1. Hexose content of the saccharide peaks obtained by gel filtration of carbohydrate fractions of milk samples from eastern grey kangaroo**

Values are calculated from the data of Figs 1 and 2

| Saccharide | Sephadex peak No. | Hexose (% of total) Sample A | Sample B | Saccharide(s) | Sephadex peak No. | Hexose (% of total) Sample A | Sample B |
|---|---|---|---|---|---|---|---|
| Mono- | 1 | 3·4 | 0·2 | Nona- | 9 | 3·5 | 5·1 |
| Di- | 2 | 4·6 | 2·8 | Deca- | 10 | 3·4 | 4·5 |
| Tri- | 3 | 9·9 | 11·4 | Neutral | | | |
| Tetra- | 4 | 12·1 | 14·1 | polysaccharide | — | 11·6 | 13·6 |
| Penta- | 5 | 8·1 | 13·0 | Sialyl | | | |
| Hexa- | 6 | 8·7 | 9·1 | saccharides | — | 25·4 | 11·2 |
| Hepta- | 7 | 5·6 | 7·8 | | | | |
| Octa- | 8 | 4·2 | 7·1 | Total | | 100·5 | 99·9 |

During paper chromatography the contents of the di- to decasaccharide peaks, as expected, showed decreasing mobility with increase in molecular size (Fig. 4). The only disaccharide detected in peak 2 co-chromatographed with lactose, but in the case of milk sample A this peak was contaminated with *N*-acetylgalactosamine, which emerged as a small peak between peaks 1 and 2 of Figs 1*a* and 2*a*. The trisaccharide

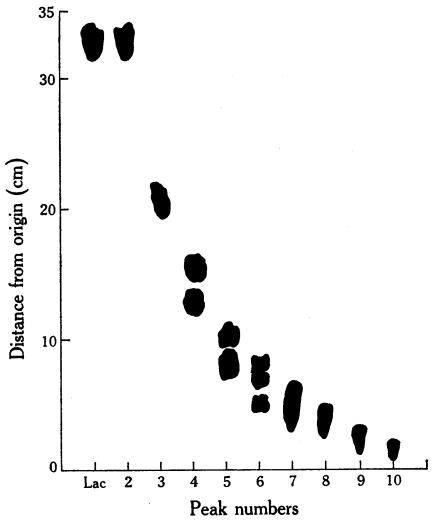


**Fig. 4.** Drawing of paper chromatogram of di- to decasaccharides of milk sample A. Descending chromatography was done on Whatman paper No. 1 for 48 h. The peak numbers are those of Fig. 1. Lac, lactose. For other details see Materials and Methods.

peaks of both milk samples gave only one spot with either thin-layer or paper chromatography, and therefore appeared to be homogeneous. The tetrasaccharide peak of milk sample A gave two spots of about equal intensity in both chromatographic systems, but that of sample B gave only the spot with lower chromatographic mobility. The pentasaccharide peak gave two spots with both milk samples. The hexasaccharide peak gave three spots, but two of these may have been due to contamination by the contents of neighbouring peaks. With the higher oligosaccharides

of peaks 7–10 the chromatographic mobilities were too low to permit definite conclusions regarding heterogeneity. However, the elongated or streaky appearance of the spots suggested that the contents of these peaks were not homogeneous.

The results of chromatography thus indicated that only the contents of the di- and trisaccharide peaks, and those of the tetrasaccharide peak of milk sample B, were homogeneous.

*Monosaccharide Composition of the Neutral Oligosaccharides*

Samples of the pooled, freeze-dried contents of peaks 2–10 (Figs 1 and 2) were hydrolysed with acid, and the hydrolysates were examined qualitatively by thin-layer chromatography as well as quantitatively by assay of the constituent monosaccharides.

Thin-layer chromatography showed that the hydrolysates of the contents of the di- and trisaccharide peaks of both milk samples, and of the tetrasaccharide peak of sample B, contained only glucose and galactose. Those of all the other peaks yielded, in addition to glucose and galactose, a hexosamine which co-chromatographed with glucosamine. The hexosamine was shown by means of the amino acid analyser to be glucosamine, and not galactosamine. None of the hydrolysates contained fucose.

**Table 2. Monosaccharide composition of acid hydrolysates of neutral oligosaccharides of milk samples from eastern grey kangaroo**

Values are based on the means of duplicate determinations of each monosaccharide, carried out as described in Materials and Methods

| Oligo-saccharide(s) | Sample A molar ratios | | | | Sample B molar ratios | | | |
|---|---|---|---|---|---|---|---|---|
| | Glucose | Galac-tose | Glucos-amine | Sum of ratios | Glucose | Galac-tose | Glucos-amine | Sum of ratios |
| Di- | 1·00 | 1·10 | 0 | 2·10 | 1·00 | 1·04 | 0 | 2·04 |
| Tri- | 1·00 | 1·90 | 0 | 2·90 | 1·00 | 2·01 | 0 | 3·01 |
| Tetra- | 1·00 | 2·54 | 0·46 | 4·00 | 1·00 | 3·15 | 0·01 | 4·16 |
| Penta- | 1·00 | 3·36 | 0·54 | 4·90 | 1·00 | 3·75 | 0·29 | 5·04 |
| Hexa- | 1·00 | 3·71 | 1·02 | 5·73 | 1·00 | 4·55 | 0·44 | 5·99 |
| Hepta- | 1·00 | 4·51 | 1·35 | 6·86 | 1·00 | 5·07 | 0·71 | 6·78 |
| Octa- | 1·00 | 5·43 | 1·43 | 7·86 | 1·00 | 6·04 | 1·11 | 8·15 |
| Nona- | 1·00 | 6·44 | 1·47 | 8·91 | 1·00 | 6·43 | 1·25 | 8·68 |
| Deca- | 1·00 | 7·30 | 1·66 | 9·96 | 1·00 | 7·58 | 1·52 | 10·1 |

Table 2 presents the results of the quantitative determination of the glucose, galactose, and glucosamine content of the acid hydrolysate of each peak. The results confirmed the absence of glucosamine from the di- and trisaccharide peaks, and its virtual absence from the tetrasaccharide peak of sample B; they also confirmed the presence of glucosamine in all other peaks. There was a marked increase in the molar ratio of galactose to glucose with increase in molecular size, and a less marked but significant increase in the ratio of glucosamine to glucose. With the di- and trisaccharide peaks and the tetrasaccharide peak of sample B, the ratios of galactose to glucose were close to whole numbers; this was consistent with the paper chromatographic evidence of homogeneity of the contents of these peaks. With most of the other peaks, on the other hand, the ratios of galactose or glucosamine to glucose were not whole numbers, which supported the evidence that these were heterogeneous. Finally, with all the saccharide peaks the sums of the molar ratios were very close

to the whole numbers expected from the molecular sizes as determined by gel filtration, which confirmed that the neutral oligosaccharides comprised the whole range of di- to decasaccharides, and that they contained no monosaccharides (such as fucose or galactosamine) other than those determined by assay.

## Discussion

Our results confirm previous findings that lactose is not the predominant milk sugar of marsupials. This disaccharide constituted less than 5% of the total hexose of milk sample A, and less than 3% of that of sample B (Table 1).

Evidence that the principal carbohydrate of marsupial milk is not lactose was first obtained by Bolliger and Pascoe (1953) with milk of the wallaroo (*Macropus robustus*); paper chromatography yielded only one spot which did not coincide with lactose, and acid hydrolysis increased the total reducing sugar by a factor greater than two. Gross and Bolliger (1958, 1959) showed that milk of the brush-tailed possum (*Trichosurus vulpecula*) contained, in addition to some lactose and free galactose, an oligosaccharide which on acid hydrolysis yielded only galactose. Jenness *et al.* (1964) examined milk of the quokka (*Setonix brachyurus*), red kangaroo (*Megaleia rufa*), and American opossum (*Didelphis virginiana*). Paper chromatography showed that the milk of all three species contained lactose, galactose, glucose, and about five oligosaccharides which yielded both glucose and galactose on hydrolysis; lactose was not the main sugar. Bergman and Housley (1968) examined the carbohydrate fraction of milk of the American opossum and found free galactose and three spots of oligosaccharides, but no lactose or free glucose. Estimation of the monosaccharide composition of acid hydrolysates of the carbohydrates showed that the oligosaccharides consisted of galactose and glucose in the approximate ratio of 5 : 1.

Our two milk samples differed considerably in their amounts of monosaccharide (Table 1) but both contained much less monosaccharide than was observed by Jenness *et al.* (1964) and Bergman and Housley (1968). Jenness *et al.* (1964) found that specimens from American opossum and red kangaroo obtained late in lactation contained higher concentrations of free glucose and galactose than samples obtained in mid-lactation. Both our milk samples from grey kangaroo were obtained relatively late in lactation, but before the young had permanently left the pouch. It would appear, therefore, that considerable variation can be expected in the monosaccharide content of marsupial milk, but the factors responsible for this variation remain to be elucidated.

In agreement with the results of Jenness *et al.* (1964) and Bergman and Housley (1968) we found that acid hydrolysates of all the neutral oligosaccharides yielded both glucose and galactose. Gross and Bolliger (1958, 1959) found only galactose in hydrolysates of the milk carbohydrate of brush-tailed possum; this may have been due to the relative insensitivity of the detection methods used, but differences between species in this regard cannot be ruled out.

All the acid hydrolysates of oligosaccharides larger than trisaccharides (sample A) or tetrasaccharides (sample B) contained glucosamine, in addition to galactose and glucose. This amino sugar presumably originated from *N*-acetylglucosamine, since the linkage by which the acetyl group is bound in *N*-acetylamino sugars is hydrolysed by acid. Jenness *et al.* (1964) had previously proposed that marsupial milk contains *N*-acetylglucosamine since the milk of the three species investigated supported the growth of *Lactobacillus bifidus* var. *pennsylvanicus,* for which bound *N*-acetylglucosamine is an essential growth factor (György 1958). Furthermore, dialysates

of the milks of all three species gave positive qualitative tests for *N*-acetylhexosamine with the Morgan–Elson reaction. However, Jenness *et al.* (1964) were unable to detect glucosamine in chromatograms of hydrolysates of their marsupial milk sugars. This was ascribed to its destruction during hydrolysis, and to its weak reaction with silver nitrate. In the present work there was no evidence for significant destruction of glucosamine during hydrolysis.

Bolliger and Pascoe (1953) reported the presence of pentose in milk of the wallaroo but, like Jenness *et al.* (1964), we found no pentose or fucose (a methylpentose) in acid hydrolysates of our milk oligosaccharides. Fucose, however, is a major constituent of the milk carbohydrates of monotremes (Messer and Kerry 1973), and is also a constituent of many human milk oligosaccharides (Kobata 1972).

A striking feature of our results was the extraordinary variety of carbohydrates found in both milk samples. In addition to monosaccharides, a disaccharide (lactose) and an unknown number of sialyl saccharides and neutral polysaccharides, we found one neutral trisaccharide, two each of neutral tetra- and pentasaccharides, and probably more than one each of neutral hexa-, hepta-, octa-, nona-, and decasaccharides. A large number of oligosaccharides, other than lactose, have been isolated from human milk (Kobata 1972) but their total quantity in the milk is only around 0·4% (György 1958) as compared with about 7% for lactose. By contrast, the hexose content of milk samples A and B was 4·4* and 5·3%* (w/w, lactose equivalents), respectively, of which less than 0·2% was lactose.

Although the chemical structures of many of the human milk oligosaccharides have been elucidated (Kuhn 1958; Kobata 1972), little can be said at present about the structures of marsupial milk oligosaccharides. The neutral trisaccharide had the same monosaccharide composition as a trisaccharide, 6′-galactosyllactose, recently isolated from human milk (Yamashita and Kobata 1974), but further studies will be required to establish whether the two are identical. The monosaccharide composition of the contents of the tetrasaccharide peak of milk sample A (Table 2) was such as to suggest that this contained 46% of a tetrasaccharide with the molecular formula $Glc\text{-}(Gal)_2\text{-}GlcNAc$ and 54% of a tetrasaccharide with the formula $Glc\text{-}(Gal)_3$. The former has the same composition as lacto-*N*-tetraose (Kuhn *et al.* 1953) or lacto-*N*-neotetraose (Kuhn and Gauhe 1962) and may therefore be identical with one of these. On the other hand, no tetrasaccharide containing galactose and glucose in the ratio 3 : 1 has been found previously in human (or any other) milk.

Comparison of the monosaccharide compositions of the contents of the penta- to decasaccharide peaks (Table 2) with those of known human milk oligosaccharides (Kobata 1972) suggests that these peaks, too, may contain novel oligosaccharides.

**Acknowledgments**

We are indebted to Professor G. B. Sharman and to Mr W. E. Poole for providing the milk samples, to Mr L. Higginbottom for assistance with the estimation of glucosamine by the amino acid analyser, and to Dr M. A. W. Thomas for helpful

* These values were calculated from the yield of carbohydrate fraction from each sample (see Materials and Methods) and the hexose content of these fractions (Fig. 1). They are actually underestimates of the total milk carbohydrate content, firstly because the anthrone method gives lower values for galactose, the predominant hexose, than for lactose (Morris 1948), and secondly because they take no account of the contributions made by sialic acid and *N*-acetylglucosamine.

advice. This work was supported by the Australian Research Grants Committee, and by a scholarship to G.S.M. from the Medical Research Committee of the University of Sydney.

## References

Aminoff, D. (1961). Methods for the quantitative estimation of *N*-acetylneuraminic acid and their application to hydrolysates of sialomucoids. *Biochem. J.* **81**, 384–92.

Andrews, P. (1964). Estimation of the molecular weights of proteins by gel filtration. *Biochem. J.* **91**, 222–33.

Bergman, H. C., and Housley, C. (1968). Chemical analyses of American opossum (*Didelphys virginiana*) milk. *Comp. Biochem. Physiol.* **25**, 213–18.

Bolliger, A., and Pascoe, J. V. (1953). Composition of kangaroo milk (wallaroo, *Macropus robustus*). *Aust. J. Sci.* **15**, 215–17.

Finch, P. R., Yuen, R., Schachter, H., and Moscarello, M. A. (1969). Enzymic methods for the micro assay of D-mannose, D-glucose, D-galactose, and L-fucose from acid hydrolysates of glycoproteins. *Anal. Biochem.* **31**, 296–305.

Griffiths, M., McIntosh, D. L., and Leckie, R. M. C. (1972). The mammary glands of the red kangaroo with observations on the fatty acid components of the milk triglycerides. *J. Zool.* **166**, 265–75.

Gross, R., and Bolliger, A. (1958). The occurence of carbohydrates other than lactose in the milk of a marsupial (*Trichosurus vulpecula*). *Aust. J. Sci.* **20**, 184–5.

Gross, R., and Bolliger, A. (1959). Composition of milk of the marsupial *Trichosurus vulpecula*. *Am. J. Dis. Child.* **98**, 768–75.

György, P. (1958). *N*-Containing saccharides in human milk. 'Chemistry and Biology of Mucopolysaccharides.' pp. 140–156. (Ciba Foundation Symp.) (J. & A. Churchill: London.)

Hansen, S. A. (1975). Thin-layer chromatographic method for identification of oligosaccharides in starch hydrolysates. *J. Chromatogr.* **105**, 388–90.

Huggett, A. S. G., and Nixon, D. A. (1957). Use of glucose oxidase, peroxidase and *o*-dianisidine in the determination of blood and urinary glucose. *Lancet* **ii**, 368–70.

Jenness, R., Regehr, E. A., and Sloan, R. E. (1964). Comparative biochemical studies of milks. II. Dialyzable carbohydrates. *Comp. Biochem. Physiol.* **13**, 339–52.

Kobata, A. (1972). Isolation of oligosaccharides from human milk. *Meth. Enzymol.* **28**, 262–71.

Kobata, A., Ginsburg, V., and Tsuda, M. (1969). Oligosaccharides of human milk. I. Isolation and characterization. *Arch. Biochem. Biophys.* **130**, 509–13.

Kuhn, R. (1958). Les oligosaccharides du lait. *Bull. Soc. Chim. Biol.* **40**, 297–314.

Kuhn, R., and Gauhe, A. (1962). Die Konstitution der Lacto-*N*-neotetraose. *Chem. Ber.* **95**, 518–22.

Kuhn, R., Gauhe, A., and Baer, H. H. (1953). Über ein *N*-haltiges Tetrasaccharid aus Frauenmilch. *Chem. Ber.* **86**, 827–30.

Messer, M. (1974). Identification of *N*-acetyl-4-*O*-acetylneuraminyl-lactose in echidna milk. *Biochem. J.* **139**, 415–20.

Messer, M., and Dahlqvist, A. (1966). A one-step ultramicro method for the assay of intestinal disaccharidases. *Anal. Biochem.* **14**, 376–92.

Messer, M., and Kerry, K. R. (1973). Milk carbohydrates of the echidna and the platypus. *Science* **180**, 201–3.

Morris, D. L. (1948). Quantitative determination of carbohydrates with Dreywood's anthrone reagent. *Science* **107**, 254–5.

Öhman, R., and Hygstedt, O. (1968). The isolation of sialyllactosides with the aid of gel filtration. *Anal. Biochem.* **23**, 391–402.

Roe, J. H. (1955). The determination of sugar in blood and spinal fluid with anthrone reagent. *J. Biol. Chem.* **212**, 335–43.

Trevelyan, W. E., Procter, D. P., and Harrison, J. S. (1950). Detection of sugars on paper chromatograms. *Nature (London)*, **166**, 444–5.

Yamashita, K., and Kobata, A. (1974). Oligosaccharides of human milk. V. Isolation and characterization of a new trisaccharide, 6′-galactosyllactose. *Arch. Biochem. Biophys.* **161**, 164–70.

Manuscript received 2 May 1977