Concentration and distribution of sialic acid in human milk and infant formulas[1–3]

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ABSTRACT

Background: In animal studies, sialic acid supplementation is associated with increases of gangliosides in the brain and improved learning ability. Only limited data are available on the sialic acid content of human milk and infant formulas.

Objective: We compared the concentrations of oligosaccharide-bound, protein-bound, and free sialic acid in milk from mothers of full-term and preterm infants and in a range of infant formulas.

Design: The milk from 20 and 14 mothers of full-term and preterm infants (mean gestational age: 31 ± 3 wk), respectively, was collected at 4 stages of lactation (colostrum, transition, 1 mo, and 3 mo) and compared with 21 different infant formulas.

Results: Total sialic acid concentrations were highest in colostrum ($\bar{x}$ ± SEM: 5.04 ± 0.21 mmol/L in full term) and decreased by nearly 80% over the next 3 mo. Human milk from mothers of preterm infants contained 13–23% more sialic acid than did milk from mothers of full-term infants at 3 of the 4 lactation stages ($P < 0.02$). The sialic acid content of most formulas was <25% of that found in mature human milk ($P < 0.01$). Most of the sialic acid in the formulas (≈70%) was bound to glycoproteins, whereas in human milk most sialic acid was bound to free oligosaccharides.

Conclusions: Human milk, including milk from mothers of preterm infants, is a rich source of oligosaccharide-bound sialic acid, which contrasts with the relatively small amounts found in infant formulas. The nutritional significance of sialic acid is presently unknown, but it is plausible that it is a conditional nutrient that contributes to sialic acid accretion in the brain. *Am J Clin Nutr* 2001;74:510–5.

KEY WORDS Sialic acid, oligosaccharides, glycoprotein, human full-term milk, human preterm milk, infant formulas, Sydney, Australia

INTRODUCTION

Early human milk is a rich source of sialic acid, *N*-acetylneuraminic (1), but the metabolic fate and nutritional significance of sialic acid are currently unknown. There is evidence from animal models that exogenous sialic acid influences brain growth and learning ability (2). Neural cell membranes contain 20 times more sialic acid than do other types of membranes (3) and brain gangliosides are an especially rich source. We found that the adult human brain contains concentrations of sialic acid that are 2- to 4-fold higher than those of other mammals, including chimpanzees (4). Sialic acid is thought to play a role in the structural and functional establishment of synaptic pathways: >40% of sialic acid in the brain is found in the synaptosomal fraction and contributes to the negative charge of the membrane (3). Because most neurotransmitters are positively charged, sialic acid may assist neurotransmission by facilitating the binding of transmitter molecules to the synaptic membrane.

Morgan and Winick (2) showed that exogenous sialic acid administered by intraperitoneal injection increased the production of ganglioside sialic acid in the brain and improved learning ability in well-nourished and malnourished rat pups. They also found that these changes persisted into maturity. Carlson and House (5) showed that both intraperitoneally and orally administered sialic acid increased brain ganglioside sialic acid in young rats. Timing appears to be critical, however, because studies in older animals do not show a significant incorporation of labeled sialic acid after acute dosing (6). The livers of all mammals, including those of humans, have the capacity to synthesize sialic acid from simple sugar precursors. However, the liver of newborn infants is relatively immature and the rapid growth and development of the brain, especially in preterm infants, may be compromised by a limited rate of de novo synthesis (1, 7). Thus, dietary sources of sialic acid may play a role in determining the final concentration of sialic acid in the brain and may possibly affect the learning ability of human infants.

Considering its potential significance, relatively little attention has been paid to determining the content and distribution of sialic acid in mammalian milks. In 1985 Carlson (1) drew attention to the high sialic acid concentrations in human milk compared with the small amounts in infant formula. There was a rapid decline over time in both oligosaccharide- and protein-bound

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Received August 7, 2000.

Accepted for publication February 13, 2001.

sialic acid and a high variability among women at the same stage of lactation. We also found a high concentration of sialic acid in the first month of lactation with concentrations decreasing by 70% over 3 mo (8). At the same stage of lactation there was a 3-fold difference in concentrations among 10 mothers, making it one of the most variable components of human milk. Cow milk contains very low concentrations of oligosaccharides and sialic acid–containing glycoproteins (9, 10).

The aim of the present study was to compare systematically the content and distribution of sialic acid in human milk at different stages of lactation from mothers of full-term and preterm infants and in a range of commercial infant formulas. We report both the total concentrations of sialic acid as well as its content in different fractions, ie, in free oligosaccharides, in glycoproteins, and in free form. Although some sialic acid also exists as part of glycolipids, particularly as gangliosides, the amount in human milk represents <0.5% of the total sialic acid content (11, 12) and is not reported here.

SUBJECTS AND METHODS

Human milk

Mothers of full-term (full-term group; n = 20) and preterm infants (preterm group; n = 14) were recruited in the maternity wards of a large teaching hospital in Sydney, Australia. The study was approved by the hospital ethics committee and all the mothers gave informed, written consent before inclusion in the study. Subjects were healthy mothers aged 20–40 y with uncomplicated perinatal histories. The ($\bar{x}$ ± SD) gestational age of the preterm infants was 31 ± 3 wk. In the full-term group, milk samples (5–20 mL) were collected by manual expression immediately before feeding at approximately the same time of the morning (0900–1200) on 4 occasions: 1–6 d (colostrum), 7–10 d (transition milk), 30–45 d (mature milk), and 92–110 d (3 mo) after birth. In the preterm group, ≈100 mL milk was collected by breast pump and a 20-mL aliquot was taken for analysis. Previous studies showed no within-feeding or diurnal variation in the sialic acid content of human milk (13). There were complete data for all 4 time points in 15 mothers of full-term infants and 5 mothers of preterm infants. Milk samples were stored in plastic sterile vials at −20°C until analyzed.

Infant formulas

Commercial infant formulas (n = 21) that were suitable for preterm and full-term infants and infants >6 mo of age were analyzed. The formulas used were manufactured by Mead Johnson Australia (Enfalac, Enfamil, O-Lac, Enfapro, Prosobee, and Enfalac for preterm infants); Wyeth Australia Pty Ltd (S26, SMA, S26 Progress, Infasoy, Infasoy Progress, and S26 for preterm infants); Douglas Pharmaceuticals Australia Ltd (Karicare, Karicare First, Karicare Progress, Karicare Soya, and Karicare Soya Follow On); and Nestle Australia Ltd (NAN 1, Lactogen, NAN 2, and Pre-NAN) . The formulas were prepared according to the manufacturer's instructions by using water treated by reverse osmosis. Aliquots were stored at −20°C and were analyzed within 4 wk.

Sialic acid assay

Milk samples were centrifuged at 3000 × g at 4°C for 30 min and the upper cream layer was removed by pipette. Trichloroacetic acid (TCA, 10%) was added to an equal volume of the skim milk to precipitate the protein. The mixture was mixed by vortex, iced for 10 min, and then centrifuged at 3000 × g at 4°C for 30 min. The pellet of protein was washed twice with cold 5% TCA to remove traces of contaminating sugars. The combined supernatant fluids containing residual milk protein were centrifuged twice at 3000 × g at 4°C for 30 min and the combined pellets were resuspended in 2 mL of 0.05 mol sulfuric acid/L and heated for 2 h at 80°C. Sialic acid was determined as described previously (4, 14) by using the routinely applied (15) colorimetric test described by Aminoff (16) modified by including a final extraction of sialic acid in dimethyl sulfoxide (17). This avoids some of the interference problems found in earlier analyses (17, 18). Absorbance was measured at 549 nm with a Shimadzu UV-160A spectrophotometer (Shimadzu, Tokyo) and the sialic acid concentration was determined by comparison with a standard curve prepared with *N*-acetylneuraminic acid.

To determine free and oligosaccharides-bound sialic acid, the supernatant fluid from deproteinated and defatted milk was applied to a Bio-gel P-2 (fine 45–90 μm; Bio-Rad Laboratories, Hercules, CA) column (75 × 2.5 cm; Pharmacia Biotech, Uppsala, Sweden) to remove lactose. Elution was done with Milli-Q highly purified water (Millipore Corp, Allen, TX) at a flow rate of 7 mL/h at 3–4°C and 25 mL fractions were collected. Aliquots (2.5 mL) of each fraction were pooled and analyzed for sialic acid. The optimal time for release of oligosaccharides-bound sialic acid was determined by prior hydrolysis of supernatant fluid with a variety of acids at varying time intervals. We found that the maximum release of oligosaccharide-bound sialic acid was obtained with an equal volume of 0.1 mol trifluoracetic acid/L for 30 min at 80°C. After hydrolysis, the sample was adjusted to a pH of 2–6 with 1 mol NaOH/L and the solution was applied to the Bio-gel P-2 column. Sialic acid was determined as described above. All samples were analyzed in duplicate and the final concentration of sialic acid in each fraction was expressed as mmol/L milk.

Statistical analysis

Data were analyzed by a two-factor repeated measures analysis of variance model with 4 time points by using the Greenhouse-Geisser adjustment for asphericity. To investigate the different time trends for the human milk of full-term and preterm infants, we checked for an interaction between the dependent variable and grouping factor. The overall comparison of sialic acid between human milk of full-term and preterm infants and infant formula products was obtained from the repeated measures analysis of variance models, and comparisons at individual time points were conducted by using t tests. All analyses were completed by using SPSS for WINDOWS 8.0 (SPSS Inc, Chicago). Values were considered significant at P = 0.05.

RESULTS

Human milk

The oligosaccharide-bound, protein-bound, and free sialic acid concentrations in human milk at 4 different stages are shown in **Table 1**. Total sialic acid concentrations were highest in colostrum and decreased over time. The downward time trend was evident in the full data set, in 15 of the full-term group, and in 5 of the preterm group who had complete data for all time points

TABLE 1
Oligosaccharide-bound, protein-bound, and free sialic acid concentrations in human milk as a function of the duration of lactation[1]

	Human milk			
	Colostrum	Transition	Mature, 1 mo	Mature, 3 mo
	mmol/L			
Oligosaccharide-bound sialic acid				
Full-term group	3.72 ± 0.15 [20]	2.64 ± 0.14 [18]	1.48 ± 0.07 [17]	0.73 ± 0.05 [15]
Preterm group	4.27 ± 0.15 [14]	3.16 ± 0.18 [10]	1.89 ± 0.11 [10]	0.91 ± 0.12 [5]
P[2]	0.018	0.03	0.003	0.13
Protein-bound sialic acid				
Full-term group	1.18 ± 0.09	0.73 ± 0.03	0.43 ± 0.03	0.29 ± 0.02
Preterm group	1.30 ± 0.08	1.01 ± 0.08	0.58 ± 0.05	0.37 ± 0.04
P[2]	0.36	0.001	0.008	0.03
Free sialic acid				
Full-term group	0.14 ± 0.01	0.09 ± 0.01	0.07 ± 0.003	0.03 ± 0.001
Preterm group	0.19 ± 0.02	0.11 ± 0.02	0.10 ± 0.01	0.03 ± 0.01
P[2]	0.03	0.37	0.01	0.22
Total sialic acid				
Full-term group	5.04 ± 0.21	3.46 ± 0.14	1.98 ± 0.08	1.04 ± 0.06
Preterm group	5.76 ± 0.19	4.27 ± 0.24	2.56 ± 0.15	1.30 ± 0.13
P[2]	0.02	0.005	0.001	0.062

[1] $\bar{x}$ ± SE; *n* in brackets (applies to protein-bound, free, and total sialic acid values also).
[2] Difference between groups at the same stage of lactation (Bonferroni adjustment for multiple comparisons).

($P < 0.001$; **Figure 1**). By 3 mo, only ≈20% of the initial content of sialic acid was present in both groups. The difference in time trend between groups (ie, interaction) was marginally significant ($P = 0.09$), with a more pronounced decrease of sialic acid in milk from the preterm group than in milk from the full-term group.

Most of the sialic acid in human milk (69–76%), whether from the preterm or full-term groups and irrespective of the stage of lactation, was bound to free oligosaccharides (**Figure 2**). A smaller fraction of the sialic acid (21–28%) was bound to glycoproteins and only 3% was present in the free form.

Milk from the preterm group contained ≈13–23% more total sialic acid than did milk from the full-term group at all stages. The overall difference between the 2 groups was highly significant, regardless of whether all data available were included in the analysis (as shown in Table 1) or whether the 15 mothers from the full-term group and the 5 mothers from the preterm group with complete data for all time points were analyzed separately (Figure 1). By 3 mo of lactation, the difference between milk from the full-term and preterm groups was no longer significant for oligosaccharide-bound or free sialic acid. Except for colostrum, protein-bound sialic acid was significantly higher in milk from the preterm group than from the full-term group (Table 1). Besides temporal changes, there were large interindividual differences in sialic acid content. At 1 mo lactation, there was a 2-fold variation in the milk from the full-term group (range: 1.29–2.62 mmol/L) and a 3-fold variation in the milk from the preterm group (range: 1.37–4.04 mmol/L).

Infant formulas

All of the infant formulas studied contained <25% of the concentration of total sialic acid of mature human milk (**Table 2**). There were also 10-fold differences in sialic acid concentrations

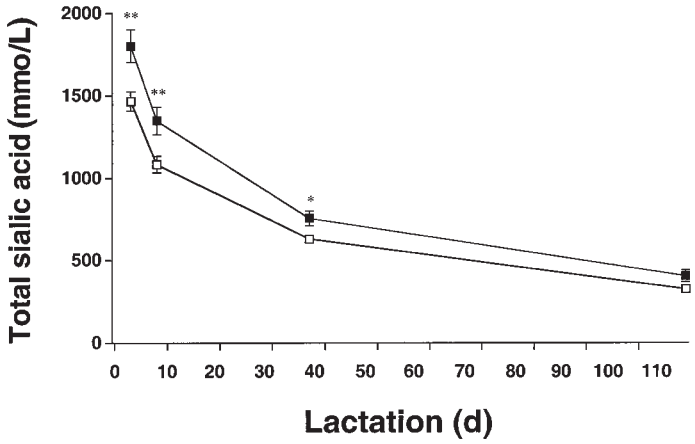

FIGURE 1. Time trend in total sialic acid concentrations ($\bar{x}$ ± SE) in the milk of mothers of full-term (□; $n = 15$) and preterm (■; $n = 5$) infants throughout the first 3 mo of lactation. Only those mothers whose data were complete for all 4 time points were included. $P < 0.0001$ for time trend in both groups. *,**Significantly different from full-term infants: *$P < 0.05$, ** $P < 0.01$. The difference between groups (ie, interaction) was marginally significant ($P = 0.07$ without adjustment, $P = 0.09$ after the Greenhouse-Geisser adjustment for asphericity).

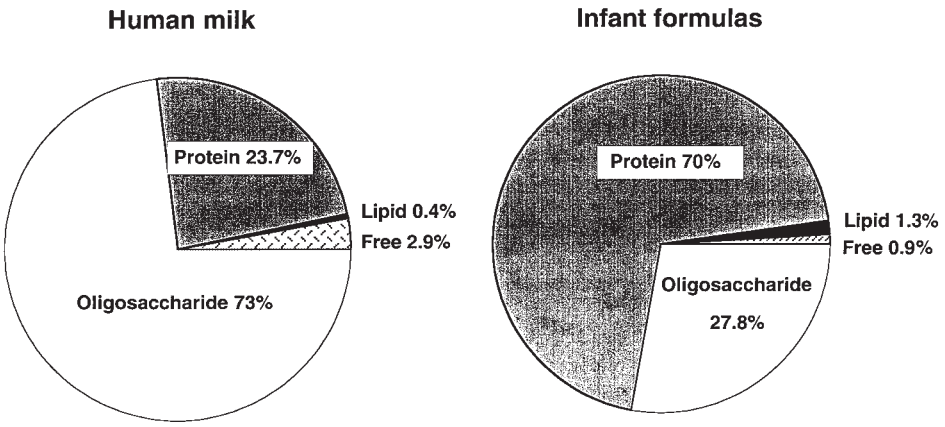

FIGURE 2. The average proportion of sialic acid bound to oligosaccharides, protein, or lipids, or in free form in human milk and infant formula (excluding soy formulas). The values for human milk are the average of all samples analyzed. The values for the lipid-bound fraction are from the literature (11, 12).

among the different types of formula. Formulas for preterm infants contained the most sialic acid (0.63 ± 0.12 mmol/L; $n = 3$) followed by follow-on formulas, ie, formulas that are suitable for feeding infants aged >6 mo (0.43 ± 0.03 mmol/L; $n = 4$). Soy formulas appeared to contain some sialic acid (0.05 ± 0.003 mmol/L; $n = 5$), although this likely represents the presence of interfering substances such as quinic acid. Cow-milk-based formulas containing 60% whey (ie, the whey-to-casein ratio in the protein component was 60:40) contained significantly more sialic acid (0.37 ± 0.01 mmol/L; $n = 5$) than did those containing 20% whey (0.21 ± 0.01; $n = 4$; $P < 0.05$–0.001). Most of the sialic acid in infant formulas was bound to protein (70%), some was bound to free oligosaccharides (27.8%), and only 0.9% was found in the free form (Figure 2).

DISCUSSION

In the present study, we confirmed that human milk is a very rich source of sialic acid compared with currently available infant formulas. Despite advances in the design and formulation of formulas to better mimic human milk, no formulas contained sialic acid >25% of that found in mature human milk. There were also significant differences among the formulas. Cow-milk-based formulas with a 60:40 whey:casein ratio contained 2 times more sialic acid than did the 20:80 whey:casein products, suggesting that bovine whey is a better source of sialic acid than is bovine casein. Two preterm formulas were higher in sialic acid when compared with the other formulas, consistent with the fact that they were also whey enriched and higher in protein. However, one formula for preterm infants with a whey:casein ratio of 78:22 paradoxically contained much less sialic acid than the other formulas.

Human milk showed significant declines in sialic acid concentrations with duration of lactation; sialic acid concentrations in colostrum were 5 times higher than were concentrations in mature milk at 3 mo. However, there were also large interindividual variations confirming the findings of earlier studies (8, 19). Concentrations of sialic acid in mature milk (1 mo of lactation) varied 2-fold among full-term and 3-fold among the preterm group. Sialic acid, therefore, is one of the most variable fractions of human milk. The reason for the wide range of values remains unknown, but may represent genetic differences in synthetic capacity or in environmental exposure to infective microorganisms.

Milk from the preterm group was an even greater source of sialic acid than was milk from the full-term group at all stages. This is similar to our finding from an earlier study with a different group of mothers in which we used a different method (8). Milk from mothers of preterm infants has a higher concentration of protein and nitrogen, immunoglobulins, and lipids when compared with milk from mothers of full-term infants at similar stages of lactation (20–22). Nonetheless, we should be extremely

TABLE 2
Oligosaccharide-bound, protein-bound, and free sialic acid concentrations in infant formulas prepared according to manufacturers' instructions[1]

Formula	Sialic acid			
	Oligosaccharide-bound	Protein-bound	Free[2]	Total
		mmol/L		
60% whey ($n = 5$)	0.10 ± 0.01[b]	0.27 ± 0.02[b,c]	0.001 ± 0.001[a]	0.37 ± 0.01[b,c]
20% whey ($n = 4$)	0.05 ± 0.01[a,b]	0.16 ± 0.01[a,b]	0[a]	0.21 ± 0.01[b]
Follow-on ($n = 4$)	0.11 ± 0.03[b]	0.32 ± 0.01[b,c]	0.006 ± 0.003[a]	0.43 ± 0.03[c]
Soy milk ($n = 5$)	0[a]	0.05 ± 0.01[a]	0[a]	0.05 ± 0.003[a]
Preterm ($n = 3$)	0.26 ± 0.02[c]	0.36 ± 0.13[c]	0.01 ± 0.003[b]	0.63 ± 0.12[d]

[1] $\bar{x}$ ± SE. Values in the same row with different superscript letters are significantly different, $P < 0.05$ (post hoc test with Tukey's adjustment).
[2] Seven of 21 formula products contained trace amounts of free sialic acid recorded as zero in the calculation of the mean.

cautious when interpreting the differences between milk from mothers of the preterm and full-term groups because of the present study's small sample size (only 14 preterm infants) and because there were differences in the method of sample collection. In the full-term group, the milk collected was generally foremilk; in the preterm group, the milk samples potentially contained hindmilk because the mothers used a breast pump and emptied the breast each time. There may have been slightly different fat contents in the milk samples because of this. However, only trace (microgram) quantities of sialic acid were found in the lipid fraction of the samples (11, 12). Previous studies showed no within-feeding or diurnal variation in the sialic acid concentration of human milk (13). Further studies are clearly needed to confirm this in addition to our finding of apparently higher concentrations of sialic acid in milk from the preterm group.

The present study allowed comparisons not only of concentrations of total sialic acid in human milk with those of infant formula but also of the form in which sialic acid was present, ie, its distribution between oligosaccharide-bound, glycoprotein-bound, and free forms (Figure 2). In human milk, most sialic acid (≈73%) is bound to free oligosaccharides and this proportion remains fairly constant throughout the duration of lactation, despite an overall decrease in absolute amounts. In contrast, most sialic acid is bound to glycoproteins (70%) in infant formulas. Carlson (1) made similar observations.

The larger amount of sialic acid in human milk than in infant formulas that are based on cow milk results from major differences in both the amount and types of glycoproteins and oligosaccharides in the milk of the 2 species (23, 24). Human κ-casein, the major glycoprotein in human milk, contains more carbohydrate by weight (40–60%) than bovine κ-casein (10%) (23). Furthermore, the oligosaccharide moiety of human κ-casein is rich in sialic acid compared with that of bovine κ-casein (25). Human milk is also a rich source of many free and varied oligosaccharides (26, 27). High concentrations of oligosaccharides (≈13 g/L) are still found in human milk at 3–4 mo lactation (28), and sialyl-oligosaccharides are some of the most common. In cow milk there is very little carbohydrate in the form of free oligosaccharides and few contain sialic acid (9, 10, 26). Last, the sialic acid in human milk appears to be almost exclusively *N*-acetylneuraminic acid (29, 30), whereas in bovine milk, between 5% and 35% (depending on the stage of lactation) of total sialic acid is *N*-glycoloylneuraminic acid (Neu5Gc) rather than Neu5Ac (30). We also found that soy-milk formulas apparently contained minor amounts of protein-bound sialic acid (0.05 mmol/L). However, it is highly unlikely that this protein-bound sialic acid found in soy milk formulas actually is sialic acid because colorimetric methods of analysis do not discriminate between sialic acid and quinic acid and other compounds found in plant tissues (31).

Sialic acid was also found in small but significant amounts in the free form. Average concentrations of 0.14 and 0.19 mmol/L were found in the colostrum of mothers from the full-term and preterm groups, respectively, but by 3 mo lactation, the concentrations had decreased to 0.026 and 0.03 mmol/L. By contrast, infant formulas contained <0.01 mmol free sialic acid/L. The significance of the free form is not known but may reflect the metabolic status of sialic acid or its availability in the mammary gland because concentrations dropped with decreases in the oligosaccharide-bound and protein-bound forms of sialic acid in human milk. The amounts of free sialic acid are higher than that of published data for glycolipid-bound sialic acid in human colostrum ($\bar{x}$: 0.02 mmol/L) and mature milk ($\bar{x}$: 0.006 mmol/L) (11, 12). It was also suggested that free sialic acid is an artifact of storage and processing. Tests to determine interassay variation over a 4-wk storage period at −20°C showed that the CV of the 3 fractions of sialic acid were 4.5%, 1.2%, and 0.8% for the free, oligosaccharide-bound, and protein-bound forms, respectively. Thus, free sialic acid is a more variable form but we did not observe any trend for free sialic acid to increase in concentration at the expense of the other forms.

The differences in the form of sialic acid found between human milk and infant formulas are relevant to the biological role and eventual fate of sialic acid in the gut in so far as one form may be more resistant to digestion than another. The most important role of free human milk oligosaccharides is to protect infants from intestinal infection by acting as host receptor decoys (32). As such, they must remain intact within the small intestine. Sialylated oligosaccharides are known to inhibit specific organisms, including *Escherichia coli* (33). However, the question of whether some sialic acid is cleaved and absorbed within the small or large intestine remains unanswered. Our group showed with breath-hydrogen methods that most human milk oligosaccharides resist digestion in the small intestine of breast-fed infants and undergo fermentation in the colon (34). Studies in vitro also confirmed that sialic acid is not released from the incubation of human milk oligosaccharides with pancreatic and mucosal enzyme mixtures (35). However, small-molecular-weight oligosaccharides may be absorbed intact (36) and lysosomal sialidases within the epithelial cell may release free sialic acid. In several species, small intestinal sialidase activity is highest during the suckling period and positively correlated with the sialic acid content of the milk (37). Radiolabelled forms of both sialic acid and sialyl-lactose were found to be well absorbed by rat pups (38).

This study contributes to the knowledge of human milk composition and the changes that occur over the course of early lactation. It allows systematic comparisons of the total amounts of sialic acid present and also its distribution among the different nutrient fractions of human milk and infant formulas. Our finding that there are higher concentrations of sialic acid in preterm milk than in full-term milk remains uncertain and requires confirmation by using sample collection methods that are identical in both cases. Further studies are also warranted to identify the biological and nutritional roles of sialic acid in human milk and the importance of the individual fractions.

We thank all of the mothers who kindly participated, the nursing staff of King George V Hospital who helped recruit the mothers for this study, and thank AS Truswell and M Messer for their comments on the manuscript.

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