

Ribonucleosides in Human Milk.

Concentration Profiles of These Minor Constituents as a Function of the Nursing Time

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Z. Naturforsch. **50c**, 105–113 (1995); received August 15/September 23, 1994

Ribonucleosides, RNA Catabolites, Human Milk, Nursing Time

Ribonucleosides are secreted as products of cellular RNA and ribonucleotide metabolism into physiological fluids such as blood, milk and urine. Unmodified and modified ribonucleosides have been detected in the micromolar range as minor constituents in the milk of different mammals. In addition to the common nucleosides adenosine, cytidine, guanosine, inosine and uridine, modified components such as 1-methyladenosine, 1-methylguanosine, 1-methyl-inosine, N2-methylguanosine, N2-dimethylguanosine, N6-carbamoyl-L-threonyl-adenosine, pseudouridine and 5-aminoimidazole-4-carboxamide-N-ribofuranoside (AICAR) have been identified and most of them quantified in samples of human and/or bovine and/or goat's milk. From these investigations it is known that nucleosides, in analogy to nucleotides, show a typical species-specific pattern. Longitudinal studies have been carried out to determine the concentration profiles of the individual ribonucleosides in the milk of humans as a function of the nursing time.

Introduction

Ribonucleosides are secreted as products of cellular RNA and ribonucleotide metabolism into physiological fluids such as blood, milk and urine. In a series of investigations over the past two decades, different working groups have detected a great number of modified and common nucleosides as intrinsic compounds. In particular, the concentration profiles of these compounds in normal and pathological human urine have been studied (Chheda, 1975; Davis *et al.*, 1977; Schlimme *et al.*, 1986; Gehrke and Kuo, 1989, 1990; Boos *et al.*, 1988; Schlimme *et al.*, 1990; Schwarzenau *et al.*, 1990). According to Schöch and co-workers (Sander *et al.*, 1985, 1986; Schöch *et al.*, 1988, 1990), for example, urine concentrations, in particular of some modified ribonucleosides generated from tRNA catabolism are suitable for measuring alterations of the metabolic status of the whole body.

Although specific compositions of milk nucleotides have been described for several mammalian species by different authors since the late fifties

(Manson, 1956; Deutsch and Nilsson, 1960; Johke and Goto, 1962; Larson, 1976; Gil and Sanchez-Medina, 1981), and their role as dietary modulators is of current interest concerning compositions of infant formula (Gil *et al.*, 1988; Quan *et al.*, 1990), it is only during the last decade that the ribonucleosides have become an object of research (Tiemeyer *et al.*, 1984; Schlimme *et al.*, 1986, 1991, 1993; Raetzke *et al.*, 1988, Raetzke and Schlimme, 1990; Schnee-hagen and Schlimme, 1992; Groß *et al.*, 1992; Topp *et al.*, 1993). In principle, the ribonucleosides can enter milk by two pathways: (1) secretion as metabolic products from the lactating cell into the alveolar lumen, and (2) transfer as blood metabolites across the blood-milk barrier (Ziv and Sulman, 1975; Ziv and Heavner, 1984).

In addition to the common nucleosides adenosine (Ado), cytidine (Cyd), guanosine (Guo), inosine (Ino) and uridine (Urd), modified components such as 1-methyladenosine (m1Ado), 1-methylguanosine (m1Guo), N2-methylguanosine (m2Guo), N2-dimethylguanosine (m2,2Guo), N6-carbamoyl-L-threonyl-adenosine (t6Ado), pseudouridine (φ) and 5-aminoimidazole-4-carboxamide-N-ribofuranoside (AICAR) have been identified in different mammalian species (Fig. 1).

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Most of them have been quantified in individual and bulk samples of human, bovine and/or goat's milk. N6-methyladenosine (m6Ado), which is neither present in raw human, bovine and goat's milk nor formed from 1-methyladenosine (m1Ado) by Dimroth rearrangement under the pH and temperature conditions prevailing in thermally untreated milk (Ott and Schlimme, 1990), was characterized for the first time in heat treated milk (Schlimme *et al.*, 1994).

These investigations indicate that nucleosides in analogy to nucleotides (Johke and Goto, 1962; Larson, 1976; Gil and Sanchez-Medina, 1981), occur in a typical species-specific pattern (Schlimme *et al.*, 1986, 1991, 1993; Schneehagen and Schlimme, 1992; Schneehagen, 1993). The present paper firstly reports on longitudinal studies carried out to determine the concentration profiles of the individual ribonucleosides in the milk of humans as a function of the nursing time.

Materials and Methods

High performance liquid chromatography (HPLC): Merck-Hitachi system with UV detection model 655 A-22; photodiode array detector 994, Waters-Millipore. The two-column HPLC system used allows selective separation and analysis of ribonucleosides from the biological matrix. For ribonucleoside analysis, milk samples were adjusted to pH 3.4 with concentrated formic acid immediately after milking and stored at -20°C until investigation, *i.e.* without subjecting the milk serum sample to a concentration treatment. The determination limit/run (detection at 260 nm) was in the range between 1.5 pmol (uridine) and 6.4 pmol (guanosine). Further details of the arrangement, the automated operation of the ribonucleoside analyzer, the validation of the HPLC system and the structural characterization of the

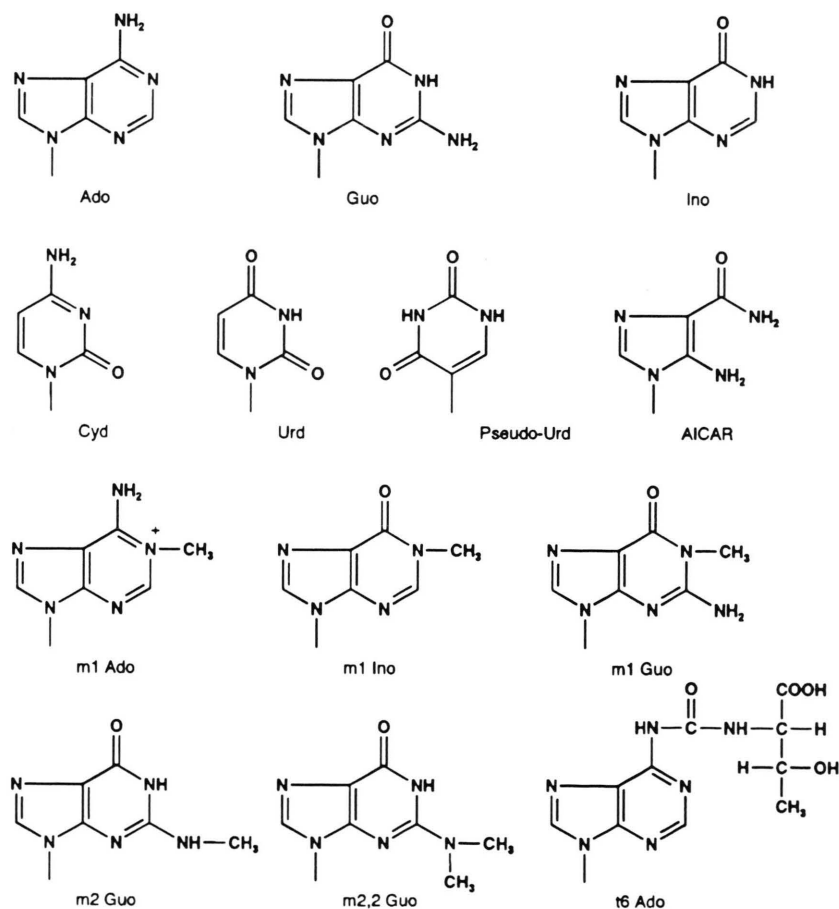


Fig. 1. Formulae (nucleobases) of ribonucleosides characterized in different mammalian milks; see legend of Fig. 2 for abbreviations.

ribonucleosides have been previously described (Boos *et al.*, 1988; Raezke and Schlimme, 1990; Schlimme and Boos, 1990; Schlimme *et al.*, 1991).

Fig. 2 shows HPLC diagrams of the analyses of 100 μ l samples of human milk without and with spiking with authentic ribonucleosides. The peaks in the HPLC chromatogram were examined before and after treatment of the milk with periodate. Such treatment oxidizes the ribonucleosides so that peaks attributable to this class of substance disappear from the chromatogram. Peaks were assigned to particular ribonucleosides by comparing the chromatographic behavior and UV ab-

sorption spectra to those of pure substances. The UV spectra were measured with a photodiode array detector (Model 994, Waters-Millipore, Eschborn). Characteristic peak shifting and quenching in HPLC peak analysis, caused by pre-chromatographic enzymic (adenosine deaminase, EC 3.5.4.4; nucleoside phosphorylase, EC 2.4.2.1; Boehringer Mannheim) and chemical modifications such as Dimroth rearrangement of 1-methyladenosine to N6-methyladenosine (Brookes and Lawley, 1960; Schlimme *et al.*, 1981) as well as addition of glyoxal (Tiffany *et al.*, 1957) to modify guanosine, N2-methylguanosine and

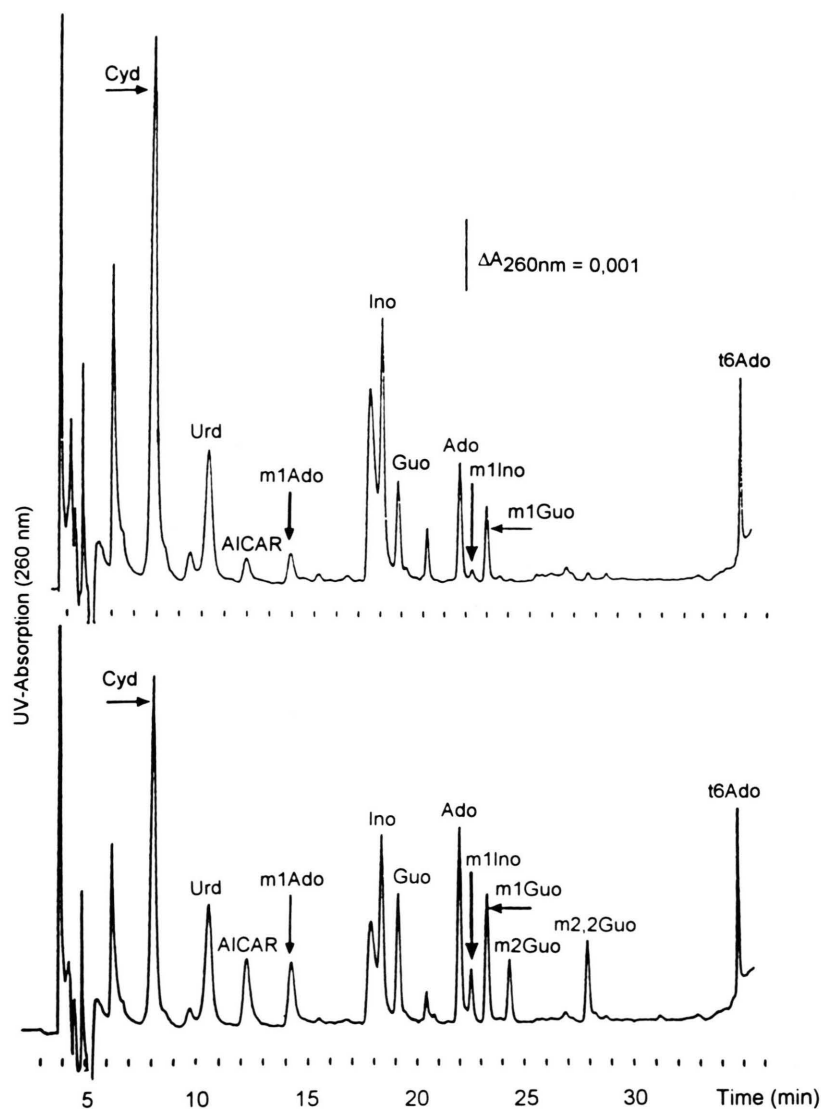


Fig. 2. HPLC diagrams of the analyses of 100 μ l of human milk non-spiked (top) and spiked with standard nucleosides (bottom). Cyd, cytidine; Urd, uridine; AICAR, 5-aminoimidazole-4-carboxamide-N-ribofuranoside; m1 Ado, 1-methyladenosine; Ino, inosine; Guo, guanosine; Ado, adenosine; m1 Ino, 1-methylinosine; m1 Guo, 1-methylguanosine; m2 Guo, N2-methylguanosine; m2,2 Guo, N2-dimethylguanosine; t6 Ado, N6-carbamoyl-L-threonyl-adenosine.

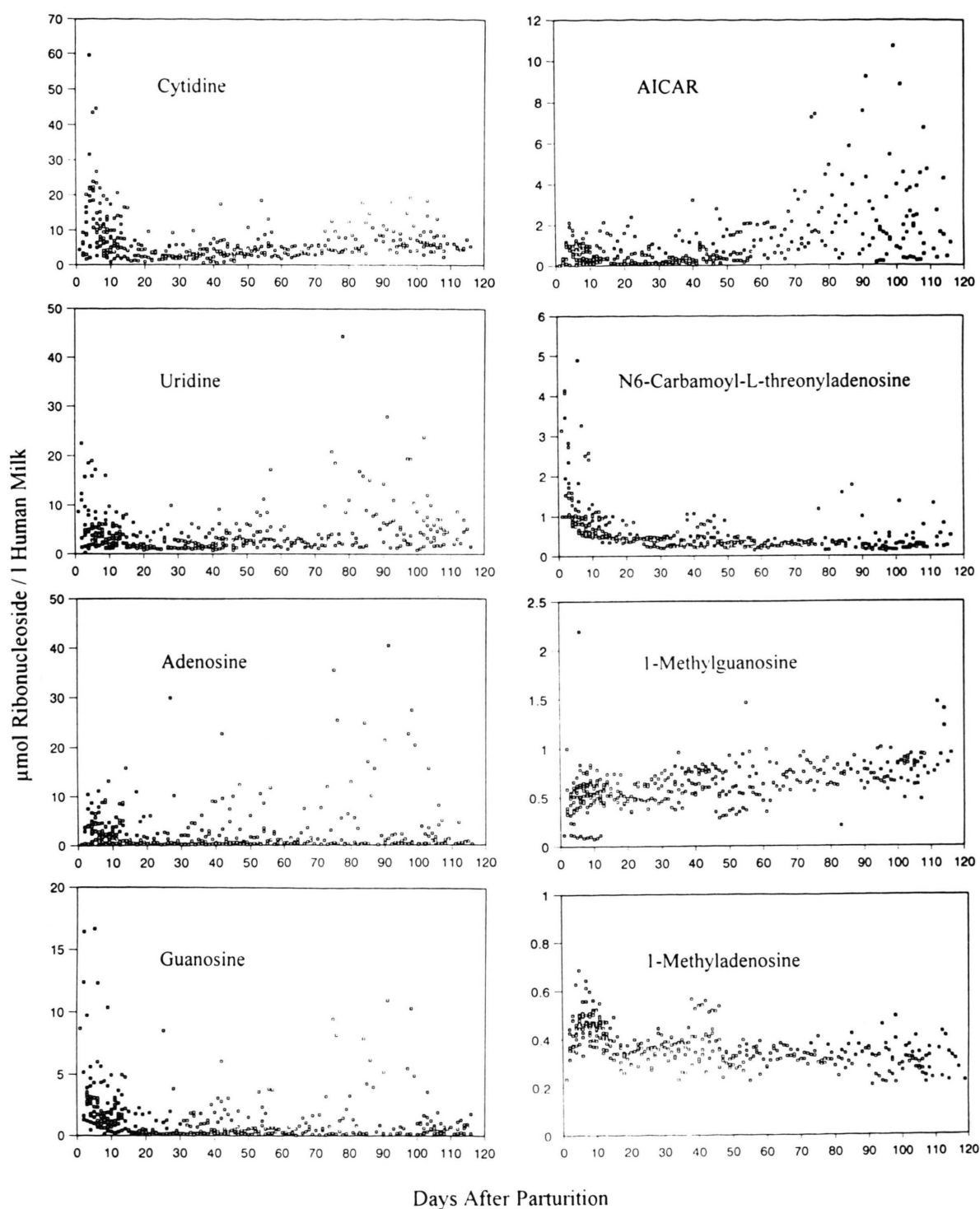


Fig. 3. Concentration profiles of ribonucleosides characterized in human milk throughout the nursing period.

AICAR were also used – for the first time in chromatographic peak analysis – for identifying the ribonucleosides.

Standards of the pure nucleosides were purchased from Sigma Chemie with the exception of m2,2Guo (PL-Biochemicals) and t6Ado (synthesized according to Chheda and Hong, 1971); the t6Ado isomers bearing D-, allo-D- and allo-L-threonine side chains were prepared as described (Martin and Schlimme, 1994).

Results and Discussion

Few publications deal with the identification of ribonucleosides in human milk (Schlimme *et al.*, 1986; Schneehagen and Schlimme, 1992; Groß *et al.*, 1992; Schneehagen, 1993; Schlimme *et al.*, 1993; Topp *et al.*, 1993). The concentration levels and profiles of these minor constituents in the milk of 20 nursing mothers have been investigated as a function of the nursing time. Fig. 3 shows, for example, the interindividual concentration profiles with advancing lactation of the eight identified and quantified ribonucleosides cytidine, uridine, adenosine, guanosine, 1-methyladenosine, 1-methylguanosine, N6-carbamoyl-L-threonyladenosine and AICAR. The values shown in the graphs include daily samples from a longitudinal study of two nursing mothers as well as milk samples taken randomly from 18 individuals over a nursing time of at least 120 days *post partum* including the colostrum phase.

The interindividual daily mean values calculated from the above mentioned ribonucleoside concentrations measured (Fig. 3) tend to decrease – with the exception of 1-methylguanosine – without distinct maximum values in the course of lactation. The concentration profiles of the human milk ribonucleosides throughout a nursing time of at least four months are similar to those reported for bovine milk (Raezke and Schlimme, 1990; Schlimme *et al.*, 1991).

Table I summarizes the interindividual mean values of the eight ribonucleosides quantified during mature stages of lactation over a nursing time of at least 120 days, starting with the 15th day after parturition. The minimum and maximum values measured throughout this nursing period are also given. The appropriate values found in bovine milk (Raezke and Schlimme, 1990) are given in comparison.

The interindividual variation – expressed as relative standard deviation in percent – arises mainly from the biological variation, with a small contribution from the analysis imprecision of maximally 7% (Raezke and Schlimme, 1990). The variation found for cytidine and uridine in human milk are twofold and those for adenosine and guanosine up to 4-fold larger than the variations of these compounds in bovine milk (Raezke and Schlimme, 1990; Schlimme *et al.*, 1991). Whereas the relative standard deviation for AICAR is between that observed for pyrimidine and purine nucleosides, the relative standard deviations of the modified ribonucleosides are markedly smaller. The variation is 22% for 1-methyladenosine and 26% for 1-methylguanosine, *i.e.* in the range for modified compounds in bovine milk.

The concentrations of 1-methyladenosine and N6-carbamoyl-L-threonyladenosine agree well with our findings of one mother throughout a nursing period of 1 month, 28th–58th days *post partum* (Schneehagen and Schlimme, 1992) as well as with results reported by Schöch's group (Groß *et al.*, 1992; Topp *et al.*, 1993). These workers analyzed milk samples taken from 9 nursing mothers on the 20th day *post partum* and quantified the following nucleosides: pseudouridine, 1-methyladenosine, 1-methylinosine, 1-methylguanosine, N2-methylguanosine, N2-dimethylguanosine and N6-carbamoyl-L-threonyladenosine (Groß *et al.*, 1992; Topp *et al.*, 1993). The concentrations of N2-methylguanosine, N2-dimethylguanosine and 1-methylinosine in our samples were found to be less than 0.05 $\mu\text{mol/l}$. A more precise determination of these human milk constituents was not possible under the automated analytical conditions used, *i.e.* without preconcentration treatment of the milk samples as carried out by Topp *et al.* (1993). The molar ratio of N2-dimethylguanosine and N6-carbamoyl-L-threonyladenosine, which is 2.7 in eukaryotic tRNA (Schöch *et al.*, 1990), is of particular interest in body fluids. Both of these non-reutilizable tRNA catabolites are quantitatively excreted in human urine (Schlimme *et al.*, 1990; Schwarzenau *et al.*, 1990; Schöch *et al.*, 1990) with a concentration ratio (m2,2Guo/t6Ado) of 2.7 in normal subjects (Schlimme *et al.*, 1987, 1990; Schwarzenau *et al.*, 1990). The ratio of 0.08 in human milk (Topp *et al.*, 1993) agrees with our findings as well as with

Table I. Interindividual values of ribonucleosides ($\mu\text{mol/l}$) in human milk (h) compared to bovine milk (b)^a.

Characteristic values	Adenosine		Cytidine		Guanosine		Inosine		Interindividual values of				1-Methyl-guanosine		N6-Carb-amoyl-L-threonyl-adenosine		AICAR	
	h	b	h	b	h	b	h	b	h	b	h	b	h	b	h	b	h	b
Arithmetic mean c ^b	2.96	1.36	5.14	2.44	1.01	0.83	— ^f	0.97	4.17	14.69	0.34	0.40	0.68	n.d. ^g	0.40	0.71	1.34	n.d.
Standard deviation s ^c	5.88	0.67	3.49	0.78	1.59	0.32	—	0.42	4.75	4.73	0.07	0.08	0.18	—	0.23	0.14	1.66	—
Relative standard deviation sr ^d	199	49	68	32	159	39	—	43	114	32	22	20	26	—	59	20	124	—
Minimum value min ^e	0.12	0.06	0.55	0.53	0.70	0.10	—	0.01	0.72	3.63	0.21	0.17	0.21	—	0.12	0.40	0.04	—
Maximum value max ^e	40.44	3.93	19.25	10.76	10.95	2.22	—	3.25	44.24	68.22	0.92	1.58	1.47	—	1.78	3.40	10.75	—

^a Analysis of the ribonucleosides was performed as described in Materials and Methods.

^b Human milk (h): Each mean value is the arithmetic mean of determinations (in duplicate) carried out on milk samples collected daily from a longitudinal study of two nursing mothers as well as randomly from eleven individuals over a nursing time of at least 120 days *post partum*, starting with the 15th day after parturition. Bovine milk (b): Each mean value is the arithmetic mean of determinations (in duplicate) carried out on milk collected on 3 mornings per week (Monday, Wednesday and Friday) from each of eight cows (race: German black pied) throughout the whole lactation period, with the exception of the first 3 weeks *post partum* (Raezke and Schlimme, 1990).

^c Standard deviation in $\mu\text{mol/l}$.

^d Relative standard deviation in % (including the contribution of the analytical procedure, *i.e.* the imprecision, to the standard deviation of maximally 7%).

^e Minimum and maximum values measured during the course of lactation (see note b).

^f Not determined due to poor analytical peak resolution.

^g n.d. = not detectable.

the ratio of these tRNA catabolites in galactorrhoea fluids of about 0.25 (Schlimme *et al.*, 1987). This implies that others than normal whole body tRNA turnover processes (Topp *et al.*, 1993) influence the formation and secretion of ribonucleosides in the mammary gland. In this context it should be mentioned that a strong correlation was found between the amount of immunoglobulins and the concentration of t6Ado in human milk during the first two weeks after parturition.

In Table II, the interindividual mean concentrations of ribonucleosides in human milk during the first and second week *post partum* are given, *i.e.* the colostral and the transitorial stages of lactation are compared to the values obtained for bovine colostrum.

The results in Tables I and II indicate that the composition of ribonucleosides in human milk differs from that found in bovine milk and in the milk of other ruminants, *e.g.* goat's milk (Schlimme *et al.*, 1991), not only quantitatively but also qualitatively. AICAR and 1-methylguanosine, both of which are absent in bovine and goat's milk, are minor constituents in human milk. Whether the

findings concerning the inosine content in human and bovine colostrum depend on different adenosine desaminase activities is not yet known. The concentration of cytidine in mature human milk exceeds that in the milk of ruminants by a factor of 2. On the other hand, the uridine content in mature human milk is only 20 to 30% of that determined in mature bovine or goat's milk. The small size of the uridine pool in human milk is unexpected, in view of the lactobiological importance of UDP-activated hexoses for lactose biosynthesis and the larger lactose contents of human (7.0 g/100 ml) relative to bovine milk (4.7 g/100 ml). The larger lactose content of human milk, however, may be the result of greater reutilization of uridine for supplying the lactating cell with uracil nucleotides. The larger cytidine concentration in mature human milk is difficult to explain on the basis of the importance of cytidine ribonucleosides as co-factors in the biosynthesis of phospholipids (fat globule membranes), because the fat contents of human and bovine milk are very similar.

Summarizing up, the experimental findings indicate that concentration profiles exist for each of

Table II. Interindividual values of ribonucleosides ($\mu\text{mol/l}$) in human milk during the colostral and transitorial stages of lactation compared to bovine colostrum^a.

Characteristic	Adenosine		Cytidine		Guanosine		Inosine		Interindividual values of Uridine		1-Methyladenosine		1-Methylguanosine		N6-Carbamoyl-L-threonyl-adenosine		AICAR	
	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II
Human																		
Arithmetic mean c ^b	3.2	3.1	14.3	7.6	3.3	1.8	13.7	— ^c	6.1	4.1	0.44	0.44	0.47	0.53	1.4	0.75	0.66	0.50
Relative standard deviation sr ^c	84	107	80	64	104	91	82	—	84	62	18	12	41	32	70	54	76	92
Cow																		
Arithmetic mean c ^d	2.0	2.7	11.9	6.0	2.1	2.0	4.7	2.9	131.6	58.2	1.0	0.54	n.d. ^f		2.9	1.1	n.d. ^f	
Relative standard deviation sr ^e	25	52	42	22	31	19	18	34	31	15	61	14	—		39	22	—	

^a Analysis of the ribonucleosides was performed as described in Materials and Methods.

^b Each mean value is the arithmetic mean of determinations (in duplicate) carried out on milk samples collected daily from a longitudinal study of two nursing mothers as well as randomly from eleven individuals during the colostral (first (I) week *post partum*) and transitorial (second (II) week *post partum*) stages of lactation; amount of samples: 112.

^c Standard deviation in $\mu\text{mol/l}$.

^d Each mean value is the arithmetic mean of determinations (in duplicate) carried out on milk collected on 3 mornings per week (Monday, Wednesday and Friday) from each of eight cows (race: German black pied) in the first (I) and second (II) lactation weeks *post partum* (Raezke and Schlimme, 1990).

^e Not determined due to poor analytical peak resolution.

^f n.d. = not detectable.

the quantified ribonucleosides in mammalian milks. The largest amounts of ribonucleoside were measured directly after parturition. Generally, the concentrations of most of the ribonucleosides tend to decrease gradually with advancing lactation. The species-specific pattern of these minor constituents in milk from different mammals is a remarkable property which corresponds to findings on nucleotides (Johke and Goto, 1962; Larson, 1976; Gil and Sanchez-Medina, 1981).

Studies carried out on human milk by Schöch's group (Topp *et al.*, 1993) and by us demonstrate markedly differing ratios of the metabolically non-reutilizable tRNA catabolite N2-dimethylguanosine and N6-carbamoyl-L-threonyl-adenosine which strongly implies that the tRNA metabolism in the epithelial cells of the mammary gland differs from that of the whole body.

Furthermore, another criterion observed is the up to 4-fold greater variation of the interindividual means, especially of the unmodified ribonucleosides, in human milk relative to those in bovine milk. The cows investigated were genetically far more homogeneous than the nursing mothers and the food-supply of the mothers was very probably more varied and less well defined than the feeding conditions of the cows. Whether these marked differences in variation of the interindividual mean ribonucleoside contents are genetically and/or nutritionally influenced remains to be clarified.

Acknowledgements

We wish to thank all the nursing mothers for kindly supplying us with milk samples. Dr. G. Haase and Dr. D. Tait are gratefully acknowledged for valuable criticism.

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