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The Lactose and Neuraminlactose Content of Rat Milk and Mammary Tissue

By N. J. KUHN
Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, U.K.

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Specific analyses of rat milk sugar revealed lactose and *N*-acetylneuraminyl-lactose. The latter showed multiple, interconvertible forms in certain paper-chromatographic systems. Mean lactose concentrations rose from 33.1 μmol/ml at day 0 to 100 μmol/ml at day 20 of lactation. Mean *N*-acetylneuraminyl-lactose concentrations rose from 7.1 μmol/ml at day 0 to 25.9 μmol/ml at day 4 and thereafter declined, decreasing to zero by day 20 of lactation. Similar data are given on the concentrations of these sugars in mammary tissue of rats. Neuraminyl-lactose was also detected in mouse mammary tissue.

During studies on lactogenesis it became necessary to have accurate values for the concentration of lactose in rat milk. It was noticed that analyses performed with the specific method of Reithel (1963), in which hexokinase and glucose 6-phosphate dehydrogenase are used to determine the glucose released by β-galactosidase, gave lower lactose values than published values based on non-specific chemical methods of sugar determination (see the Discussion section for references). However, further lactose appeared when milk samples were incubated with neuraminidase, suggesting the presence of lactose combined with sialic acid. Such material does not react in the enzymic assay but would still be detected in chemical determinations of reducing sugar.

Concentrations of lactose and of sialic acid-bound lactose were therefore measured in rat milk and mammary tissue at different stages of lactation, and in the mammary tissue of two mice and a guinea pig. Evidence is given for the identity of the sialic acid-bound lactose as *N*-acetylneuraminyl-lactose (neuraminlactose).

Methods

Primiparous rats of a Wistar-derived colony were used. Litter sizes averaged about 12 pups. Milk was collected from all nipples while the rats were under ether or Nembutal anaesthesia, in some cases after intraperitoneal injection of 0.1 i.u. of oxytocin. Milk samples from a total of 27 rats were diluted 200-fold with water and 0.1 ml samples were assayed for lactose (Reithel, 1963) before and after incubation with 4 munits of neuraminidase in 50 mM-citric acid–sodium phosphate buffer, pH 5.6, for 2h at 37°C. A trace of toluene was added to prevent contamination by micro-organisms. The extra lactose released by neuraminidase is referred to in Table 1 as 'neuraminlactose'. Inguinal mammary tissue, extracted by the method of Kuhn & Lowenstein (1967), was assayed similarly.

Acidic sugars of pooled rat milk samples (2–10 ml) were isolated by dialysis for 36h against approx. 100 vol. of water at 0–4°C. The diffusate was passed through a 1 cm × 5 cm column of Dowex 1 resin (formate form; X8; 200 mesh), which was then washed with several litres of water and eluted slowly at 0–4°C with 30 ml of 0.15 M-pyridine–acetic acid buffer, pH 4.9. The eluate was freeze-dried and the white sugar residue weighed and stored at −18°C until required.

Descending paper chromatography was carried out with the following solvent systems: (1) propan-1-ol – ethyl acetate – water (7:1:2, by vol.) (Baar & Bull, 1953); (2) butan-1-ol – propan-1-ol – 0.1 M-HCl (1:2:1, by vol.) (Svennerholm & Svennerholm, 1958); (3) butan-1-ol – pyridine – water (6:4:3, by vol.) (Kuhn & Brossmer, 1958); (4) pyridine – ethyl acetate – acetic acid – water (5:5:1:3, by vol.) (Kuhn & Brossmer, 1956); (5) pyridine – ethyl acetate – water (5:10:6, by vol.) (Carubelli *et al.*, 1961); (6) propan-2-ol – water (4:1, v/v) (Choi & Carubelli, 1968); (7) butan-2-one – acetone – water – formic acid (3:1:1:0.1, by vol.) (Schneir & Winzler, 1962). Whatman no. 4 paper was used with all except system (3), for which Whatman no. 1 paper was impregnated with sodium borate (Cardini & Leloir, 1957). Lactose and neuraminlactose were detected by spraying with 4% diphenylamine in ethanol – 4% aniline in ethanol – syrupy phosphoric acid (5:5:1, by vol.), followed by heating at 80°C for 10 min. *N*-Acetylneuraminic acid was detected with Ehrlich's reagent (0.5 g of *p*-dimethylaminobenzaldehyde and 5 g of trichloroacetic acid in 20 ml of aq. 50%, v/v, ethanol and 60 ml of butan-1-ol) and heated at 100°C for 10 min. For the detection of *N*-acetylamino sugar papers were first

dipped in ethanolic 0.5M-NaOH and heated at 100°C for 10min and then dipped in the *p*-dimethylaminobenzaldehyde reagent of Reissig *et al.* (1955) and, after being air-dried, were heated at 100°C for 10min.

Colorimetric methods for the determination of sialic acids are given in the text.

O-Acetyl groups were determined as the ferric hydroxamate. A portion (0.1ml) of sample (0–1.0μequiv. of ester) was mixed with 0.2ml of 1M-hydroxylamine, pH9.0, and incubated at 37°C for 2h. After the addition of 1ml of 1M-HCl and 0.1ml of 1M-$FeCl_3$ the resulting colour was read at 540nm. A standard curve was prepared with α-D-glucose penta-acetate.

Incubations with *N*-acetylneuraminic acid aldolase were performed at 37°C for 4h and contained *N*-acetylneuraminic acid (0.5μmol), potassium phosphate buffer, pH7.2 (60μmol), *N*-acetylneuraminic acid aldolase (0.05units) and toluene (10μl), in a final volume of 0.6ml.

Enzymes and nucleotides were from Boehringer Corp. (London) Ltd. (London W.5, U.K.) and from Sigma (London) Chemical Co. Ltd. (London S.W.6, U.K.). *N*-Acetylneuraminic acid reference standard was from Sigma or from Koch–Light Laboratories, Colnbrook, Bucks., U.K., and a standard solution was standardized by total nitrogen determination (Strauch, 1965). α-D-Glucose penta-acetate microanalytical reagent was from British Drug Houses Ltd., Poole, Dorset, U.K.

Results

Lactose and neuraminlactose concentrations

Table 1 shows that the mean lactose concentration of rat milk rises from 33.1μmol/ml at parturition to 100.1μmol/ml on day 20 of lactation. Neuraminlactose concentrations are low at the beginning of lactation, rise to a maximum of 25μmol/ml 4 days later and become negligible by the end of lactation.

The absolute tissue concentrations of these sugars in the mammary gland (Table 1) are less meaningful since they partly reflect the extent of previous suckling by the litter. However, the ratios of neuraminlactose to lactose closely match the same ratios in milk sampled at the corresponding stage of lactation, indicating that both sugars occupy the same tissue compartment(s). Neither sugar could be detected by the routine assay in mammary gland removed 2–3 days before parturition.

Mammary tissue removed from two mice 7 days after parturition contained respectively 10.6 and 4.2μmol of lactose/g and 5.7 and 3.7μmol of neuraminlactose/g. Mammary tissue of a single lactating guinea pig contained 60μmol of lactose/g and no detectable neuraminlactose.

Table 1. *Lactose and neuraminlactose content of rat milk and mammary tissue during lactation*

Milk samples collected at '0 days' were obtained within 3h of parturition in the absence of suckling by the newborn. Mammary tissue collected at '0 days' was obtained during the course of parturition, before the suckling had begun. Values are means±S.E.M. with the number of samples in parentheses.

	Milk			Mammary tissue		
Time *post partum* (days)	Lactose (μmol/ml)	Neuraminlactose (μmol/ml)	Neuraminlactose/lactose ratio	Lactose (μmol/ml)	Neuraminlactose (μmol/g)	Neuraminlactose/lactose ratio
0	33.1±0.7 (5)	7.1±1.4 (5)	0.20	12.6±1.2 (5)	1.0±0.3 (4)	0.08
1	38.8±3.3 (7)	11.6±1.6 (7)	0.30	9.0±3.2 (3)	2.6±3.5 (3)	0.29
4	42.4±4.0 (5)	25.9±3.2 (5)	0.61	13.3±3.1 (3)	7.0±1.2 (3)	0.53
8	43.4±4.0 (7)	22.3±2.3 (7)	0.51	10.6±2.3 (4)	5.2±0.7 (4)	0.49
12	77.2±1.8 (3)	22.8±3.5 (3)	0.30			
16	96.8±5.9 (7)	4.1±8.7 (7)	0.04			
18				36.1±2.9 (4)	2.1±0.6 (4)	0.06
20	100.1±5.3 (5)	2.4±2.4 (5)	0.02			

Identity of neuraminlactose

Analysis by paper chromatography suggests that the acidic sugar of rat milk contains a single substance, which can, however, exist in multiple and partially interconvertible forms. Thus system (1) revealed two spots of $R_{lactose}$ approx. 0.50 and 0.18, here designated components 1 and 2 respectively. One pooled milk sample that was examined, containing milk from various stages of lactation, also contained substantial amounts of an even slower-moving sugar. This is designated component 3, although its banded appearance suggested there were in fact several components. After elution and storage as the solid at −18°C component 3 gradually changed into faster-moving material, which, after 1 year, moved as two spots indistinguishable from components 1 and 2. Further, components 1 and 2 were themselves readily interconvertible since, when eluted from the chromatogram and re-run in the same system, each gave again two spots moving identically with the original components 1 and 2. Components 1 and 2, and a stored preparation of component 3, gave the same two spots in system (6), but in systems (4), (5) and (7) they moved as single, apparently identical spots.

To confirm the impression that components 1 and 2 only co-existed in solvents of ill-defined pH, they were chromatographed in system (1) that had been modified by the use of 0.1 M-HCl or aq. 0.1 M-NH_3 soln. in place of water. Only a single spot appeared in both cases. The chromatographic behaviour of components 1 and 2 is summarized in Table 2, where it is seen that all systems showed a relatively fast-moving spot, of $R_{lactose}$ 0.42–0.71, whereas systems (1) and (6) showed an additional slower-moving spot of $R_{lactose}$ 0.18–0.23,

It was not possible to demonstrate the presence of lactones. Thus none of the components reacted typically to a hydroxamate spray (with gluconolactone used for comparison). Paper electrophoresis at pH values 8.5, 7.0, 5.5, 3.5 and 2.6 each revealed only a single component, which moved towards the anode relative to lactose, used as electro-endosmotic marker.

After elution from paper chromatograms developed in system (1), components 1 and 2 were both identified as *N*-acetylneuraminyl lactose by the following analysis. After incubation of the samples with neuraminidase, paper chromatography in system (1) showed the disappearance of the original material and the appearance of lactose and sialic acid, detected by their mobilities and staining characteristics. When samples treated with neuraminidase were assayed for lactose (Reithel, 1963) and sialic acid (Warren, 1959) these were present in the ratio 1:1. Components 1 and 2 did not react significantly with ninhydrin, indicating the absence of free amino groups. Ester analysis revealed less than 0.07 ester equiv./mol of sugar, implying the absence of lactones and of *O*-acyl groups.

Components 1 and 2 were also hydrolysed with 0.025 M-H_2SO_4 at 80°C for 1 h, and the sialic acid released was isolated by using anion-exchange resin. In each case the product behaved identically with authentic *N*-acetylneuraminic acid on paper chromatography in system (2). Solutions were also of identical concentration when assayed by three different chemical methods, the thiobarbituric acid method (Warren, 1959), the Ehrlich test (Werner & Odin, 1952) and the resorcinol method (Svennerholm, 1957). The final colours had absorption spectra identical with those given by authentic *N*-acetylneuraminic acid. Use of Eegriwie's reagent (Klenk & Uhlenbruck, 1957) showed the absence of *N*-glycolyl groups. Incubation with *N*-acetylneuraminic acid aldolase released pyruvate, determined enzymically (Bücher *et al.*, 1963), and *N*-acetylmannosamine, identified by comparison with authentic *N*-acetylmannosamine and *N*-acetylglucosamine on paper chromatography in system (3).

Component 3 was less thoroughly analysed. However, it was completely digested by neuraminidase to give lactose and *N*-acetylneuraminic acid in the ratio 0.87:1. These were also identified chromatographically.

Table 2. *Relative mobilities of N-acetylneuraminyl lactose on Whatman no. 4 paper*

Component 3, referred to in the text, is not included. References to the solvent systems used are given in the Methods section.

Solvent system	$R_{lactose}$
Propan-2-ol–water (4:1, v/v)	0.23, 0.46
Propan-1-ol–ethyl acetate–water (7:1:2, by vol.)	0.18, 0.50
Propan-1-ol–ethyl acetate–0.1 M-HCl (7:1:2, by vol.)	0.71
Propan-1-ol–ethyl acetate–aq. 0.1 M-NH_3 soln. (7:1:2, by vol.)	0.57
Pyridine–ethyl acetate–water (5:10:6, by vol.)	0.67
Pyridine–ethyl acetate–acetic acid–water (5:5:1:3, by vol.)	0.57
Butan-2-one–acetone–water–formic acid (3:1:1:0.1, by vol.)	0.42

Discussion

It is well known that the milk of several species contains a variety of oligosaccharides (Stacey & Barker, 1962), and several authors have detected multiple components other than lactose on chromatography of rat milk (Roberts *et al.*, 1954; Jenness *et al.*, 1964). The evidence presented above suggests that these multiple forms occur only under certain conditions of chromatography, that they are largely interconvertible and that they are all forms of *N*-acetylneuraminyl lactose. This substance was detected in rat mammary tissue by Trucco & Caputto (1954) and Heyworth & Bacon (1957), and was subsequently shown by Choi & Carubelli (1968) to be *N*-acetylneuraminyl(2→3)lactose. It is therefore likely that the *N*-acetylneuraminyl lactose of rat milk also has the 2→3 linkage. The related sugar *NO*-diacetylneuraminyl-lactose has also been characterized with both the 2→3 linkage (bovine milk; Kuhn & Brossmer, 1959) and the 2→6 linkage (human milk; Kuhn & Gauhe, 1965).

In the present study, which is mainly concerned with establishing the quantities of lactose and neuraminlactose in rat milk, it has not been possible to establish the nature of the multiple forms of the latter. Since electrophoresis over a wide pH range showed a single anionic component, it is probable that the commonest form of neuraminlactose is the free acid or its salt. It therefore appears necessary to explain the slower-migrating forms that appear in chromatographic systems of ill-defined pH (Table 2). These are unlikely to be lactones, since they would migrate faster than the free form. However, their mobilities would be consistent with polymeric forms, possibly not unlike the lactides readily formed by α-hydroxy acids.

Milk is generally considered to be a low-carbohydrate food and, among other species, rat milk is thought to have an exceptionally low lactose content. The mean value of 82 μmol/ml (2.8%) given by Cox & Mueller (1937) is widely quoted, and a similar mean value of 88 μmol/ml (3.0%) was reported by Dymsza *et al.* (1964). However, the results of Cox & Mueller (1937) and that of Luckey *et al.* (1954) indicated that the lactose content may change with the stage of lactation. None of these authors used a specific assay.

The present data show that the true lactose content of rat milk rises threefold during lactation, the first milk containing the unusually low concentration of 33 μmol/ml (1.1%). This situation contrasts with that in the rabbit, where lactose concentrations fall markedly as lactation proceeds (Cowie, 1969). In both cases the change is such as to approach a carbohydrate content similar to that of the adult diet, and may therefore serve an adaptive purpose. During part of lactation, however, neuraminlactose occurs in relatively large amounts. By day 4 of lactation it comprises over 30% on a molar basis, or over 50% by wt., of the total milk sugar, although by the end of lactation it is barely detectable. The function of neuraminlactose is unclear, but its presence has the effect of raising the carbohydrate content of milk without undue osmotic contribution.

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References

Baar, S. & Bull, J. P. (1953) *Nature (London)* **172**, 414–415
Bücher, T., Lamprecht, W. & Latzko, E. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), p. 253, Academic Press, New York and London
Cardini, C. E. & Leloir, L. F. (1957) *J. Biol. Chem.* **225**, 317–324
Carubelli, R., Ryan, L. C., Trucco, R. E. & Caputto, R. (1961) *J. Biol. Chem.* **236**, 2381–2388
Choi, H. U. & Carubelli, B. (1968) *Biochemistry* **7**, 4423–4430
Cowie, A. T. (1969) *J. Endocrinol.* **44**, 437–450
Cox, W. M. & Mueller, A. J. (1937) *J. Nutr.* **13**, 249–260
Dymsza, H. A., Czajka, D. M. & Miller, S. A. (1964) *J. Nutr.* **84**, 100–106
Heyworth, R. & Bacon, J. S. D. (1957) *Biochem. J.* **66**, 41–47
Jenness, R., Regehr, E. A. & Sloan, R. E. (1964) *Comp. Biochem. Physiol.* **13**, 339–352
Klenk, E. & Uhlenbruck, G. (1957) *Hoppe-Seyler's Z. Physiol. Chem.* **307**, 266–271
Kuhn, N. J. & Lowenstein, J. M. (1967) *Biochem. J.* **105**, 995–1002
Kuhn, R. & Brossmer, R. (1956) *Chem. Ber.* **89**, 2013–2025
Kuhn, R. & Brossmer, R. (1958) *Justus Liebigs Ann. Chem.* **616**, 221–225
Kuhn, R. & Brossmer, R. (1959) *Chem. Ber.* **92**, 1667–1671
Kuhn, R. & Gauhe, A. (1965) *Chem. Ber.* **98**, 395–413
Luckey, T. D., Mende, T. J. & Pleasants, J. (1954) *J. Nutr.* **54**, 345–359
Reissig, J. L., Strominger, J. L. & Leloir, L. F. (1955) *J. Biol. Chem.* **217**, 959–966
Reithel, F. J. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), p. 103, Academic Press, New York and London
Roberts, H. R., Pettinati, J. D. & Bucek, W. (1954) *J. Dairy Sci.* **37**, 538–545
Schneir, M. & Winzler, R. J. (1962) *Biochem. Prep.* **9**, 1–4
Stacey, M. & Barker, S. A. (1962) *Carbohydrates of Living Tissues*, Van Nostrand, London, New York and Toronto
Strauch, L. (1965) *Z. Klin. Chem.* **5**, 165–167
Svennerholm, E. & Svennerholm, L. (1958) *Nature (London)* **181**, 1154–1155
Svennerholm, L. (1957) *Biochim. Biophys. Acta* **24**, 604–611
Trucco, R. E. & Caputto, R. (1954) *J. Biol. Chem.* **206**, 901–909
Warren, L. (1959) *J. Biol. Chem.* **234**, 1971–1975
Werner, I. & Odin, L. (1952) *Acta Soc. Med. Upsal.* **57**, 230–241