tained on products formed by reaction of these polymers with nitrous acid. Finally, the results obtained here introduce a new and complementary procedure for selective cleavage of CR-heparins which is based upon the extreme acid lability of the glycosidic bonds of the 2-O-sulfated L-idosyl residues (Lloyd and Forrester, 1971). There remains the complete characterization of the segments of these polymers derived from regions which contain D-glucuronic acid and lower levels of *N*- and *O*-sulfate, but the approaches developed here provide clearly defined directions for the continuing studies.

References

Conrad, H. E., Varboncoeur, E., and James, M. E. (1973), *Anal. Biochem. 51,* 486.

Danishefsky, I., Steiner, H., Bella, A., and Friedlander, A. (1969), *J. Biol. Chem. 244,* 1741.

DeJongh, D. C., Radford, T., Hribar, J. D., Hanessian, S., Bieber, M., Dawson, G., and Sweeley, C. C. (1969), *J. Am. Chem. Soc. 91,* 1728.

Durette, P. L., and Horton, D. (1971), *Adv. Carbohydr. Chem. 26,* 49.

Erbing, C., Lindberg, B., and Svensson, S. (1973), *Acta Chem. Scand. 27,* 3699.

Foster, A. B., Harrison, R., Inch, T. D., Stacey, M., and Weber, J. M. (1963), *J. Chem. Soc.,* 2279.

Helting, T., and Lindahl, U. (1971), *J. Biol. Chem. 246,* 5442.

Helting, T., and Lindahl, U. (1972), *Acta Chem. Scand. 26,* 3515.

Höök, M., Lindahl, U., Bäckström, G., Malström, A., and Fransson, L.-A. (1974), *J. Biol. Chem. 249,* 3908.

Höök, M., Lindahl, U., Hallén, A., and Bäckström, G. (1975), *J. Biol. Chem. 250,* 6065.

Isbell, H. S., and Pigman, W. (1969), *Adv. Carbohydr. Chem. 24,* 14.

Kochetkov, N. K., and Chizhov, O. S. (1966), *Adv. Carbohydr. Chem. 21,* 39.

Lindahl, U., and Axelsson, O. (1971), *J. Biol. Chem. 246,* 74.

Lindahl, U., Bäckström, G., Jansson, L., and Hallén, A. (1973), *J. Biol. Chem. 248,* 7234.

Lloyd, P. F., and Forrester, P. F. (1971), *Carbohydr. Res. 19,* 430.

Ögren, S., and Lindahl, U. (1975), *J. Biol. Chem. 250,* 2690.

Perlin, A. S., Casu, B., Sanderson, G. R., and Johnson, L. F. (1970), *Can. J. Chem. 48,* 2260.

Perlin, A. S., Mackie, D. M., and Dietrich, C. P. (1971), *Carbohydr. Res. 18,* 185.

Pigman, W., and Isbell, H. S. (1968), *Adv. Carbohydr. Chem. 23,* 11.

Shively, J. E. (1975), Structural Analysis of Heparins and Heparan Sulfates, Ph.D. Dissertation, University of Illinois, Urbana, Ill.

Shively, J. E., and Conrad, H. E. (1976), *Biochemistry,* preceding paper in this issue.

Silbert, J. E. (1967), *J. Biol. Chem. 242,* 5153.

Taylor, R. L., and Conrad, H. E. (1972), *Biochemistry 11,* 1383.

Taylor, R. L., Shively, J. E., Conrad, H. E., and Cifonelli, J. A. (1973), *Biochemistry 12,* 3633.

Wolfrom, M. L., Wang, P. Y., and Honda, S. (1969), *Carbohydr. Res. 11,* 179.

Oligosaccharides of Human Milk: Isolation and Characterization of Two New Nonasaccharides, Monofucosyllacto-*N*-octaose and Monofucosyllacto-*N*-neooctaose†

Katsuko Yamashita, Yoko Tachibana, and Akira Kobata*

ABSTRACT: Two new nonasaccharides, fucosyllacto-*N*-octaose and fucosyllacto-*N*-neooctaose, were isolated from human milk. By sequential enzymic degradation and by quantitative methylation analysis, their structures were elucidated as shown in Figure 4 of this article.

A series of higher oligosaccharides derived from lacto-*N*-hexaose and lacto-*N*-neohexaose was found in human milk (Kobata and Ginsburg, 1972b). During the structural study of N-3, a mixture of difucosyllacto-*N*-hexaose and lacto-*N*-neohexaose, we found that this oligosaccharide fraction is contaminated with about 10% of a nonaose. Unlike other major components of N-3, this sugar liberates octaose instead of hexaose upon removal of fucose by mild acid hydrolysis. This paper describes the isolation and structural studies of the nonaose.

Materials and Methods

Enzyme. β-Galactosidase (Arakawa et al., 1974) and β-*N*-acetylhexosaminidase (Li and Li, 1972) were purified from jack bean meal by the method of cited references. α-L-Fucosidase from *Charonia lampas* (Nishigaki et al., 1974) was kindly provided by Dr. T. Okuyama, Seikagaku Kogyo Co. α-L-Fucosidase was also purified from *Bacillus fulminans* (Kochibe, 1973). This enzyme cleaves only Fucα1→2Gal linkage, in contrast to the *Charonia lampas* enzyme which cleaves all fucosyl linkages. The *Bacillus* strain was kindly

† From the Department of Biochemistry, Kobe University School of Medicine, Ikuta-ku, Kobe, Japan. *Received February 11, 1976.* This work has been supported by research grants from the Scientific Rese :ch Fund (1975-1976) of the Ministry of Education, Science and Culture, of Japan. This paper is part VIII of a series.

Chart I: Structures of Oligosaccharides.[a]

Lacto-*N*-tetraitol,	Galβ1→3GlcNAcβ1→3Galβ1→4sorbitol
Lacto-*N*-neotetraitol,	Galβ1→4GlcNAcβ1→3Galβ1→4sorbitol
Lacto-*N*-fucopentaitol II,	Galβ1→3GlcNAcβ1→3Galβ1→4sorbitol 4 ↑ Fucα1
Lacto-*N*-hexaitol,	Galβ1→4GlcNAcβ1 ↓ 6 Galβ1→3GlcNAcβ1→3Galβ1→4sorbitol
Lacto-*N*-neohexaitol,	Galβ1→4GlcNAcβ1 ↓ 6 Galβ1→4GlcNAcβ1→3Galβ1→4sorbitol
N-2-2_{OH},	Mixtures of monofucosyl lacto-*N*-hexaitols and monofucosyl lacto-*N*-neohexaitols

[a] All monosaccharides have a D configuration except for fucose which has an L configuration.

supplied by Dr. S. Yamamoto, National Institute of Police Science.

Standard Oligosaccharides from Human Milk and Partially O-Methylated Sugar Alcohol Acetates. Tritium-labeled monofucosylhexaitols mixture (*N*-2-2_{OH}),[1] lacto-*N*-hexaitol, lacto-*N*-neohexaitol, lacto-*N*-fucopentaitol-II, lacto-*N*-neotetraitol, and lacto-*N*-tetraitol were obtained by reduction of human milk oligosaccharides, isolated according to the method previously described (Kobata, 1972), with NaB^3H_4 (154 mCi/mmol, New England Nuclear Co. Boston, Mass.) as described previously (Takasaki and Kobata, 1974). Galβ1→4GlcNAcβ1→6Galβ1→4[^{3}H]sorbitol, GlcNAcβ1→6Galβ1→4[^{3}H]sorbitol, and GlcNAcβ1→3Galβ1→4[^{3}H]sorbitol were prepared from tritium-labeled lacto-*N*-hexaitol by partial acid hydrolysis (in 0.1 N HCl, 100 °C, 40 min) and isolated by paper chromatography with solvent II (Kobata and Ginsburg, 1972a). GlcNAcβ1→3(GlcNAcβ1→6)Galβ1→4[^{3}H]sorbitol was prepared by β-galactosidase digestion of tritium-labeled lacto-*N*-neohexaitol (Kobata and Ginsburg, 1972b).

Partially *O*-methylated hexoses were prepared from methyl α-galactopyranoside and methyl α-glucopyranoside by limited time methylation with methyl iodide using BaO-$Ba(OH)_2$ as catalyst (Kuhn and Egge, 1963) followed by acid hydrolysis in 1 N HCl, 100 °C for 3 h. Methylation was performed at 0 °C for 18 h and then at room temperature for 1 h. The hydrolysates were reduced with $NaBH_4$ and acetylated with a mixture of acetic anhydride and pyridine (1:1) at 100 °C for 1 h.

Partially O-methylated *N*-methyl aminosugars were prepared according to the method of Tai et al. (1975).

Paper Chromatography. Descending paper chromatography was performed with the following solvents: I, ethyl acetate–pyridine–acetic acid–water (5:5:1:3); II, ethyl acetate–pyridine–water (12:5:4). Sugars were located either with alkaline–silver nitrate or with periodate–benzidine reagent (Gordon et al., 1956). Radioactive sugars were located with Packard radiochromatogram scanner Model 7201.

Analysis of Carbohydrate Composition. Sugar composition was determined using the micromethods recently devised in our laboratory (Takasaki and Kobata, 1974); 10 μg of oligosaccharide sample was hydrolyzed with 0.01 (for fucose), 1 (for hexoses), and 2 N HCl (for hexosamine) at 100 °C for 2 h and reduced with NaB^3H_4 and followed by paper electrophoresis in 0.06 M borate buffer, pH 9.5. Radioactivity was determined with a Packard Tri-Carb liquid scintillation spectrometer using Bray's solution (Bray, 1960). Radioactivity on paper was determined after incubation of the paper pieces with 1 ml of water in the counting vials and addition of 7 ml of the scintillation fluid.

Methylation Analysis. Oligosaccharide alcohols were dissolved in 0.5 ml of dimethyl sulfoxide, and methylated with sodium methylsulfinyl carbanion and methyl iodide (Hakomori, 1964). The permethylated sugar alcohol was purified by silica gel column (Yamashita et al., 1976) and was hydrolyzed, reduced, and acetylated as described by Stellner et al. (1973). The acetylation time was extended to 4 h. The partially O-methylated sugar alcohol acetates were analyzed by gas chromatographer–mass spectrometer, Model JMS-D 100 (Japan Electron Optics Laboratory), using a glass column (2 mm × 1.5 m) of 2% OV-17 on Gas-chrom Q. The temperature was programmed from 150 to 240 °C at a rate of 2 °C per min.

Conditions for mass spectrometry were: ion source temperature, 210 °C; separator temperature, 280 °C; ionizing potential, 29 eV; ionization current, 300 μA; ion accelerating voltage, 1.9 kV; sensitivity, 1×10^{-6} A/V; and ion source pressure, 1×10^{-6} Torr.

Results

Isolation of Nonaose. Oligosaccharide fraction from human milk was obtained as previously reported (Kobata, 1972). The fraction obtained from 1 l. of milk was further fractionated into four groups (A, B, C, and lactose) by passage through a column of fine grade Sephadex G-25 (8 × 160 cm). Fraction A, which contains higher neutral oligosaccharides than heptasaccharide and most of sialyl oligosaccharides, was subjected to paper electrophoresis on Whatman No. 3MM in pyridine–acetate buffer, pH 5.4, for 2 h at 80 V per cm. Neutral oligosaccharide fraction, thus separated from sialyl oligosaccharides, was eluted from paper and mounted on Whatman No. 3MM papers as a band so that 2 mg was loaded in 1-cm width. The papers were subjected to paper chromatography with solvent I for 4 days to get N-3 fraction (Kobata and Ginsburg, 1972b). When N-3 fraction, thus obtained from a Le (a^+b^-), nonsecretor individual, was rechromatographed with solvent I for 9 days at 30 °C under the relative humidity of 70–80%, it was separated into three oligosaccharide components.[2] These three components were eluted separately from papers and rechromatographed again with solvent I (Figure 1) to make sure that each component was free from mutual contaminations. Components 2 and 3, which are called N-3-2_{OH} and N-3-1_{OH} in this paper, respectively, were both isomeric mixtures of difucosyl derivatives of lacto-*N*-hexaose and lacto-*N*-neohexaose as already reported (Kobata and Ginsburg, 1972b), and component 1 was the newly found nonaose. This new nonaose was found irrespective of the secretor status and Lewis blood types of the milk donor. The contents of this oligosaccharide in milk of Le(a^-b^-), secretor and Le(a^+b^-), nonsecretor individuals were 12.4 and 15.1 mg per l. of milk, respectively, which were approximately one-eighth of the difucosylhexaoses contents.

Monosaccharide Composition and Reducing Terminal.

[1] The structures of oligosaccharides mentioned in this paper are as shown in Chart I.

[2] This chromatographic condition was required for the successful separation of the three components.

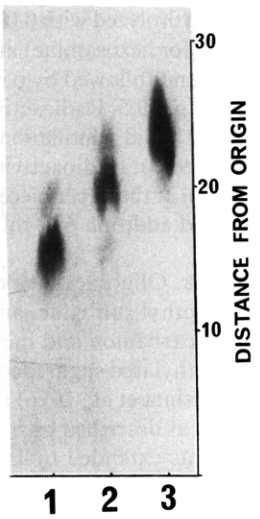


FIGURE 1: Paper chromatography of three components separated from N-3 fraction. The chromatogram was developed with solvent I for 9 days, and the sugars were visualized with alkaline-$AgNO_3$ reagents. Lane 1, component 1; lane 2, component 2; lane 3, component 3.

Complete acid hydrolysis of the new nonaose (in 1 N HCl, 100 °C for 2 h) followed by paper chromatography revealed the liberation of fucose, glucose, galactose, and glucosamine with alkaline–silver nitrate staining.

Monosaccharide ratio, as determined by Takasaki's method (Takasaki and Kobata, 1974), was 1 fucose, 4 galactoses, and 3 glucosamines for each glucose.

An aliquot (500 μl) of the nonaose was labeled with NaB^3H_4 according to the method previously reported (Takasaki and Kobata, 1974), and the radioactive product was subjected to paper chromatography on Whatman No. 1 paper for 9 days with solvent I. Radioautoscanning gave a single radioactive peak which moved slightly slower than the original nonaose. The radioactive component was eluted from paper to get tritium-labeled nonaitol (4.6×10^6 cpm).

An aliquot (2×10^4 cpm) of the radioactive nonaitol was hydrolyzed in 1 N HCl at 100 °C for 2 h. Analysis of the hydrolysate by paper electrophoresis in 0.06 M borate buffer, pH 9.5 (Takasaki and Kobata, 1974), showed sorbitol as a sole radioactive component. This result indicated that glucose is located at the reducing end of the nonaose as in the case of other known milk oligosaccharides.

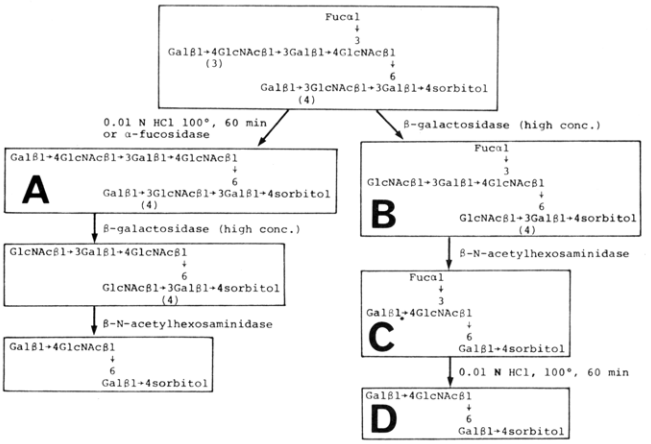
Partial Hydrolysis of the Tritium-Labeled Nonaitol. When the tritium-labeled nonaitol was partially hydrolyzed by heating in 0.1 N HCl at 100 °C for 120 min and chromatographed with solvent II, radioactive lactitol, GlcNAcβ1→6Galβ1→4sorbitol, and GlcNAcβ1→3Galβ1→4sorbitol were detected (Figure 2) on the chromatogram together with higher radioactive sugar alcohols.

Identity of the two radioactive triitol peaks as GlcNAcβ1→6Galβ1→4sorbitol and GlcNAcβ1→3Galβ1→4sorbitol was further confirmed by their complete conversion to lactitol by jack bean β-*N*-acetylhexosaminidase digestion. This evidence suggested that the nonaose has GlcNAcβ1→3(GlcNAcβ1→6) Galβ1→4Glc grouping at the reducing end as in the cases of lacto-*N*-hexaose and lacto-*N*-neohexaose derivatives.

Enzymic Degradation. Sequential enzymic degradation of nonaitol was performed according to the procedures shown in Scheme I.

Tritium-labeled nonaitol was heated in 0.01 N HCl at 100 °C for 1 h, and the reaction mixture was freed from HCl by

Scheme I: Sequential Degradation Scheme of the Nonaitol.



repeated evaporation with distilled water. When the reaction mixture was analyzed by paper chromatography with solvent I for 7 days, a single radioactive peak corresponding to an octaitol was detected. The same octaitol was obtained by *Charonia lampas* α-L-fucosidase (0.1 unit, 16 h) digestion of the nonaitol. However, α-L-fucosidases from *Bacillus fulminans* did not act on the nonaitol, indicating that no Fucα1→2Gal grouping is contained in the nonaitol. When the octaitol (A in Scheme I) was incubated with jack bean β-galactosidase (1.5 units/ml), it was converted to a hexaitol. Although the octaitol was resistant to β-*N*-acetylhexosaminidase action, this hexaitol was converted to a tetraitol with the same mobility as Galβ1→4GlcNAcβ1→6Galβ1→4sorbitol. Identity of the tetraitol as Galβ1→4GlcNAcβ1→6Galβ1→4sorbitol was further confirmed by sequential digestion with β-galactosidase and β-*N*-acetylhexosaminidase as already reported in the previous paper (Kobata and Ginsburg, 1972a). From the results of these sequential enzymic degradations, the monosaccharide sequence of the nonaose was estimated as Fuc·[2Gal·2GlcNAc·(Galβ1→4GlcNAcβ1→6Galβ1→4-Glc)].

In order to determine the location of fucosyl residue, the following sequential enzymic degradation was performed. The tritium-labeled nonaitol was incubated with jack bean β-galactosidase (1.5 units/ml) and the product was analyzed by paper chromatography. The nonaitol was completely converted to a heptaitol by this treatment. This heptaitol was completely degraded into a pentaitol with the same paper chromatographic mobility as lacto-*N*-fucopentaitol II by digestion with jack bean β-*N*-acetylhexosaminidase. By heating with 0.01 N HCl at 100 °C for 60 min, the radioactive pentaitol was converted to a tetraitol with the same chromatographic mobility as Galβ1→4GlcNAcβ1→6Galβ1→4sorbitol. The identity of the radioactive tetraitol as Galβ1→4GlcNAcβ1→6Galβ1→4sorbitol was also confirmed by sequential digestion with β-galactosidase and β-*N*-acetylhexosaminidase as described already.

These results indicated that the fucosyl residue is located on Galβ1→4GlcNAcβ1→6Galβ1→4Glc grouping of the nonaose.

The fucose-containing pentaitol (C in Scheme I) was resistant to even high concentration of jack bean β-galactosidase. Since fucose-free tetraitol (D in Scheme I) was easily converted to GlcNAcβ1→6Galβ1→4sorbitol by low concentration (0.03 unit/ml) of the β-galactosidase, the fucosyl residue is most possibly located on the Galβ1→4GlcNAc grouping of the tetrasaccharide protecting the nonreducing terminal galactose from the action of β-galactosidase (Arakawa et al., 1974).

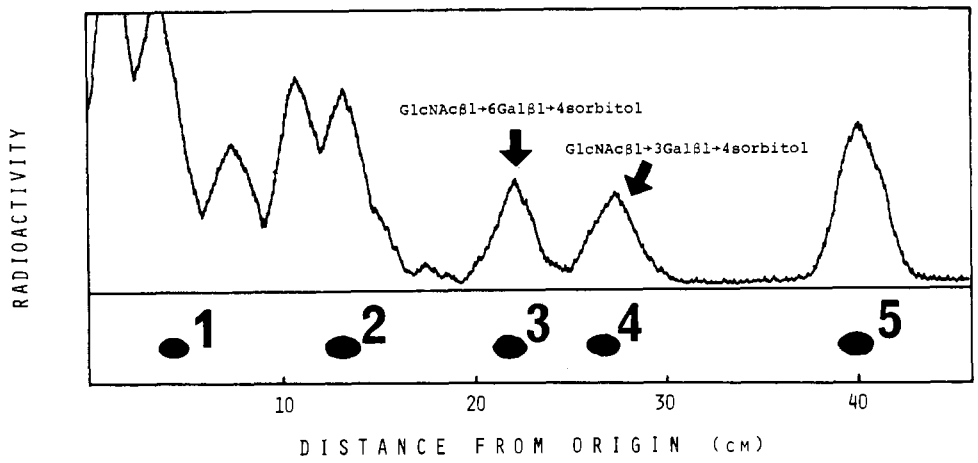


FIGURE 2: Products of partial acid hydrolysis of the tritium-labeled nonaitol (0.1 N HCl at 100 °C for 2 h). The product of hydrolysis was chromatographed with solvent II for 2 days. Standard sugars are as follows: 1, lacto-*N*-neohexaitol; 2, lacto-*N*-tetraitol; 3, GlcNAcβ1→6Galβ1→4sorbitol; 4, GlcNAcβ1→3Galβ1→4sorbitol; 5, lactitol.

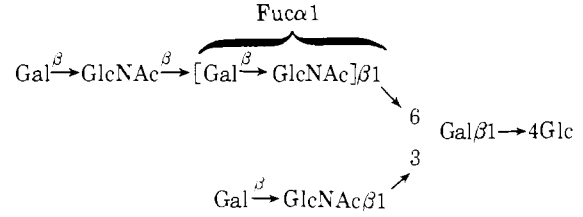
TABLE I: Molar Ratio of the Alditol Acetates Obtained from the Hydrolysates of Permethylated Nonaitol, Defucosylnonaitol (A), and β-Galactosidase-Digested Nonaitol (B) Shown in Scheme I.

Methylated Sugars	Molar Ratio: Nonaitol	Nonaitol (-1Fuc) (A)	Nonaitol (-2Gal) (B)
Sorbitol			
1,2,3,5,6-Penta-*O*-methyl-(4-mono-*O*-acetyl)	1.0	1.0	1.0
Fucitol			
2,3,4-Tri-*O*-methyl-(1,5-di-*O*-acetyl)	0.9		0.9
Galactitol			
2,3,4,6-Tetra-*O*-methyl-(1,5-di-*O*-acetyl)		2.0	2.0
2,4,6-Tri-*O*-methyl-(1,3,5-tri-*O*-acetyl)	1.1	1.0	0.9
2,4-Di-*O*-methyl-(1,3,5,6-tetra-*O*-acetyl)	0.8	0.9	0.8
2-*N*-Methylacetamido-2-deoxyglucitol			
3,4,6-Tri-*O*-methyl-(1,5-di-*O*-acetyl)			2.1
3,6-Di-*O*-methyl-(1,4,5-tri-*O*-acetyl)	0.8	2.1	
4,6-Di-*O*-methyl-(1,3,5-tri-*O*-acetyl)	1.1	1.0	
6-Mono-*O*-methyl-(1,3,4,5-tetra-*O*-acetyl)	1.0		1.0

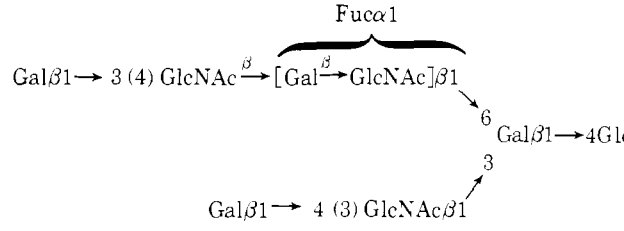
Summarizing the results of partial acid hydrolysis and sequential enzymic degradations, the monosaccharide sequence of the nonaose is now estimated as follows.

Methylation Analysis. In order to determine the position of each glycosidic linkage in the nonaose, methylation studies were performed with the nonaose and key oligosaccharide alcohols obtained by sequential glycosidase digestion of the nonaitol. The molar ratios of all partially O-methylated monosaccharides obtained from the hydrolysate of the permethylated nonaitol and two oligosaccharide alcohols (A and B in Scheme I) were summarized in Table I, making the value for 1,2,3,5,6-penta-*O*-methyl-4-*O*-acetylsorbitol 1.0. The data of nonaitol indicated that 1 mol of fucose and 2 mol of galactose of this sugar are located at the nonreducing terminal. These data are consistent with the structure presented in the foregoing section.

The positions of links of two galactose residues occupying nonreducing ends to *N*-acetylglucosamines were estimated as follows.



By comparing the data of nonaitol and heptaitol (B in Scheme I) obtained by β-galactosidase digestion of the nonaitol, it was realized that 2 mol of 2,3,4,6-tetra-*O*-methylgalactitol acetate and 0.8 mol of 3,6-di-*O*-methyl- and 1.1 mol of 4,6-di-*O*-methyl-2-*N*-methylacetamido-2-deoxyglucitol acetate disappeared in the latter. The disappearance of approximately 2 mol of glucosaminitol derivatives can be compensated by the appearance of 2.1 mol of 3,4,6-tri-*O*-methyl-2-*N*-methylacetamido-2-deoxyglucitol acetate in the heptaitol. This evidence indicated that the 2 mol of galactose forming the nonreducing ends of the nonaose are linked at C-3 and C-4 positions of 2 mol of *N*-acetylglucosamine residues as shown:



It was suggested that fucose is located at the 6-linked Galβ1→4GlcNAc grouping from enzymic degradation study. The position of the fucosyl residue was determined as follows by comparing the data of nonaitol and of octaitol (A in Scheme I) obtained by defucosidation of the nonaitol.

Together with 2,3,4-tri-*O*-methylfucitol acetate, 1 mol of 6-mono-*O*-methyl-2-N-methylacetamido-2-deoxyglucitol acetate disappeared in the octaitol. The disappearance of the glucosaminitol derivative was compensated by the increase of approximately 1 mol of 3,6-di-*O*-methyl-2-*N*-methylacetamido-2-deoxyglucitol acetate in the octaitol. These data in-

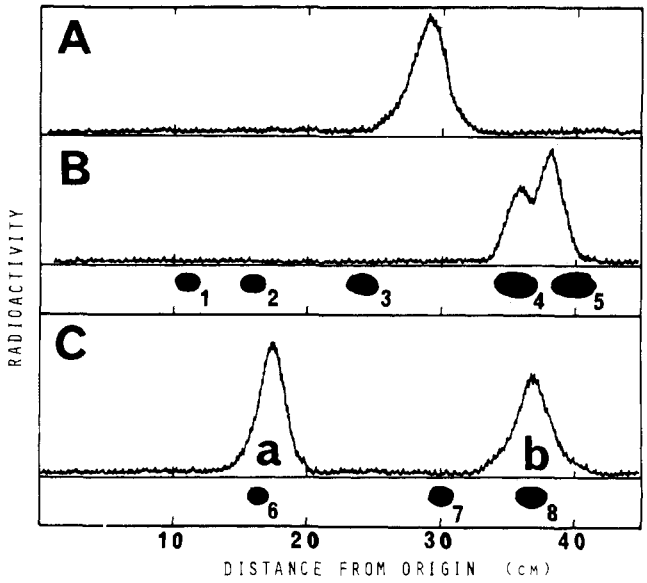


FIGURE 3: Action of jack bean glycosidases on [^{3}H]defucosylnonaitol. (A) Defucosylnonaitol treated with low concentration (0.03 unit/ml) of β-galactosidase; (B) the radioactive peak in A treated with β-*N*-acetylhexosaminidase; (C) products in B treated with high concentration (1.5 units/ml) of β-galactosidase. A and B were developed with solvent I for 7 days, and C, with solvent II for 9 days. Standard sugars: 1, nonaitol; 2, defucosylnonaitol; 3, N-2-2_{OH}; 4, lacto-*N*-hexaitol; 5, lacto-*N*-neohexaitol; 6, lacto-*N*-fucopentaitol II; 7, lacto-*N*-tetraitol; 8, GlcNAcβ1→3(GlcNAcβ1→6)Galβ1→4sorbitol.

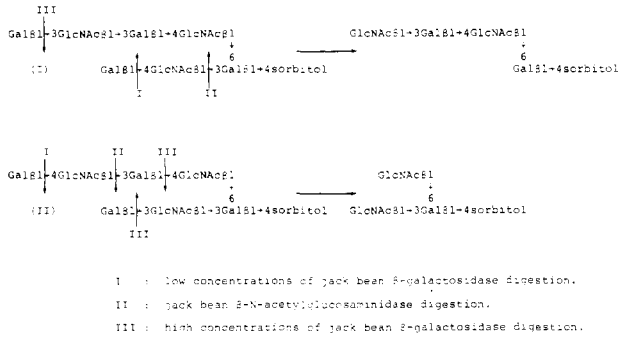
dicated that the fucose in the nonaose is located at the C-3 position of *N*-acetylglucosamine residue of 6-linked Galβ1→4GlcNAc grouping.

The data, so far obtained, indicated that the structure of the nonaose is either I or II as shown in Figure 4.

Controlled Sequential Degradation of Tritium-Labeled Octaitol. As already reported in the previous paper (Arakawa et al., 1974), low concentration (0.03 unit/ml) of jack bean β-galactosidase hydrolyzes Galβ1→4GlcNAc linkage but not Galβ1→3GlcNAc linkage. This character of the enzyme was used for the final determination of the nonaose structure.

As indicated in Scheme II, treatment of the octaitol first

Scheme II: Sequential Degradation Scheme of Octaitol (A).



with low concentration of jack bean β-galactosidase, followed by β-N-acetylhexosaminidase and final treatment with high concentration (1.5 units/ml) of jack bean β-galactosidase should produce pentaitol from I and tetraitol from II.

When tritium labeled octaitol was incubated with a low concentration of jack bean β-galactosidase, it was completely converted to a heptaitol (Figure 3A). This heptaitol was recovered from paper and incubated with jack bean β-*N*-acetylhexosaminidase. Analysis of the incubation mixture by paper chromatography showed that two types of hexaitols were

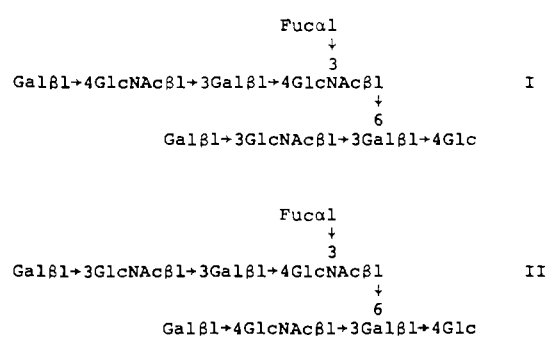


FIGURE 4: Proposed structures of two nonasaccharides.

produced by this digestion (Figure 3B). This hexaitol mixture was finally converted by high concentration of β-galactosidase to a mixture of a pentaitol and a tetraitol with the same chromatographic mobility as GlcNAcβ1→3(GlcNAcβ1→6)-Galβ1→4sorbitol (Figure 3C). These two oligosaccharides were recovered separately from paper by elution with water, and subjected further to sequential glycosidase digestion.

When the pentaitol (peak a in Figure 3C) was incubated with β-*N*-acetylhexosaminidase, it was completely converted to a tetraitol with the same chromatographic mobility as Galβ1→4GlcNAcβ1→6Galβ1→4sorbitol. The identity of the radioactive tetraitol as Galβ1→4GlcNAcβ1→6Gal-β1→4sorbitol was further confirmed by sequential digestion with β-galactosidase and β-*N*-acetylhexosaminidase as described already.

Another tetraitol (peak b in Figure 3C) was identified as GlcNAcβ1→3(GlcNAcβ1→6)Galβ1→4sorbitol by paper chromatography with solvent II. By incubation with β-*N*-acetylhexosaminidase, it was completely hydrolyzed to lactitol.

These experimental results indicated that the nonaose is a mixture of I and II shown in Figure 4. The ratio of I and II in the nonaose sample was estimated approximately as 1 to 1 from the radioactivity distribution in peaks a and b of Figure 3C.

Discussion

Human milk contains various kind of oligosaccharides. Structurally, these oligosaccharides can be classified into five groups. Namely, lactose, lacto-*N*-tetraose, lacto-*N*-neotetraose, lacto-*N*-hexaose, and lacto-*N*-neohexaose are the parent sugars of each group (Kobata, 1972).

The nonasaccharides reported in this paper are considered to be monofucosyl derivatives of two parent octasaccharides which do not fall into the classification described above.

According to the trivial names given by Kuhn and Egge (1963), these sugars will be called lacto-*N*-octaose and lacto-*N*-neooctaose. Therefore, nonasaccharides I and II in Figure 4 will be called fucosyllacto-*N*-octaose and fucosyllacto-*N*-neooctaose, respectively.

Structurally, lacto-*N*-octaose and lacto-*N*-neooctaose are related to lacto-*N*-hexaose and lacto-*N*-neohexaose. The reciprocal occurrence of two nonreducing terminal Galβ1→3GlcNAc and Galβ1→4GlcNAc groupings is interesting. We have looked over the fraction A (Kobata, 1972) of five different human milk samples for the occurrence of these two octasaccharides using defucosyl nonasaccharides as standards. So far, we have not succeeded in detecting the free form of lacto-*N*-octaose and lacto-*N*-neooctaose.

The absence of the octasaccharides is rather a curious phenomena since other parent oligosaccharides were always found to occur more or less in all milk samples.

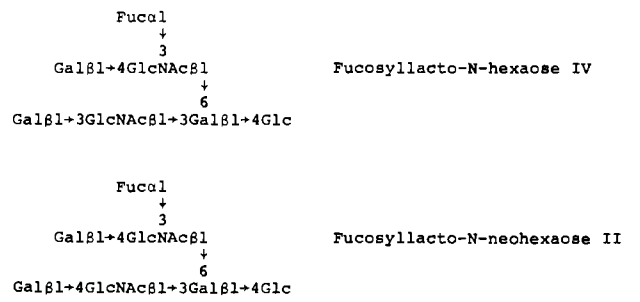


FIGURE 5: Structures of two monofucosyl hexaoses.[2]

Another curious phenomenon is that only single fucosyl linkage, Fucα1→3GlcNAc, is found in both fucosyllacto-*N*-octaose and fucosyllacto-*N*-neooctaose. In monofucosyl derivatives of lactose, tetraoses, and hexaoses, multiple fucosyl linkages are always found.

A structural study of N-2 fraction, a mixture of monofucosyllacto-*N*-hexaoses and monofucosyllacto-*N*-neohexaoses, has recently been performed, and the structures of six isomeric heptasaccharides were elucidated.[3] Among them, two oligosaccharides as shown in Figure 5 were found. Their structural relation to fucosyllacto-*N*-octaose and fucosyllacto-*N*-neooctaose is obvious. This evidence strongly suggests that fucosyllacto-*N*-hexaose IV and fucosyllacto-*N*-neohexaose II may work as precursors of fucosyllacto-*N*-octaose and fucosyllacto-*N*-neooctaose.

In preliminary experiments, we found di- and trifucosyl derivatives of lacto-*N*-octaose and lacto-*N*-neooctaose in human milk.

In the hope of verifying the biosynthetic mechanism proposed above, the structural studies of these sugars are now being performed in our laboratory.

[3] Yamashita, K., Tachibana, Y., and Kobata, A., manuscript in preparation.

Acknowledgments

We thank Miss M. Inohara for her skillful secretarial assistance.

References

Arakawa, A., Ogata, S., Muramatsu, T., and Kobata, A. (1974), *J. Biochem. (Tokyo) 75,* 707–714.

Bray, G. (1960), *Anal. Biochem. 1,* 279–285.

Gordon, H. T., Thornburg, W., and Werum, L. N. (1956), *Anal. Chem. 28,* 849.

Hakomori, S. (1964), *J. Biochem. (Tokyo) 55,* 205–208.

Kobata, A. (1972), *Methods Enzymol. 28,* 262–271.

Kobata, A., and Ginsburg, V. (1972a), *J. Biol. Chem. 247,* 1525–1529.

Kobata, A., and Ginsburg, V. (1972b), *Arch. Biochem. Biophys. 150,* 273–281.

Kochibe, N. (1973), *J. Biochem. (Tokyo) 74,* 1141–1149.

Kuhn, R., and Egge, H. (1963), *Chem. Ber. 96,* 3338–3348.

Li, Y.-T., and Li, S.-C. (1972), *Methods Enzymol. 28,* 702–713.

Nishigaki, M., Muramatsu, T., Kobata, A., and Maeyama, K. (1974), *J. Biochem. (Tokyo) 75,* 509–517.

Stellner, K., Saito, H., and Hakomori, S. (1973), *Arch. Biochem. Biophys. 155,* 464–472.

Tai, T., Yamashita, K., and Kobata, A. (1975), *J. Biochem. (Tokyo) 78,* 679–686.

Takasaki, S., and Kobata, A. (1974), *J. Biochem. (Tokyo) 76,* 783–789.

Yamashita, K., Tachibana, Y., and Kobata, A. (1976), *Arch. Biochem. Biophys. 174,* 582–591.