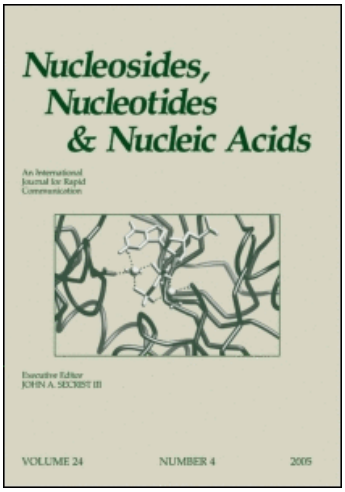

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Determination of N^6-Threoninocarbonyladenosine, N^2, N^2-Dimethylguanosine, Pseudouridine and Other Ribonucleosides in Human Breast Milk

H. Topp[a]; H. Groß[a]; G. Heller-Schöch[a]; G. Schöch[a]
[a] Research Institute of Child Nutrition, Dortmund, FRG

To cite this Article Topp, H. , Groß, H. , Heller-Schöch, G. and Schöch, G.(1993) 'Determination of N^6-Threoninocarbonyladenosine, N^2, N^2-Dimethylguanosine, Pseudouridine and Other Ribonucleosides in Human Breast Milk', Nucleosides, Nucleotides and Nucleic Acids, 12: 6, 585 — 596
To link to this Article: DOI: 10.1080/07328319308019013
URL: http://dx.doi.org/10.1080/07328319308019013

NUCLEOSIDES & NUCLEOTIDES, 12(6), 585-596 (1993)

DETERMINATION OF N^6-THREONINOCARBONYLADENOSINE, N^2,N^2-DIMETHYLGUANOSINE, PSEUDOURIDINE AND OTHER RIBONUCLEOSIDES IN HUMAN BREAST MILK

H. Topp*, H. Groß, G. Heller-Schöch and G. Schöch

Research Institute of Child Nutrition, Heinstück 11, 4600 Dortmund 50, FRG

ABSTRACT *- Seven modified ribonucleosides from degraded tRNA and rRNA were quantified in milk from mothers with preterm infants. The amounts of N^6-threoninocarbonyladenosine, N^2,N^2-dimethylguanosine and pseudouridine supplied in the milk have been estimated and related to the respective urinary amounts excreted by preterm infants.*

INTRODUCTION

Modified ribonucleosides from degraded RNA have been determined in urine and serum by several groups as potential markers of malignant growth in mammals [e.g. 1-5].

We have developed a noninvasive method to determine the whole body degradation rates of tRNA, rRNA and mRNA in mammals by measuring the renal excretion rates of some modified ribonucleosides and nucleobases which are liberated in the course of RNA degradation and which are virtually quantitatively excreted in urine [6-13].

Investigations concerning the content of unmodified and modified ribonucleosides in cow's and goat's milk were first carried out by the group of E. Schlimmme [14-18]. From their findings they concluded that ribonucleosides may be useful for characterizing milk of different species and different technological treatment. Recently, the same group published data concerning the concentrations of adenosine (A), cytidine (C), guanosine (G), uridine (U), 1-methyladenosine (m^1A), 1-methylinosine (m^1I), 1-methylguanosine (m^1G) and N^6-threoninocarbonyladenosine (t^6A) in human milk [19, 20].

In the present study we investigated the mean concentrations of t^6A, N^2,N^2-dimethylguanosine (m_2^2G) and pseudouridine (Ψ) along with that of m^1A, m^1I, m^1G, N^2-methylguanosine (m^2G), A, C, G and U in the milk from 9 mothers with preterm infants on day 20 of lactation.

Special attention has been paid to the concentrations of t^6A, m_2^2G and Ψ with the aim to estimate to what extent the amounts of these RNA catabolites supplied in breast milk could affect the amounts of t^6A, m_2^2G and Ψ excreted in urine of preterm infants on the assumption that these catabolites are completely absorbed.

This question is of interest because our determinations of the whole body degradation rates of tRNA and rRNA in preterm infants (e.g. as potential parameters to assess metabolic stress) are based on measuring the urinary excretion of t^6A, m_2^2G and Ψ [6-10].

Furthermore, the molar pattern of t^6A, m_2^2G and Ψ in milk is compared with the respective pattern in urine and possible explanations for the observed difference in these patterns are given.

MATERIALS AND METHODS

Aliquots of 24 h breast milk samples stemmed from 9 mothers with preterm infants (gestational age: 34.5 ± 2 weeks; birth weight 1758 ± 300 g) on day 20 of lactation. Standards of A, G, U, Ψ, m^1A, m^1G, m^2G were purchased from Sigma C, m^1I from Serva and m_2^2G from Pharmacia P-L Biochemicals. t^6A was a kind gift from Professor Dr. Dr. E. Schlimme, Dept. Chem. & Physics, Fed. Dairy Res. Inst., Kiel, F.R.G..

After lyophilisation of a 15 ml milk sample the fat was extracted with 10 ml acetone/dichloromethane (9:1). The sample was centrifuged for 5 min at 27000*g* and the supernatant was discarded. This procedure was repeated once. Ribonucleosides were then extracted from the sediment with 12 ml 70 % ethanol in an ultrasonic bath for 10 min. The sample was centrifuged for 5 min at 6000*g* and the supernatant was transferred. This procedure was repeated twice. Proteins in the combined supernatants were then sedimented by the addition of 1 volume of acetone at 4 °C over 16 h. After centrifugation for 30 min at 13000*g* the supernatant was concentrated to approximately 1 ml by rotary evaporation.

The ribonucleosides were then selectively enriched on a boronate gel (Affi-Gel 601, Bio Rad) modified after Kuo et al [5]. 2 ml 2.5 M

TABLE 1
Elution gradient for ribonucleosides

Step no.	Step time (min)	Buffer composition, % A	B	C	Gradient type
1	20	100	0	0	isocratic
2	15	90	10	0	linear
3	10	80	20	0	linear
4	5	70	30	0	linear
5	7.5	40	60	0	linear
6	6	0	100	0	linear
7	21	0	0	100	linear
8	7	0	0	100	isocratic
9	10	100	0	0	isocratic

ammonium acetate pH 9.5 and H_2O were added to the sample to a final volume of 6 ml. The sample was loaded onto a column (15 x 75 mm, packed with 1 g Affi-Gel 601) previously equilibrated with 20 ml 0.25 M ammonium acetate pH 8.8 by gravity. Under these alkaline conditions the ribonucleosides bind to the gel. The sample vial was washed with in total 8 ml 0.25 M ammonium acetate pH 8.8 and the washes were also loaded onto the column. The ribonucleosides were eluted by gravity with 25 ml 0.1 M formic acid from which the first 5 ml were discarded. The 20 ml ribonucleoside fraction was lyophilised and resuspended in 1 ml 10 mM ammonium phosphate pH 5.3.

A 20 μl aliquot was analysed by reversed phase HPLC (Nucleosil 120-5 C_{18}, Macherey-Nagel; pre-column 4.6 x 40 mm, main-column 4.6 x 250 mm; flow rate 0.75 ml/min; column temperature 33 °C) using a ternary elution gradient (Table 1) made up of the following components (modified from Gehrke and Kuo [21]):

A: 0.01 M ammonium phosphate, pH 5.3;
B: 20% methanol in 0.01 M ammonium phosphate, pH 5.1;
C: 35% acetonitrile in 0.01 M ammonium phosphate, pH 4.9.

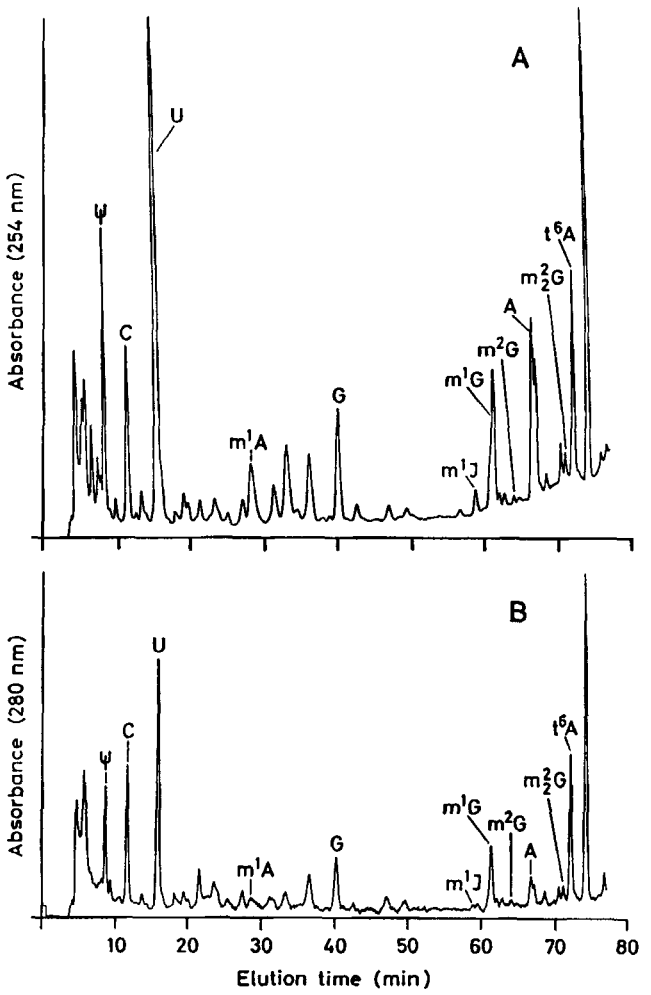

FIG. 1. HPLC separation of the ribonucleoside fraction from human breast milk; detection at 254 nm (A) and 280 nm (B); detector sensitivity was 0.002 AUFS; the signal was attenuated 128-fold for the first 18 min and 64-fold thereafter. The injected sample of 20 μl corresponds to 0.3 ml original milk. Compounds (pmol): Ψ (370.3), C (576.7), U (1252.0), m^1A (81.8), G (70.7), m^1I (19.7), m^1G (98.5), m^2G (3.5), A (74.2), $m_2{}^2G$ (8.7), t^6A (79.1).

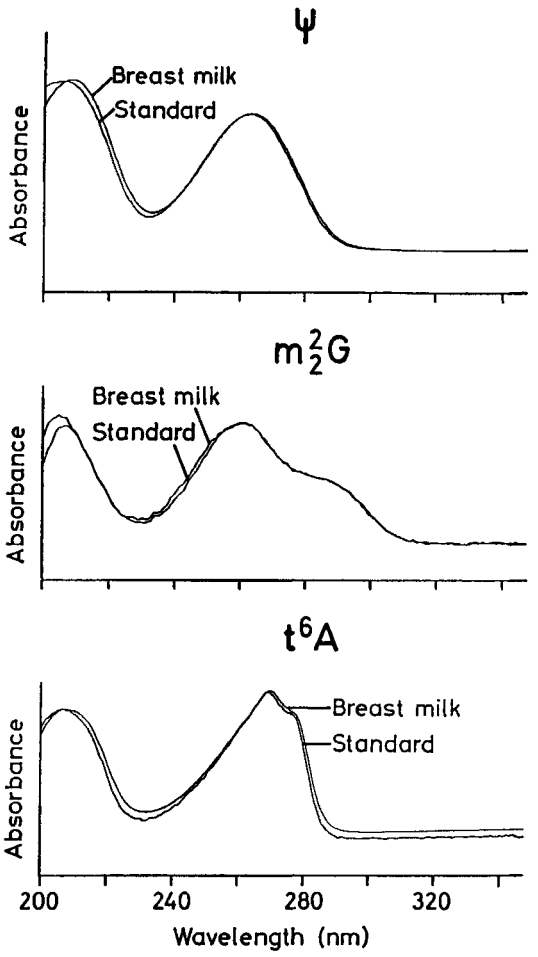

FIG. 2. UV spectra of Ψ, $m_2{}^2G$ and t^6A from breast milk and of corresponding standards taken with a diode array detector.

The ribonucleosides were identified by their characteristic retention times and by their UV-spectra taken with an LC-480 Auto Scan Diode Array Detector (Perkin Elmer). Quantification of the ribonucleosides was done by measuring their absorbance at 254 nm and 280 nm using Absorbance Detectors 160 (Beckman) and an Integrator SP 4270 (Spectra-Physics).

RESULTS

Figure 1 shows chromatograms of the ribonucleoside fraction from human breast milk taken at 254 and 280 nm.

TABLE 2
Recovery of ribonucleosides added to human breast milk and subsequent preparation of the sample

Ribonucleoside	Recovery, %		
	Mean[a]	SD	RSD, %[b]
U	70.7	3.9	5.5
C	91.2	4.6	5.0
G	72.8	2.6	3.6
A	65.0	3.6	5.6
Ψ	96.1	8.3	8.6
m^1A	86.3	8.0	9.3
m^1I	82.4	0.8	1.0
m^1G	86.0	2.1	2.5
m^2G	94.2	6.1	6.5
m_2^2G	92.7	5.1	5.5
t^6A	58.3	7.8	13.3

a Four identical additions of all ribonucleoside standards were made to a breast milk sample. The amounts added were for the different ribonucleosides in the range of half to twice the amount determined without addition.
b Relative standard deviation: standard deviation as a percentage of the mean value.

Figure 2 shows that the UV spectra of Ψ, m_2^2G and t^6A from breast milk agree with the spectra of the corresponding standard ribonucleosides. The spectra of the other determined ribonucleosides from milk not shown here also agree with those of the respective standards.

In Table 2 the recovery of standard ribonucleosides after addition to a milk sample and complete preparation of the sample is given. The values for the different ribonucleosides are in the range of 96.1 to 58.3 percent. The relative standard deviation is in most cases below 10 percent.

TABLE 3
Average concentrations of ribonucleosides in human breast milk on day 20 of lactation

Ribonucleoside	Average concentration (pmol/ml)[a]	SD (pmol/ml)	RSD, %[b]
U	5343.6	1277.1	23.9
C	4257.1	1498.5	35.2
G	207.3	91.6	44.2
A	529.5	212.3	40.1
Ψ	1648.9	331.4	20.1
m^1A	293.7	32.9	11.2
m^1I	88.8	28.5	32.1
m^1G	378.8	113.3	29.9
m^2G	16.6	3.2	19.4
m_2^2G	36.5	6.0	16.4
t^6A	461.9	81.8	17.7

[a] Mean values were from milk samples of 9 mothers. The values have been corrected for recovery (Tab. 2).
[b] Relative standard deviation.

Table 3 shows average concentrations of the identified ribonucleosides in the breast milk from 9 mothers on day 20 of lactation. It is obvious that the interindividual variation is relatively small for the modified ribonucleosides Ψ, m_2^2G and t^6A, known to be virtually quantitatively excreted in urine of humans [8-10, 22-24] and relatively high for the unmodified ribonucleosides known to be partly reutilized and further catabolized.

Starting from the average concentrations of Ψ, m_2^2G and t^6A in the milk samples (Tab. 3) we have calculated the amounts supplied in breast milk to a preterm infant on day 20 of lactation assuming a drinking volume of 400 ml (Tab. 4). Taking these values we have estimated to what extent the excreted amounts of Ψ, m_2^2G and t^6A in the urine of 20 day old preterm infants with an average body weight of 2 kg could be

TABLE 4
Estimated amounts of Ψ, m_2^2G and t^6A supplied to preterm infants in breast milk on day 20 of lactation in relation to daily excretion values in the urine of preterm infants

Ribonucleoside	Average amount supplied in milk[a] (nmol/d)	Average amount excreted in urine[b] (nmol/d)	Amount supplied in milk as a percentage of the excreted amount
Ψ	659.6	26731 ± 5761	2.5
m_2^2G	14.6	1341 ± 297	1.1
t^6A	184.8	739 ± 157	25.0

[a] Drinking volume assumed: 400 ml/d.
[b] Excretion values of the ribonucleosides were determined in 54 urine samples from 31 preterm infants (mean body weight 2006 ± 58 g). Samples were collected by precise timing (collection period 596 ± 58 min); values were extrapolated to 24 h [9].

affected by the amounts of these ribonucleosides supplied in the milk if one assumes that they are completely absorbed (Tab. 4).

The data reveal that in the case of urinary Ψ and m_2^2G a possible contribution to these ribonucleosides from milk is negligible. In contrast, t^6A supplied in milk could affect urinary excretion values by approximately 25 percent.

DISCUSSION

In the present study we have quantified 7 modified ribonucleosides from RNA degradation as well as A, G, C and U in human breast milk of mothers with preterm infants on day 20 of lactation. Determinations of ribonucleosides in human milk were first carried out by the group of E. Schlimme [19, 20]. In addition to m^1A, m^1G, m^1I and t^6A determined by this group we have quantified Ψ, m^2G and m_2^2G.

It has been described that Ψ orally given to human adults is completely absorbed [23]. The absorption of m_2^2G has not been investigated systematically.

However, our determinations of the whole body degradation rates of tRNA and rRNA in preterm infants by measuring the urinary excretion of the RNA catabolites m_2^2G and Ψ [8-10] cannot be significantly affected by an assumed absorption of the relatively small amounts of m_2^2G and Ψ supplied in breast milk (Tab. 4). This is an important fact if the whole body degradation rates of tRNA and rRNA in preterm infants are to be used diagnostically, e.g. to assess the metabolic status.

Furthermore, the fact that the contribution of breast milk to the urinary excretion of m_2^2G and Ψ is negligible, confirms our previous determinations of the whole body degradation rates of tRNA and rRNA in preterm infants via urinary m_2^2G and Ψ. These determinations have revealed that the degradation rates of these RNA classes are highly correlated with the metabolic activity [8-10, 12].

On the other hand, t^6A in milk could affect the urinary excretion of t^6A in preterm infants by about 25 percent if one assumes that it is absorbed. However, it has been previously demonstrated that t^6A given orally to rats and man is virtually not excreted in urine [24] which could be due to the fact that t^6A is not absorbed. In addition, tRNA degradation rates in preterm infants determined via urinary t^6A can be easily checked by the determinations via m_2^2G.

Our measurements of the concentrations of t^6A, m_2^2G and Ψ in breast milk makes it possible for the first time to compare molar ratios of these catabolites in milk with the respective ratios in urine. All three catabolites are known to be virtually quantitatively excreted in urine of man [8-10, 22-24].

Urinary t^6A and m_2^2G originate exclusively from tRNA. Ψ in urine originates by about 30 % from tRNA, the major part around 70 % stems from rRNA and a negligible fraction stems from UsnRNAs [8-10].

It is interesting that the average molar quotient t^6A/m_2^2G in the urine of adults as well as preterm infants is about 0.5 (Tab 4; [8-10]) whereas the respective ratio in milk is 12.6 (Tab 4), i.e. the excretion of t^6A in milk in relation to m_2^2G is 23 times that in urine. The quotient t^6A/m_2^2G in urine is very similar to the respective quotient that we have determined on average for cytoplasmic tRNA [8-10]. Therefore, it can be concluded that in the milk producing breast cells tRNA species containing t^6A but no m_2^2G are turned over more rapidly than on average in the whole body.

Searches for eucaryotic cytoplasmic tRNAs containing t^6A but no m_2^2G in the published sequence data [25] reveal only four species which fulfil this criterion. These are two tRNAs for the amino acid arginine, one tRNA for lysine and initiator tRNA.

Assuming an elevated turnover of initiator tRNA in the breast as compared to the total body one could explain the altered quotient of t^6A/m_2^2G in milk. However, the molar ratio of Ψ to m_2^2G is also about twofold higher in breast milk than in urine but initiator tRNA contains no Ψ. Therefore, a higher turnover of initiator tRNA alone could not explain the higher quotient Ψ/m_2^2G in milk. However, it can not be ruled out that the higher relative amount of Ψ originates from an elevated turnover of ribosomes in the breast cells as compared to the average situation in the body. Ribosomes contain in their 28S, 18S and 5.8S RNA components in total about 95 residues of Ψ [8-10].

Assuming an elevated turnover of $tRNA^{Lys}$ (with the anticodon CUU) which contains Ψ [25] one could also explain the higher quotient Ψ/m_2^2G. However, $tRNA^{Lys}$ contains no m^1G, but the quotient m^1G/m_2^2G is also approximately higher by a factor of 10 in milk (Table 3) than on average in cytoplasmic tRNA [8].

The tRNA of which an elevated turnover could at present best explain the observed altered ratios of the modified ribonucleosides t^6A, m_2^2G, Ψ and m^1G in milk is the $tRNA^{Arg}$ (anticodon UCU) whose sequence [25] has been described to contain 1 residue of t^6A, no m_2^2G, 1 residue of m^1G and 5 residues of Ψ. Therefore, one can speculate that the abundance of this tRNA species might be very high in breast cells and/or that this species might be turned over very rapidly. It would be interesting to investigate if this tRNA species has a special function in breast cells.

However, it is also possible that a high turnover of another till now not identified tRNA species in the breast cells may contribute to the relative large amount of t^6A in the milk.

This paper includes data from the Ph.D. thesis of H. Groß.

ACKNOWLEDGEMENTS

This work has been supported by the Ministerium für Wissenschaft und Forschung des Landes Nordrhein-Westfalen and by the Bundesministerium

für Gesundheit. N^6-threoninocarbonyladenosine standard was a kind gift from Professor Dr. Dr. E. Schlimme, Dept. Chem. & Physics, Fed. Dairy Res. Inst. Kiel, F.R.G..

REFERENCES

1. Borek, E., Sharma, O.K., Waalkes, T.P. *Recent Results Cancer Res. 84*, 303-316 (1983).
2. Borek, E. Trends Biochem. Sci. *10*, 182-184 (1985).
3. Schöch, G., Heller-Schöch, G. Helv. *Paediatr. Acta (Suppl.) 38*, 3-171 (1977).
4. Zumwalt, R.W., Waalkes, T.P., Kuo, K.C., Gehrke, C.W. in: *Chromatography and Modification of Nucleosides* (Gehrke, C.W., Kuo, K.C., eds.) *Part C*, Modified Nucleosides in Cancer and Normal Metabolism, Elsevier, Amsterdam-Oxford-New York-Tokyo 15-40 (1990).
5. Kuo, K.C., Phan, D.T., Williams, N., Gehrke, C.W. in: *Chromatography and Modification of Nucleosides* (Gehrke, C.W., Kuo, K.C., eds.) *Part C*, Modified Nucleosides in Cancer and Normal Metabolism, Elsevier, Amsterdam-Oxford-New York-Tokyo 41-113 (1990).
6. Sander, G., Topp, H., Wieland, J., Heller-Schöch, G., Schöch, G. *Human Nutr. Clin. Nutr. 40C*, 103-118 (1986).
7. Sander, G., Topp, H., Heller-Schöch, G., Wieland, J., Schöch G. *Clin. Sci. 71*, 367-374 (1986).
8. Schöch, G., Sander, G., Topp, H., Heller-Schöch, G. in: *Chromatography and Modification of Nucleosides* (Gehrke, C.W., Kuo, K.C., eds.) *Part C*, Modified Nucleosides in Cancer and Normal Metabolism, Elsevier, Amsterdam-Oxford-New York-Tokyo 389-441 (1990).
9. Schöch, G., Topp, H., Held, A., Heller-Schöch, G., Ballauff, A., Manz, F., Sander, G. *Eur. J. Clin. Nutr. 44*, 647-658 (1990).
10. Topp, H., Sander, G., Jöhren, O., Fenselau, S., Fuchs, E., Gädeken, D., Heller-Schöch G., Schöch, G. in: *Metabolism and Enzymology of Nucleic Acids Including Gene and Protein Engineering* (Balan, J., ed.) *Vol. 7*, Institute of Molecular Biology, Slovak Academy of Sciences, Bratislava, 375-384 (1991).
11. Topp, H., Kikillus, K., Heller-Schöch, G., Schöch, G. *Biol. Chem. Hoppe-Seyler 372*, 770 (1991).

12. Schöch, G., Sander, G., Fuchs, E., Jöhren, O., Heller-Schöch, G., Topp, H. *Biol. Chem. Hoppe-Seyler 372*, 749 (1991).
13. Topp, H., Duden, R., Heller-Schöch, G., Schöch, G. *Biol. Chem. Hoppe-Seyler 373*, 830 (1992).
14. Schlimme, E., Raezke, K.P., Peters, K.H. *Kieler Milchwirtschaftliche Forschungsberichte 41*, 243-251 (1989).
15. Raezke, K.P., Schlimme, E. *Z. Naturforsch. 45c*, 655-662 (1990).
16. Boos, K.S., Wilmers, B., Schlimme E., Sauerbrey, R. *Nucleosides & Nucleotides 9*, 389-393 (1990).
17. Schlimme, E., Schneehagen, K., Ott, F.G. *Milchwissenschaft 45*, 654-657 (1990).
18. Schlimme, E., Raezke, K.P., Ott, F.G. *Z. Ernährungswiss. 30*, 138-152 (1991).
19. Schneehagen, K., Schlimme, E. *Kieler Milchwirtschaftliche Forschungsberichte 44*, 67-74 (1992).
20. Schneehagen, K., Schlimme, E. *Ernährungs-Umschau 40, 94* (1993)
21. Gehrke, C.W., Kuo, K.C. in: *Chromatography and Modification of Nucleosides* (Gehrke, C.W., Kuo, K.C., eds.) *Part A*, Analytical Methods for Major and Modified Nucleosides, HPLC, GC, MS, NMR, UV, and FT-IR, Elsevier, Amsterdam-Oxford-New York-Tokyo, 3-71 (1990).
22. Weissman, S., Eisen, A.Z., Lewis, M., Karon, M., Clark, P. *J. Lab. Clin. Med. 60*, 40-47 (1962).
23. Dugaiczyk, A., Eiler, J.J. *Nature 212*, 611-612 (1966).
24. Hong, C.I., Chheda, G.B., Murphy G.P., Mittelman, A. *Biochem. Pharmacol. 22*, 1927-1936 (1973).
25. Sprinzl, M., Hartmann, T., Weber, J., Blank, J., Zeidler, R. *Nucleic Acids Res. 17 Suppl.*, r1-r172 (1989).

Received 11/4/92
Accepted 4/22/93