THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 260, No. 2, Issue of January 25, pp. 714-719, 1985
Printed in U.S.A.

Branch Specificity of Bovine Colostrum CMP-Sialic Acid: *N*-Acetyllactosaminide α2→6-Sialyltransferase

INTERACTION WITH BIANTENNARY OLIGOSACCHARIDES AND GLYCOPEPTIDES OF *N*-GLYCOSYLPROTEINS*

(Received for publication, June 11, 1984)

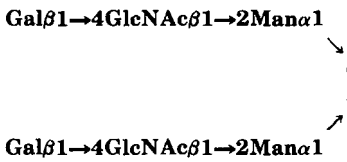
David H. Joziasse, Wietske E. C. M. Schiphorst, and Dirk H. van den Eijnden‡

From the Department of Medical Chemistry, Vrije Universiteit, Van der Boechorststraat 7, NL-1007 MC Amsterdam, The Netherlands

J. Albert van Kuik, Herman van Halbeek, and Johannes F. G. Vliegenthart

From the Department of Bio-Organic Chemistry, University of Utrecht, Croesestraat 79, NL-3522 AD Utrecht, The Netherlands

By use of 500-MHz ^{1}H NMR spectroscopy, the branch specificity of bovine colostrum CMP-NeuAc:Galβ1→4GlcNAc-R α2→6-sialyltransferase towards a biantennary glycopeptide and oligosaccharides of the *N*-acetyllactosamine type, differing in completeness and structure of their core portion, was investigated. In agreement with earlier reports (Van den Eijnden, D. H., Joziasse, D. H., Dorland, L., Van Halbeek H., Vliegenthart, J. F. G., and Schmid, K. (1980) *Biochem. Biophys. Res. Commun.* 92, 839–845), it appears that the enzyme strongly prefers the galactosyl residue at the Manα1→3Man branch of the biantennary glycopeptide for attachment of the first sialic acid residue. This branch specificity is fully preserved with the structure



Reduction of the reducing *N*-acetylglucosaminyl residue in this structure, however, leads to a decreased branch specificity, whereas removal of this residue results in a random attachment of sialic acid to the galactoses at both branches. The decrease in branch specificity is accompanied by a reduction in the rate of sialic acid transfer to the galactose at the α1→3 branch. Our results indicate that the presence of the aforementioned *N*-acetylglucosaminyl residue is a minimal structural requirement for branch specificity of the sialyltransferase. We propose that in the interaction of the sialyltransferase with its substrates, this *N*-acetylglucosaminyl residue functions as a recognition site mediating the correct positioning of the substrate on the enzyme.

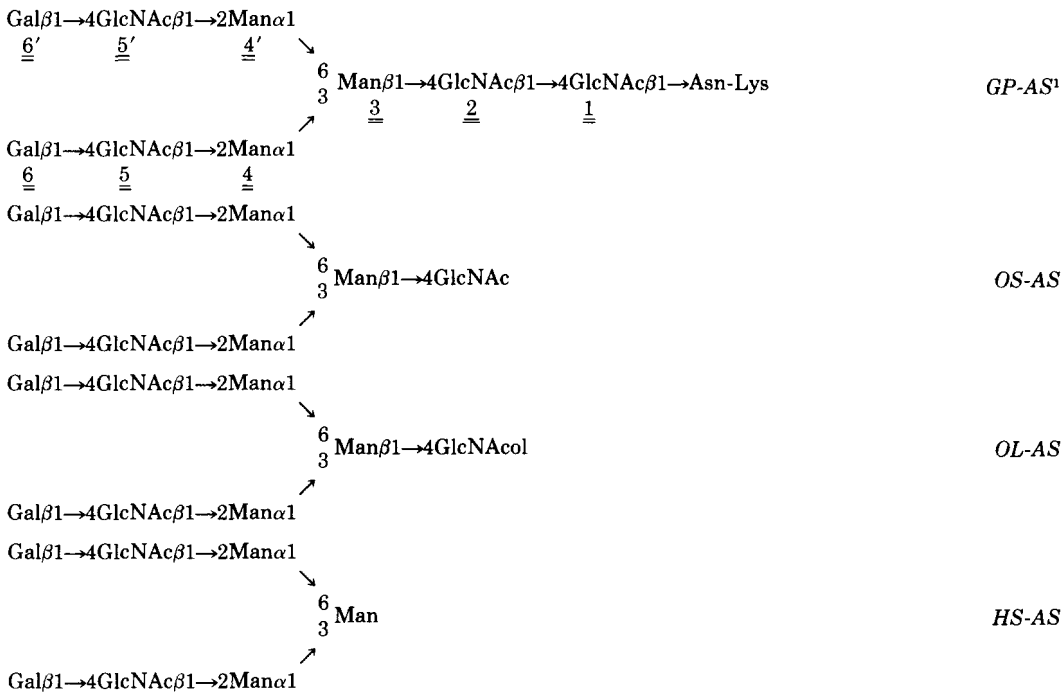
Glycosyltransferases are known to be highly specific for their acceptor substrate (1). Not only the acceptor sugar residue is recognized, but also the enzymes often interact with other parts of the glycan substrate, remote from the point of action of the enzyme (2–4). Apparently, particular features of the spatial structure of the glycan play an important role in the recognition of the acceptor by glycosyltransferases.

The first proposals for the conformation of biantennary glycans of *N*-glycosylproteins were based on simple molecular model building (5). More advanced studies involving x-ray diffraction analysis (6) suggested that the α1→3 branch of biantennary glycans has a fixed orientation in space with respect to the Manβ1→4GlcNAcβ1→4GlcNAc portion of the core. These results were confirmed in studies in which potential energy hard-sphere *exo*-anomeric calculations (7), and NMR measurements (7–9) were employed. The α1→6 branch, however, possesses a greater flexibility with respect to the rigid core. This flexibility is due to the additional freedom of rotation around the C5-C6 linkage of the β-mannose and allows an interconversion between two different energetically favorable orientations. The difference in energy between these forms as determined from HSEA calculations (10) indicates that the conformation in which the α1→6 branch folds back onto the di-*N*-acetylchitobiose unit of the core is favored (7, 10, 11).

Previously, we have shown that the β-galactoside α2→6-sialyltransferase from bovine colostrum is able to distinguish between the different branches of bi- and triantennary glycopeptides of the *N*-acetyllactosamine type (2). Sialylation *in vitro* of a biantennary glycopeptide yielded a monosialo derivative which for over 95% consisted of the isomer with sialic acid α2→6-linked to galactose on the Manα1→3 branch. In view of the potential interactions of the branches of the biantennary acceptor, or of the colostrum enzyme itself, with the Manβ1→4GlcNAcβ1→4GlcNAc core, we extended our studies on the specificity of the sialyltransferase to the following biantennary structures.

* This work was supported in part by the Netherlands Foundation for Chemical Research (SON-ZWO) and by Grant UUKC 83-13 from the Netherlands Foundation for Cancer Research (KWF). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed.



As in our previous study (2), high-resolution ^{1}H NMR was the method of choice for the characterization of the products formed by sialylation of the various biantennary substrates. This technique, especially at 500 MHz, enables us to determine in a rapid and nondestructive way the acceptor sugar and the linkage by which sialic acid is introduced as well as the branch in which the latter is located. Even when a mixture of two monosialylated biantennary species is produced, the ^{1}H NMR spectrum reveals the structures of the compounds in addition to the ratio in which they are present (11, 12).

MATERIALS AND METHODS AND RESULTS[2]

DISCUSSION

Many naturally occurring glycoproteins and oligosaccharides are known, which show specific sialylation patterns at the different branches of their carbohydrate moieties (19–27). It appears that when on biantennary glycans both α2→6 and α2→3-linked sialic acid residues are present, the Galβ1→4GlcNAcβ1→2Manα1→3Man branch almost invariably carries its sialic acid residue in α2→6 linkage, whereas the α2→3-linked residue occurs at the Galβ1→4GlcNAcβ1→2Manα1→6Man branch. This general sialylation pattern seems to indicate that sialyltransferases from different tissues are capable of distinguishing between the different branches of glycans of the *N*-acetyllactosamine type.

In our previous study, we have demonstrated that the *N*-acetyllactosaminide α2→6-sialyltransferase from bovine colostrum indeed preferentially attaches sialic acid to galactose 6 (at the 1→3 branch) in a biantennary glycopeptide (2). This result could be confirmed in the present study by use of 500-MHz ^{1}H NMR. The extension of our investigations on the branch specificity of the sialyltransferase towards biantennary structures differing in size and nature of the oligosaccharide core, however, indicates that there is a minimal structural requirement for this specificity (Table II). It appears that it is fully preserved with structures ending in *N*-acetylglucosamine 2. Thus, the presence of *N*-acetylglucosamine 1 as well as asparagine and other amino acids does not seem to be essential. Reduction of *N*-acetylglucosamine 2 to *N*-acetylglucosaminitol or removal of this sugar residue, however, dramatically affects the enzyme specificity (Table II). This shows that the presence of *N*-acetylglucosamine 2 is a key factor in the feature of branch specificity of α2→6-sialyltransferase towards biantennary glycan structures.

For the branch specificity of the sialyltransferase, at least two factors seem to be of importance. First, it is possible that the specificity is a result of a difference in accessibility of the two substrate sites (galactose 6 and 6′). It is conceivable that the access of the sialyltransferase to galactose 6′ (at the α1→6 branch) is somehow restricted because of steric hindrance by *N*-acetylglucosamine 2. Modification or removal of the latter residue would result in an increased exposure of galactose 6′ and, as a consequence, in a more random attachment of sialic acid residues to the branches, which in fact is observed with the acceptor substrates *OL-AS* and *HS-AS* (Table II). Biantennary glycans have been proposed to occur in two rotameric forms in a ratio 1:1, in one of which the α1→6 branch is folded back on the core and galactose 6′ indeed is brought in the close proximity of *N*-acetylglucosamine 2 (4, 10). In the other form, however, the α1→6 branch and consequently galactose 6′ are freely exposed. Although our NMR data indicate that the rotameric ratio is rather in

[1] The abbreviations used are: *GP-AS*, biantennary glycopeptide (peptide moiety Asn-Lys); HPLC, high-pressure liquid chromatography; ^{1}H NMR, proton nuclear magnetic resonance; *GP-MS*, monosialylated glycopeptide; *GP-BS*, bisialylated glycopeptide; *OS-AS*, biantennary octasaccharide (ending in GlcNAc-2); *OS-MS*, monosialylated octasaccharide; *OS-BS*, bisialylated octasaccharide; *OL-AS*, reduced biantennary octasaccharide (ending in GlcNAc-2-ol); *OL-MS*, monosialylated octasaccharide-alditol; *OL-BS*, bisialylated octasaccharide-alditol; *HS-AS*, biantennary heptasaccharide; *HS-MS*, monosialylated heptasaccharide; *HS-BS*, bisialylated heptasaccharide; NOE, nuclear Overhauser enhancement; HSEA hard-sphere *exo*-anomeric; BSA, bovine serum albumin.

[2] Portions of this paper (including "Materials and Methods," "Results," Tables I–III, and Figs. 1–5) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-1769, cite the authors, and include a check or money order for $6.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

favor of the backfolded conformation, which is in good agreement with potential energy calculations (10, 28), it seems that a significant part of the galactose 6′ residues occurs in the exposed conformation. It is therefore hard to understand that the extreme branch specificities as observed with *GP-AS* and *OS-AS* merely are the result of a limited accessibility of galactose 6′. Moreover, it is not unlikely that galactose 6 will be at least as accessible in *HS-AS* as in *GP-AS* and *OS-AS*. Nevertheless, the rate of sialic acid transfer to this galactose residue was much lower in the heptasaccharide than in the larger acceptor substrates (Table III). Thus, the degree of exposure of Gal-6 and Gal-6′ does not seem to be the essential factor in the reaction with the α2→6-sialyltransferase. An explanation of branch preference from different accessibilities of the substrate sites only therefore seems less plausible to us.

Perhaps a more important factor may be the interaction of the sialyltransferase with *N*-acetylglucosamine 2. It is tempting to presume that this interaction is essential for a correct positioning of the enzyme on the substrate: *N*-acetylglucosamine 2 might serve as a recognition site for the sialyltransferase bringing the catalytic center of the enzyme close to galactose 6 but not to galactose 6′. Change or removal of this recognition site then results in a decreased rate of sialic acid transfer to the 1→3 branch and a more random attachment of sialic acid to both branches (Table II).

Different rates of sialic acid incorporation into *N*-acetyllactosaminide-type acceptors differing in structure beyond the Galβ1→4GlcNAc unit have also been reported in a recent study on α2→6-sialyltransferase of embryonic chicken liver (29). It was suggested that the core structure of the acceptors is not recognized by the sialyltransferase but serves as a support for the display of the *N*-acetyllactosaminyl units in an appropriate orientation to the enzyme. More than one Galβ1→4GlcNAc-R terminus of an acceptor molecule would be bound by the sialyltransferase. It might be that α2→6-sialyltransferases indeed recognize two or more branches at the same time, using one of the branches as a point of orientation. Our results, however, suggest that bovine colostrum sialyltransferase in addition recognizes *N*-acetylglucosamine residue 2.

Analysis of the kinetics of sialic acid transfer to the biantennary oligosaccharides *OS-AS* (transfer to the 1→3 branch) and *OS-MS* (transfer to the 1→6 branch) indicated that galactose 6 is a ten times more efficient acceptor site than galactose 6′. It should be noted that the entry of sialic acid into the 1→6 branch could only be detected with the substrate *OS-MS*, which already possesses a sialic acid residue at the 1→3 branch. Therefore, it is possible that the presence of sialic acid at the 1→3 branch has a stimulatory effect on the sialic acid incorporation at the 1→6 branch.

The phenomenon of branch specificity is not restricted to sialyltransferases. Other studies have shown that *N*-acetylglucosaminide β1→4-galactosyltransferases (30–32), *N*-acetyllactosaminide β1→3-*N*-acetylglucosaminyltransferase (33), and *N*-acetyllactosaminide α1→3-galactosyltransferase[3] show a preferred site of sugar attachment with branched acceptor structures. Elucidation of the branch specificities of these enzymes and of the α2→6-sialyltransferases may help to explain the observed patterns of glycosylation in glycans of the *N*-acetyllactosamine type. Correlations between branch specificities of glycosyltransferases and oligosaccharide conformations will soon become possible in view of the rapid progress in studies on the spatial structures of *N*-linked glycans.

[3] W. M. Blanken, personal communication.

REFERENCES

1. Schachter, H., and Roseman, S. (1980) in *The Biochemistry of Glycoproteins and Proteoglycans* (Lennarz, W. J., ed), pp. 85–160, Plenum Press, New York
2. Van den Eijnden, D. H., Joziasse, D. H., Dorland, L., Van Halbeek, H., Vliegenthart, J. F. G., and Schmid, K. (1980) *Biochem. Biophys. Res. Commun.* **92,** 839–845
3. Schachter, H., Narasimhan, S., Gleeson, P., and Vella, G. (1983) *Can. J. Biochem. Cell Biol.* **61,** 1049–1066
4. Brisson, J.-R., and Carver, J. P. (1983) *Can. J. Biochem. Cell Biol.* **61,** 1067–1078
5. Montreuil, J. (1975) *Pure Appl. Chem.* **42,** 431–477
6. Warin, V., Baert, F., Fouret, R., Strecker, G., Spik, G., Fournet, B., and Montreuil, J. (1979) *Carbohydr. Res.* **76,** 11–22
7. Bock, K., Arnarp, J., and Lönngren, J. (1982) *Eur. J. Biochem.* **129,** 171–178
8. Brisson, J.-R., and Carver, J. P. (1983) *Biochemistry* **22,** 3671–3680
9. Homans, S. W., Dwek, R. A., Fernandes, D. L., and Rademacher, T. W. (1983) *FEBS Lett.* **164,** 231–235
10. Brisson, J.-R., and Carver, J. P. (1983) *Biochemistry* **22,** 3680–3686
11. Vliegenthart, J. F. G., Dorland, L., and Van Halbeek, H. (1983) *Adv. Carbohydr. Chem. Biochem.* **41,** 209–374
12. Vliegenthart, J. F. G., Dorland, L., Van Halbeek, H., and Haverkamp, J. (1982) *Cell Biol. Monogr.* **10,** 127–172
13. Fournet, B., Montreuil, J., Strecker, G., Dorland, L., Haverkamp, J., Vliegenthart, J. F. G., Binette, J. P., and Schmid, K. (1978) *Biochemistry* **17,** 5206–5214
14. Van den Eijnden, D. H., and Van Dijk, W. (1972) *Hoppe Seyler's Z. Physiol. Chem.* **353,** 1817–1820
15. Paulson, J. C., Beranek, W. E., and Hill, R. L. (1977) *J. Biol. Chem.* **252,** 2356–2362
16. Burke, D., and Keegstra, K. (1979) *J. Virol.* **29,** 546–554
17. Joziasse, D. H., Blanken, W. M., Koppen, P. L., and Van den Eijnden, D. H. (1983) *Carbohydr. Res.* **119,** 303–309
18. Vliegenthart, J. F. G., Van Halbeek, H., and Dorland, L. (1981) *Pure Appl. Chem.* **53,** 45–77
19. Schut, B. L., Dorland, L., Haverkamp, J., Vliegenthart, J. F. G., and Fournet, B. (1978) *Biochem. Biophys. Res. Commun.* **82,** 1223–1228
20. Zinn, A. B., Marshall, J. S., and Carlson, D. M. (1978) *J. Biol. Chem.* **253,** 6768–6773
21. Dorland, L., Haverkamp, J., Vliegenthart, J. F. G., Strecker, G., Michalski, J. C., Fournet, B., Spik, G., and Montreuil, J. (1978) *Eur. J. Biochem.* **87,** 323–329
22. Nilsson, B., Nordén, N. E., and Svensson, S. (1979) *J. Biol. Chem.* **254,** 4545–4553
23. Yamamoto, K., Tsuji, T., Irimura, T., and Osawa, T. (1981) *Biochem. J.* **195,** 701–713
24. Yamashita, K., Liang, C.-J., Funakoshi, S., and Kobata, A. (1981) *J. Biol. Chem.* **256,** 1283–1289
25. Paulson, J. C., Weinstein, J., Dorland, L., van Halbeek, H., and Vliegenthart, J. F. G. (1982) *J. Biol. Chem.* **257,** 12734–12738
26. Mega, T., Lujan, E., and Yoshida, A. (1980) *J. Biol. Chem.* **255,** 4057–4061
27. Lowe, M., and Nilsson, B. (1983) *J. Biol. Chem.* **258,** 1885–1887
28. Meyer, B. (1983) *Proceedings of 7th International Symposium on Glycoconjugates* (Chester, M. A., Heinegard, D., Lundblad, A., and Svensson, S., eds) p. 126, Rahms i Lund
29. Bendiak, B., and Cook, G. M. W. (1983) *Biochem. J.* **213,** 253–260
30. Pâquet, M. R., Narasimhan, S., Schachter, H., and Moscarello, M. A. (1983) *Fed. Proc.* **42,** 2199 (Abstr. 2581)
31. Blanken, W. M., Hooghwinkel, G. J. M., and Van den Eijnden, D. H. (1982) *Eur. J. Biochem.* **127,** 547–552
32. Blanken, W. M., Hooghwinkel, G. J. M., and Van den Eijnden, D. H. (1983) *Proceedings of 7th International Symposium on Glycoconjugates,* (Chester, M. A., Heinegard, D., Lundblad, A., and Svensson, S., eds) pp. 757–758, Rahms i Lund
33. Van den Eijnden, D. H., and Schiphorst, W. E. C. M. (1983) *Proceedings of 7th International Symposium on Glycoconjugates,* (Chester, M. A., Heinegard, D., Lundblad, A., and Svensson, S., eds) pp. 766–767, Rahms i Lund

Supplementary Material to

BRANCH SPECIFICITY OF BOVINE COLOSTRUM CMP-SIALIC ACID:N-ACETYLLACTOSAMINIDE α2→6-SIALYLTRANSFERASE

by

David H. Joziasse, Wietske E.C.M. Schiphorst, Dirk H. van den Eijnden, J. Albert van Kuik, Herman van Halbeek and Johannes F.G. Vliegenthart

MATERIALS AND METHODS

Biantennary asialo-glycopeptide *GP-AS* (glycopeptide GPII-6 in reference 13) derived from asialo-α_1-acid glycoprotein was a gift from Dr. K. Schmid (Boston University, Boston, MA.). The biantennary octasaccharide Galβ1→4GlcNAcβ1→2-Manα1→3[Galβ1→4GlcNAcβ1→2Manα1→6]Manβ1→4GlcNAc (*OS-AS*) was kindly donated by Dr. G. Strecker (Université de Lille, Villeneuve d'Ascq, France). The reduced octasaccharide Galβ1→4GlcNAcβ1→2Manα1→3[Galβ1→4GlcNAcβ1→2Manα1→6]Manβ1→4-GlcNAcol (*OL-AS*) was derived from the parent oligosaccharide by reduction with $NaBH_4$. Heptasaccharide Galβ1→4GlcNAcβ1→2Manα1→3[Galβ1→4GlcNAcβ1→2Manα1→6]Man (*HS-AS*), was a gift from Dr. J. Lönngren (University of Stockholm, Stockholm, Sweden). CMP-[^{14}C]NeuAc (1.68 Ci/mol) and CMP-[^{3}H]NeuAc (18,900 Ci/mol) were purchased from New England Nuclear, (Boston, MA.), and diluted with unlabeled CMP-NeuAc (14) to the desired specific activity. β-Galactoside α2→6-sialyl-transferase was purified from bovine colostrum (15) and enzyme activities were assayed with asialo-α_1-acid glycoprotein as acceptor, as previously described (2).

Preparation of sialylated products for ^{1}H-NMR spectroscopy - The glycopeptide and the oligosaccharides were incubated in reaction mixtures of 500 µL containing: 500 nmol oligosaccharide or glycopeptide, 1.26 µmol CMP-[^{3}H]NeuAc (0.312 Ci/mol), 100 µmol Tris-maleate pH 6.7, 500 µg bovine serum albumin (BSA), 100 µL glycerol and 7.6 mU sialyltransferase. The mixtures were incubated at 37°C for periods chosen as to obtain approximately equal yields of mono- and bisialylated product. After incubation the mixtures were subjected to chromatography on a column (1.6 x 200 cm) of Bio-Gel P-6 equilibrated and eluted with 50 mM ammonium acetate at pH 5.2 and 37°C, in order to separate the asialo-, monosialo- and bisialo-compounds (16). Fractions containing the monosialylated product and those containing the bisialylated derivatives were pooled and lyophilized.

^{1}H-NMR spectroscopy - Prior to ^{1}H-NMR spectroscopic analysis, the biantennary substrates and their products of sialylation (100 to 200 nmoles) were repeatedly treated with D_2O at pD 7 and room temperature. After each exchange treatment the materials were lyophilized. Finally, each sample was redissolved in 400 µL D_2O (99.996 atom% D, Aldrich, Milwaukee, WI). ^{1}H-NMR spectroscopy was performed at 500 MHz on a Bruker WM-500 spectrometer (SON hf-NMR facility, Department of Biophysical Chemistry, Nijmegen University, The Netherlands) operating in the Fourier transform mode under control of an Aspect-2000 computer. Experimental details have been described (11,12). Resolution enhancement of the spectra was achieved by Lorentzian-to-Gaussian transformation. The probe temperature was kept at 27.0 (±0.1)°C. Chemical shifts are expressed in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), but were actually measured by reference to internal acetone (δ2.225 in D_2O at 27°C), with an accuracy of 0.002 ppm.

Preparation of mono-[^{14}C]sialylated biantennary octasaccharide - Mono-[^{14}C]sialylated biantennary octasaccharide (*OS-MS*) was prepared in a reaction mixture which contained in a total volume of 2.0 mL: 4.1 µmol octasaccharide (*OS-AS*), 6.50 µmol CMP-[^{14}C]NeuAc, 1.0 mg BSA, 250 µL glycerol, 400 µmol Tris-maleate pH 6.7 and 22 mU sialyltransferase. The mixture was incubated at 37°C for 200 min, the reaction was stopped on ice and the sialylated products were isolated by gel filtration on Bio-Gel P-6 as described above. The yield was 2.23 µmol monosialo product and 0.35 µmol bisialo product.

K_m and V for each of the branches of the biantennary octasaccharide - K_m and V values for the asialo- and the mono-[^{14}C]sialo octasaccharides, *OS-AS* and *OS-MS*, respectively, were determined at a concentration of 2.0 mM CMP-[^{3}H]NeuAc. Reacti. mixtures contained, in a total volume of 40 µL: 80 nmol CMP-[^{3}H]NeuAc (1.80 Ci/mol), 20 µg BSA, 5 µL glycerol, 4 µmol Tris-maleate pH 6.7, 0.14 mU sialyltransferase and *OS-AS* varying from 4 to 160 nmol or *OS-MS* varying from 4 to 400 nmol. The mixtures were incubated for 60 min at 37°C, after which the reactions were stopped on ice. From the incubation mixtures aliquots were prepared for high-pressure liquid chromatography as described previously (17). Sialic acid, mono- and bisialo-oligosaccharide were separated on a Lichrosorb-NH_2 (5 µm, Merck) column by elution with a linear gradient of increasing buffer content at a rate of 0.5%/min, starting with a mixture of acetonitrile and 0.15 M potassium phosphate pH 5.2 70:30 (v/v). A Perkin-Elmer series 2 liquid chromatograph was used equipped with a Rheodyne injection valve and a Perkin-Elmer LC-75 variable wavelength detector operating at 195 nm. Chromatograms were recorded with a Spectra-Physics SP-4100 computing integrator. Radioactive compounds were quantified by liquid-scintillation counting of the fractionated eluate. The reaction rate was estimated for each acceptor substrate concentration, and K_m and V values were calculated from plots of s/v against s. At each concentration of asialo-acceptor the amount of bisialo-product formed was less than 5% of the amount of monosialo-product.

RESULTS

A partially purified preparation of CMP-NeuAc:*N*-acetyllactosaminide α2→6-sialyltransferase was used to investigate the preferred order of incorporation of sialic acid into the different branches of the acceptor structures *GP-AS*, *OS-AS*, *OL-AS* and *HS-AS*.The kinetics of sialic acid incorporation were very similar for the four substrates, except for differences in rate. As the incorporation of the first sialic acid residue into *OS-AS* was much faster than that of the second it was easy to accomplish the formation in a high yield of monosialylated product for use in the kinetic studies. Isolation of mono- and bisialylated products was achieved by gel filtration on Bio-Gel P-6, by which it is possible to separate them from each other and from the enzyme, the acceptor substrate and CMP-[^{14}C]NeuAc. A typical separation is shown in Fig. 1 for the case in which the reducing octasaccharide (*OS-AS*) was used as an acceptor substrate for the sialyltransferase.

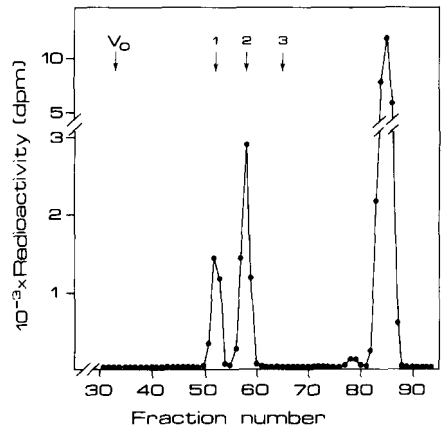


Fig. 1 - Gel filtration on Bio-Gel P-6 of the incubation mixture of the biantennary oligosaccharide *OS-AS*. Octasaccharide *OS-AS* was incubated with CMP-[^{14}C]NeuAc and bovine colostrum α2→6-sialyltransferase as described under "Methods". After 3 h at 37°C the mixture was applied to a column (1.6 x 200 cm) of Bio-Gel P-6 (200-400 mesh), equilibrated and eluted at a flow of 12 mL/h with 50 mM ammonium acetate at pH 5.2 and 37°C. Fractions of 4 mL were collected and monitored for ^{14}C radioactivity (●). Arrows indicate: 1, oligosaccharide *OS-BS*; 2, *OS-MS*; 3, *OS-AS* (detected by the phenol-sulfuric acid assay).

Structure determination of the products of sialylation - The primary structures of the mono- and bisialylated derivatives of the biantennary compounds were elucidated by 500-MHz ^{1}H-NMR spectroscopy. To illustrate how an introduced sialic acid residue manifests itself in a ^{1}H-NMR spectrum, the spectra of the reduced octasaccharide substrate (*OL-AS*) and its bisialylated derivative (*OL-BS*) are compared in Fig. 2. The chemical shifts of the structural-reporter groups of the substrates and the products are listed in Table I. The most informative regions of the ^{1}H-NMR spectra, namely, those containing the *N*-acetyl methyl resonances (2.00 < δ < 2.15), are compared in Fig. 3 for all acceptor substrates and mono- and bisialylated products.

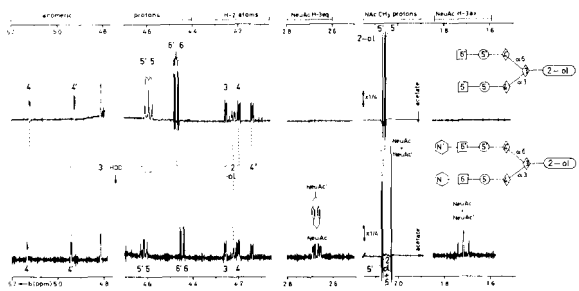


Fig. 2 - Structural-reporter-group regions of the 500-MHz ^{1}H-NMR spectra of the oligosaccharides *OL-AS* and *OL-BS*. Comparison of the structural-reporter-group regions of the 500-MHz ^{1}H-NMR spectra (D_2O; pD 7; 27°C) of the reduced octasaccharide substrate (*OL-AS*) and its bisialylated derivative (*OL-BS*), illustrating the influences of sialylation of both branches on the positions of the resonances of neighbouring protons. The relative intensity scale of the *N*-acetyl proton regions differs from that of the other parts of the spectra as indicated. The bold numbers in the spectra refer to the corresponding residues in the structures.

The ^{1}H-NMR features of the glycopeptide substrate (*GP-AS*) have been discussed extensively (11). The spectrum of the monosialylated product (*GP-MS*) showed an intensity ratio of NeuAc reporter-group signals to those of other residues of 1:1, consistent with the fact that one NeuAc residue had been introduced. This NeuAc residue is present in α2→6-linkage to a Gal residue as is evident from the typical set of chemical shifts of NeuAc structural-reporter groups: δH-3ax 1.719; δH-3eq 2.667 (and δNAc 2.029) (11,12). The shift increments observed for H-1 of Man-4 (Δδ 0.013 ppm) and for the NAc protons of GlcNAc-5 (Δδ -0.019 ppm), and the decrement for H-1 of Gal-6 (Δδ -0.021 ppm), in the step from *GP-AS* to *GP-MS* (Table I) together indicate that NeuAc is attached to Gal-6 (11,12). The absence of any such effect on the reporter groups of Manα1→6 branch residues excludes the presence in the preparation of the isomer of *GP-MS* possessing NeuAc' attached to Gal-6' instead of NeuAc to Gal-6 (Fig. 3A2). It should be noted that the ^{1}H-NMR data given in Table I for *GP-MS* are the refined values of those determined previously at 360 MHz for this compound (2). For a similar glycopeptide, having the peptide moiety Asn-Ser instead of Asn-Lys, 360-MHz NMR data have indicated that the positions of only GlcNAc-1 structural reporter groups are affected (11).

The completely sialylated product (*GP-BS*), the NMR features of which have been described (2,11,18), is the bi-α2→6-sialyl derivative of *GP-AS* as could be judged from the appearance of two equally high H-3eq doublets of doublets at δ 2.668 and 2.673, as well as from the intensity-doubling of the H-3ax triplet at δ 1.719 and the NeuAc NAc singlet at δ 2.030 (Fig. 3A3), as compared to *GP-MS*. Based on comparison with *GP-MS* (Table I), the H-3eq signal at δ 2.673 was attributed to NeuAc' α2→6-linked to Gal-6' (11). Essentially the same effects in chemical shift as described for the Manα1→3 branch reporter groups in the step from *GP-AS* to *GP-MS*, are found for H-1 and H-2 of Man-4', H-1 and NAc of GlcNAc-5', and H-1 of Gal-6' when comparing *GP-BS* with *GP-MS*, indicating that the second NeuAc residue is α2→6-linked to Gal-6'.

It has to be mentioned that sialylation of Gal-6' by NeuAc' in α2→6-linkage influences slightly but significantly the chemical shift of the NAc protons of GlcNAc-2 (Δδ 0.003 ppm, Table I). This effect is definitely not due to the inaccuracy inherent to chemical shift measurements as is illustrated by the pattern of the NAc singlets observed for a mixture of mono- and bisialylated biantennary glycopeptides in a ratio of 4:3 (Fig. 4). This ratio, which is evident from the ratio of the NAc signals of GlcNAc-5' (at δ 2.047 and 2.066), is also found for those of GlcNAc-2 (at δ 2.079 and 2.082). The appearance of this effect reflects the spatial proximity of the GlcNAc-2 NAc protons and the NeuAc' residue.

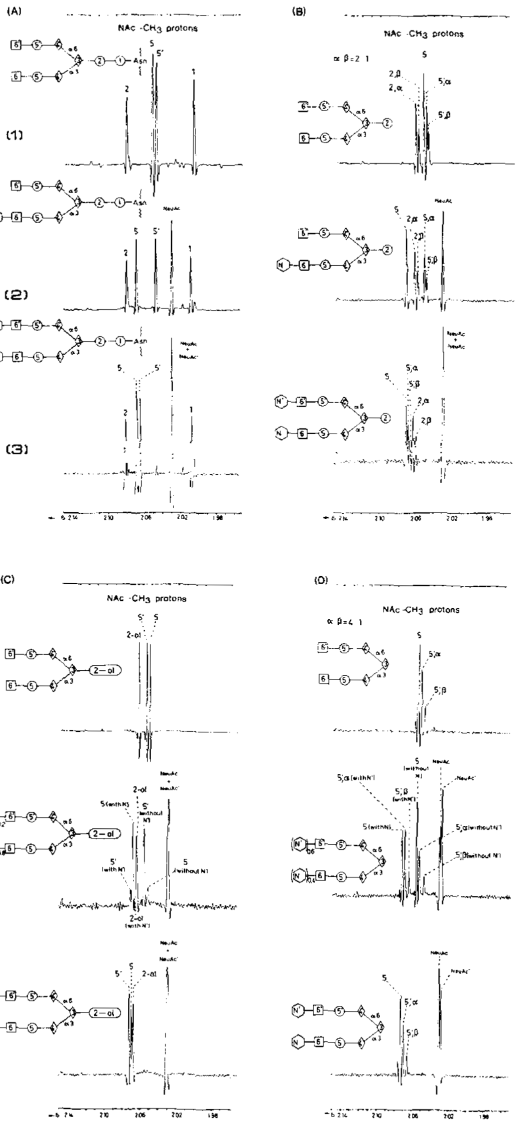


Fig. 3 - *N*-Acetyl methyl resonances in the resolution-enhanced 500-MHz ^{1}H-NMR spectra of the biantennary substrates and their sialylated derivatives. The *N*-acetyl methyl resonances in the 500-MHz ^{1}H-NMR spectra of the substrate (1), its mono- (2) and bisialylated (3) derivative, respectively, are presented for (A) the glycopeptide (*GP*); (B) the reducing octasaccharide (*OS*); (C) the reduced octasaccharide (*OL*) and (D) the reducing heptasaccharide (*HS*). The numbers in the spectra refer to the corresponding GlcNAc residues in the structure.

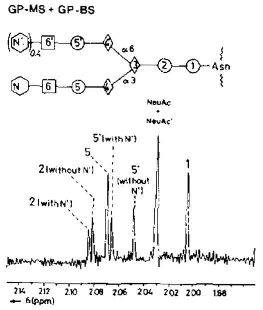


Fig. 4 - *N*-Acetyl methyl resonances in the resolution-enhanced 500-MHz ^{1}H-NMR spectrum of a mixture of *GP-MS* and *GP-BS*. From the *N*-acetyl methyl resonances in the resolution-enhanced 500-MHz ^{1}H-NMR spectrum of a mixture of *GP-MS* and *GP-BS* in the ratio of 4:3 it is obvious that sialylation of the α1→6 branch not only affects the position of the *N*-acetyl signal of GlcNAc-5' but also that of GlcNAc-2 slightly but significantly.

The 500-MHz ^{1}H-NMR characteristics of the biantennary octasaccharides ending in GlcNAc-2 (*OS-AS*, *OS-MS* and *OS-BS*) have been published (11). Under the applied measuring conditions these compounds each occur as a mixture of two anomers, containing GlcNAc-2 in the α- and β-pyranose form, in the ratio 2:1. The chemical shifts of the structural-reporter groups of GlcNAc-2, but also of Man-3, Man-4', GlcNAc-5' and Gal-6', are sensitive to this anomerisation, as is reflected by doubling of each of these signals in the anomeric intensity ratio (see for example, the NAc proton region of the spectrum of *OS-AS*, Fig. 3B1). The extent to which anomerisation affects the chemical shift of a proton ($\Delta\delta_{\alpha-\beta}$) is considered as measure of its proximity to the anomeric center of GlcNAc-2 (11).

The monosialylated (*OS-MS*) and bisialylated (*OS-BS*) products of the incubation of *OS-AS* with CMP-NeuAc in the presence of the sialyltransferase, are the oligosaccharide analogues of *GP-MS* and *GP-BS*, respectively, missing the GlcNAcβ(1→N)Asn-Lys moiety. By the same reasoning as for the monosialylated glycopeptide product (*GP-MS*) it can be concluded that in the monosialylated octasaccharide (*OS-MS*) the NeuAc residue is present in α2→6-linkage to Gal-6, exclusively (Table I, Fig. 3B2). In *OS-BS* both branches are terminated by α2→6-linked NeuAc (Table I, Fig. 3B3).

TABLE I

^{1}H chemical shifts of structural-reporter-groups of constituent monosaccharides for various *N*-acetyllactosamine-type, biantennary compounds and their in-vitro sialylated analogues

Reporter group	Residue[c]	Chemical shift[a] in[b] *GP-AS*	*GP-MS*	*GP-BS*	Anomer of oligosacch.	*OS-AS*	*OS-MS*	*OS-BS*	*OL-AS*	*OL-MS1*	*OL-MS2*	*OL-BS*	Anomer of oligosacch.	*HS-AS*	*HS-MS1*	*HS-MS2*	*HS-BS*
H-1	GlcNAc-1	5.094	5.058	5.057	-	-	-	-	-	-	-	-	-	-	-	-	-
	GlcNAc-2	4.616	4.615	4.615	α β	5.212 4.721	5.212 4.72	5.214 4.72	-	-	-	-	-	-	-	-	-
	Man-3	4.765	4.76	4.76	α β	4.775 4.765	4.77	4.77	4.809	4.804	4.804	4.813	α β	5.140 4.911	5.146 4.914	5.154 4.914	5.149 4.916
	Man-4	5.121	5.134	5.133	α β	5.123 5.121	5.137	5.135	5.118	5.132	5.120	5.130	α,β	5.146	5.158	5.146	5.159
	Man-4'	4.928	4.929	4.947	α β	4.927 4.930	4.929 4.933	4.952	4.923	4.926	4.938	4.941	α,β	4.911	4.914	4.931	4.933
	GlcNAc-5	4.582	4.605	4.604	α,β	4.582	4.604	4.606	4.583	4.606	4.583	4.606	α,β	4.583	4.608	4.583	4.607
	GlcNAc-5'	4.582	4.581	4.604	α,β	4.582	4.588	4.606	4.600	4.603	4.624	4.619	α,β	4.596	4.597	4.618	4.618
	Gal-6	4.467	4.444	4.442	α,β	4.468	4.446	4.444	4.467	4.446	4.466	4.443	α,β	4.458	4.445	4.468	4.443
	Gal-6'	4.473	4.472	4.447	α β	4.471 4.473	4.470 4.472	4.444 4.448	4.471	4.471	4.448	4.446	α,β	4.468	4.468	4.447	4.446
H-2	GlcNAc-2-ol	-	-	-	-	-	-	-	4.218	4.219	4.219	4.217	-	-	-	-	-
	Man-3	4.246	4.251	4.255	α β	4.259 4.249	4.264 4.254	4.266 4.255	4.252	4.258	4.251	4.258	α,β	4.081	4.084	4.084	4.088
	Man-4	4.190	4.195	4.196	α,β	4.193	4.199	4.198	4.194	4.198	4.198	4.199	α,β	4.194	4.199	4.194	4.201
	Man-4'	4.109	4.111	4.117	α,β	4.114	4.115	4.121	4.135	4.136	4.136	4.142	α,β	4.134	4.134	4.142	4.141
H-3ax	NeuAc	-	1.719	1.719	α,β	-	1.720	1.718	-	1.719	-	1.720	α,β	-	1.718	-	1.717
	NeuAc'	-	-	1.719	α,β	-	-	1.718	-	-	1.719	1.720	α,β	-	-	1.718	1.720
H-3eq	NeuAc	-	2.667	2.668	α,β	-	2.668	2.669	-	2.666	-	2.668	α,β	-	2.669	-	2.669
	NeuAc'	-	-	2.673	α,β	-	-	2.672	-	-	2.666	2.671	α,β	-	-	2.669	2.669
NAc	GlcNAc-1	2.004	2.008	2.008	-	-	-	-	-	-	-	-	-	-	-	-	-
	GlcNAc-2(-ol)	2.079	2.079	2.082	α β	2.060 2.057	2.061 2.058	2.063 2.060	2.064	2.065	2.065	2.069	-	-	-	-	-
	GlcNAc-5	2.050	2.069	2.069	α,β	2.052	2.071	2.071	2.051	2.069	2.053	2.069	α,β	2.055	2.072	2.056	2.073
	GlcNAc-5'	2.046	2.047	2.066	α β	2.048 2.046	2.050 2.048	2.069 2.066	2.055	2.057	2.069	2.072	α β	2.052 2.047	2.053 2.048	2.069 2.064	2.070 2.065
	NeuAc	-	2.029	2.030	α,β	-	2.030	2.030	-	2.030	-	2.029	α,β	-	2.030	-	2.030[d]
	NeuAc'	-	-	2.030	α,β	-	-	2.030	-	-	2.030	2.029	α,β	-	-	2.028	2.028[d]

[a] Data were acquired at 500 MHz for neutral solutions in D_2O at 27°C. Chemical shifts are in ppm relative to internal DSS (using internal acetone at δ 2.225).
[b] In the table heading, structures are represented by short-hand symbolic notation (compare Ref. 11): ● = GlcNAc; ◆ = Man; ○— = NeuAcα(2→6). For complete structures, see text.
[c] For numbering of monosaccharide residues, see Fig. 3; NeuAc denotes the sialic acid residue α(2→6)-linked to Gal-6; NeuAc' the one linked to Gal-6', respectively.
[d] Assignments may have to be interchanged.

The substrate octasaccharide-alditol (*OL-AS*) is the reduced analogue of *OS-AS*, so it ends in GlcNAc-2-ol. The influence of reduction on the chemical shift of GlcNAc-5', but not on GlcNAc-5, reporter groups (Table I, Fig. 3B1, C1) can be considered as additional evidence for the proximity of the former residue to the GlcNAc-2 residue. Assignments of the H-1 signals of GlcNAc-5 and -5' are based on NOE measurements (unpublished results).

The monosialylated product of the treatment of the reduced octasaccharide *OL-AS* with CMP-NeuAc in the presence of the sialyltransferase appeared to be a mixture of two compounds, differing in the branch to which the NeuAc residue is attached. The 500-MHz ^{1}H-NMR spectrum of this product showed two sets of signals, in the intensity ratio of 4:1 (Fig. 3C2). The higher-intensity signals belong to the major component *OL-MS-1* having NeuAc in α2→6-linkage to Gal-6; this could be inferred from the shift effects observed between *OL-MS-1* and *OL-AS* (Table I). The minor component of the mixture, *OL-MS-2*, is the isomer of *OL-MS-1*, carrying the NeuAc residue at the Manα1→6 branch. Its spectrum reveals only the shift effects for sialylation of Gal-6' by NeuAc', and not those for attachment of NeuAc to Gal-6. The assignments of the GlcNAc-5 and -5' H-1 signals described for *OL-AS* are corroborated by the relative intensities within the pattern of doublets at 4.58 < δ < 4.63 in the spectrum of *OL-MS*. The assignments of the *N*-acetyl signals of GlcNAc-5 and -5' for *OL-AS* were based on the specific shift increments, introduced by the presence of NeuAc in the major and NeuAc' in the minor component of the mixture (Fig. 3C1 and 3C2). It should be noted that the theoretical possibility of the presence of asialo- and/or bisialylated components in the *OL-MS* mixture can be excluded on the basis of its clear separation from these components on Bio-Gel P-6.

In the spectrum of *OL-BS*, the aforementioned shift effects for reporter groups of the Manα1→3- and Manα1→6 branch residues were found together (Table I, Fig. 3C3), indicating that both Gal-6 and Gal-6' bear an α2→6-linked NeuAc residue.

The heptasaccharide substrate (*HS-AS*) is a biantennary oligosaccharide, lacking the *N,N'*-diacetylchitobiose unit, having Man-3 as its reducing end. The assignments of the structural-reporter groups given for *HS-AS* in Table I, are refinements of those given by Bock *et al.* for this compound at 400 MHz (7). As is the case with *OS-AS*, the heptasaccharide occurs under the applied measuring conditions as a mixture of two anomers, but in the ratio of α:β ≈ 3:1. The latter is evident, for example, from the intensity ratio of the GlcNAc-5' *N*-acetyl signals (Fig. 3D). The reducing character of Man-3 does not profoundly influence the chemical shifts of any of the structural-reporter groups of the *N*-acetyllactosamine residues, except for the *N*-acetyl methyl protons of GlcNAc-5'.

As with *OL-MS*, the monosialylated product of the biantennary heptasaccharide, *HS-MS*, was found to be a mixture of two positional isomers differing in the branch in which the NeuAc residue is located. Interestingly, the ratio of the product sialylated at the 1→3 branch to that sialylated at the 1→6 branch was 2:3 as could be estimated from the intensity ratios of the various signals (e.g. those for the *N*-acetyl groups of GlcNAc-5 and -5', Fig. 3D2). Also, the complex pattern of resonances of GlcNAc-5 and -5' H-1 signals (4.58 < δ < 4.62) reflected this ratio. The shift effects in combination with the relative intensities of these signals corroborated the assignments of the GlcNAc H-1 doublets reported for *HS-AS* (Table I).

The spectrum of the completely sialylated derivative of *HS-AS*, denoted *HS-BS*, showed the features characteristic for the presence of NeuAc in both branches of the heptasaccharide, namely, two sets of NeuAc structural reporter group signals and the shift effects of several reporter groups as compared to *HS-AS* (Table I). It may be mentioned that the Gal-6 and -6' H-1 signals, which coincided in the spectrum of *HS-AS*, became separated after sialylation of both branches of the heptasaccharide. Their assignment is in line with that for other bisialylated biantennae, listed in Table I. The results obtained by ^{1}H-NMR revealed the relative preference of the sialyltransferase for each of the branches ("branch specificity") of the biantennary substrates used (Table II).

Table II - Branch specificity of bovine colostrum *N*-acetyllactosaminide α2→6-sialyltransferase

Acceptor structure	Preference ratio (1→3 branch/1→6 branch)	Relative rate of sialylation at 1→3 branch*
		%
Galβ1→4GlcNAcβ1→2Manα1→6 / Galβ1→4GlcNAcβ1→2Manα1→3 Manβ1→4GlcNAcβ1→4GlcNAc→Asn-Lys	> 40:1	100
Galβ1→4GlcNAcβ1→2Manα1→6 / Galβ1→4GlcNAcβ1→2Manα1→3 Manβ1→4GlcNAc	> 40:1	78
Galβ1→4GlcNAcβ1→2Manα1→6 / Galβ1→4GlcNAcβ1→2Manα1→3 Manβ1→4GlcNAcol	4:1	28
Galβ1→4GlcNAcβ1→2Manα1→6 / Galβ1→4GlcNAcβ1→2Manα1→3 Man	2:3	13

*Estimated from the kinetics of sialic acid incorporation and the preference ratios.

K_m and V values of *OS-AS* and *OS-MS* - To analyze the rate of formation of mono- and bisialylated products at varying concentrations of the asialo- and the monosialylated octasaccharide, *OS-AS* and *OS-MS*, respectively, use was made of HPLC. A typical example of the separation of bisialylated product from the monosialo substrate is given in Fig. 5. Analysis of the data enabled the determination of the K_m and V values for the two substrates (Table III). The affinity of the sialyltransferase for the asialo acceptor was about twice that for the monosialo substrate, whereas V with the first substrate was about five times higher than with the latter. This resulted in an about ten-fold higher kinetic efficiency for the introduction of the first sialic acid (at the α1→3 branch) compared to the second sialic acid residue (at the α1→6 branch).

Table III - K_m and V values of *N*-acetyllactosaminide α2→6-sialyltransferase for the biantennary reducing octasaccharide (*OS-AS*) and its monosialylated derivative (*OS-MS*). Values are presented ± standard deviation. K_m values are expressed in mM of oligosaccharide.

Acceptor substrate	K_m	V	Relative acceptor activity
	mM	$nmol.mg^{-1}.min^{-1}$	$V.K_m^{-1}$
OS-AS	0.69 ± 0.16	1.11 ± 0.06	1.61
OS-MS	1.39 ± 0.39	0.22 ± 0.02	0.16

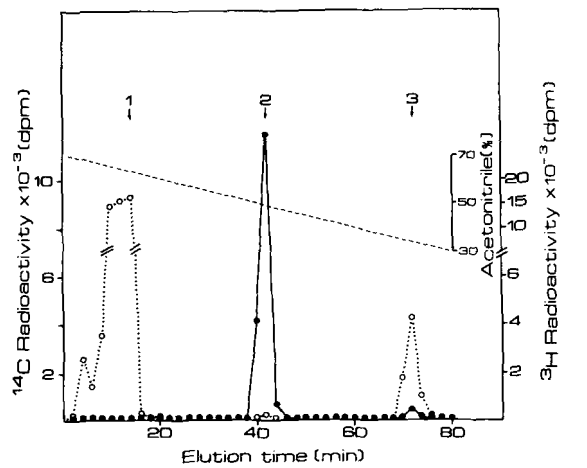


Fig. 5 - Separation of sialylated biantennary oligosaccharides by HPLC. Mono-[^{14}C]sialylated octasaccharide was incubated with α2→6-sialyltransferase and CMP-[^{3}H]NeuAc for 60 min at 37°C. From the incubation mixture aliquots were prepared for HPLC as described previously (17). HPLC was performed on a Lichrosorb-NH_2 column by elution with a linear gradient of increasing buffer content at a rate of 0.5%/min, starting with a mixture of acetonitrile and 0.15 M potassium phosphate pH 5.2 70:30 (v/v) at a flow of 2 mL/min. Radioactive compounds were quantified by liquid-scintillation counting of the eluate, which was collected in fractions of 2 mL. Arrows indicate the elution position of (1) sialic acid, (2) oligosaccharide *OS*-[^{14}C]*MS*, and (3) *OS*-[^{14}C],[^{3}H]*BS*.