THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 262, No. 5, Issue of February 15, pp. 2025-2033, 1987
Printed in U.S.A.


# Branch Specificity of Bovine Colostrum CMP-Sialic Acid: Galβ1→4GlcNAc-R α2→6-Sialyltransferase

## SIALYLATION OF BI-, TRI-, AND TETRAANTENNARY OLIGOSACCHARIDES AND GLYCOPEPTIDES OF THE *N*-ACETYLLACTOSAMINE TYPE*

(Received for publication, July 10, 1986)

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**Using 500-MHz $^1$H NMR spectroscopy we have investigated the branch specificity that bovine colostrum CMP-NeuAc:Galβ1→4GlcNAc-R α2→6-sialyltransferase shows in its sialylation of bi-, tri-, and tetraantennary glycopeptides and oligosaccharides of the *N*-acetyllactosamine type. The enzyme appears to highly prefer the galactose residue at the Galβ1→4GlcNAcβ1→2Manα1→3 branch for attachment of the 1st mol of sialic acid in all the acceptors tested. The 2nd mol of sialic acid becomes linked mainly to the Galβ1→4GlcNAcβ1→2Manα1→6 branch in bi- and triantennary substrates, but this reaction invariably proceeds at a much lower rate. Under the conditions employed, the Galβ1→4GlcNAcβ1→6Manα1→6 branch is extremely resistant to α2→6-sialylation.**

**A higher degree of branching of the acceptors leads to a decrease in the rate of sialylation. In particular, the presence of the Galβ1→4GlcNAcβ1→6Manα1→6 branch strongly inhibits the rate of transfer of both the 1st and the 2nd mol of sialic acid. In addition, it directs the incorporation of the 2nd mol into tetraantennary structures toward the Galβ1→4GlcNAcβ1→4Manα1→3 branch. In contrast, the presence of the Galβ1→4GlcNAcβ1→4Manα1→3 branch has only minor effects on the rates of sialylation and, consequently, on the branch preference of sialic acid attachment. Results obtained with partial structures of tetraantennary acceptors indicate that the Manβ1→4GlcNAc part of the core is essential for the expression of branch specificity of the sialyltransferase.**

**The sialylation patterns observed *in vivo* in glycoproteins of different origin are consistent with the *in vitro* preference of α2→6-sialyltransferase for the Galβ1→4GlcNAcβ1→2Manα1→3 branch. Our findings suggest that the terminal structures of branched glycans of the *N*-acetyllactosamine type are the result of the complementary branch specificity of the various glycosyltransferases that are specific for the acceptor sequence Galβ1→4GlcNAc-R.**

Complex- or *N*-acetyllactosamine-type glycans occur on many mammalian *N*-glycoproteins (1, 2). These glycans contain at least two branches (*N*-acetyllactosamine units) that are linked β1→2 to the 2 α-mannose residues of the core and can carry additional branches linked β1→6 to Manα1→6 and β1→4 to Manα1→3 (Fig. 1). The different branches are generally terminated by sugars such as sialic acid (1, 2), α1→3-linked galactose (3), or fucose (1, 4), or may be elongated to form polylactosaminoglycans (5). Structural studies have shown that the terminating sugars are not randomly distributed over the various branches, but occur preferentially attached to particular *N*-acetyllactosamine units (3, 4, 6, 7 and Table I). We have proposed earlier that this feature is due to the branch specificities of the glycosyltransferases involved in the chain terminations (19, 20).

With respect to sialic acid as a terminal sugar, a survey of several *N*-glycoproteins which contain both α2→3- and α2→6-linked sialic acid residues shows that specific sialylation patterns occur in nature (Table I). In these glycoproteins, the Galβ1→4GlcNAcβ1→2Manα1→3Man branch invariably carries a sialic acid in α2→6 linkage, whereas the Galβ1→4GlcNAcβ1→4Manα1→3Man branch generally is terminated by an α2→3-linked sialic acid. This study was undertaken to investigate whether these specific patterns are consistent with the *in vitro* specificity of the CMP-NeuAc:Galβ1→4GlcNAcβ1→R α2→6-sialyltransferase from bovine colostrum. Previously, we have described the branch specificity of this enzyme with biantennary substrates (20). Here, these studies are extended using tri- and tetraantennary oligosaccharides and glycopeptides as acceptors. A preliminary account of this work has appeared (21).

## MATERIALS AND METHODS[1]

## RESULTS

*Branch Specificity of Bovine Colostrum CMP-NeuAc:β-galactoside α2→6-Sialyltransferase*—The structures of the sialylated products formed enzymatically *in vitro* as established by $^1$H NMR allow determination of the branch specificity of the bovine colostrum α2→6-sialyltransferase. The results are

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[1] Portions of this paper (including "Materials and Methods," part of the "Results," Figs. 2-7, and Tables II-IV) 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. 86M-2314, cite the authors, and include a check or money order for $5.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

TABLE I

*Sialylation patterns in N-glycoproteins*

The table comprises only those structures that contain both α2→3- and α2→6-linked sialic acid residues. The denoting system used to identify the various galactose residues is indicated in Fig. 1 for *GP4*.

| Glycoprotein | Type of sialic acid linkage to | | | | Ref. |
|---|---|---|---|---|---|
| | Gal-8′ | Gal-6′ | Gal-6 | Gal-8 | |
| Calf fetuin | | α2→3/6 | α2→6 | α2→3 | (8, 9) |
| Human $\alpha_1$-protease inhibitor | (α2→3)[a] | α2→6 | α2→6 | (α2→3) | (10) |
| Human ceruloplasmin | | α2→6 | α2→6 | α2→3 | (11) |
| Human $\alpha_1$-acid glycoprotein | α2→6/3 | α2→3 | α2→6 | α2→3/6 | (12, 13) |
| Rabbit liver binding protein | | α2→6 | α2→6 | α2→3 | (14) |
| Equine pancreas ribonuclease | | α2→3 | α2→6 | | (15) |
| Porcine thyroglobulin | | α2→6 | α2→6 | α2→3 | (16) |
| Thyroxine-binding globulin | | α2→6 | α2→6 | α2→3 | (17) |
| Human serotransferrin | (α2→3) | α2→6 | α2→6 | (α2→3) | (18) |

[a] Parentheses indicate that tri- as well as tri′-antennary glycans are present on the glycoprotein.

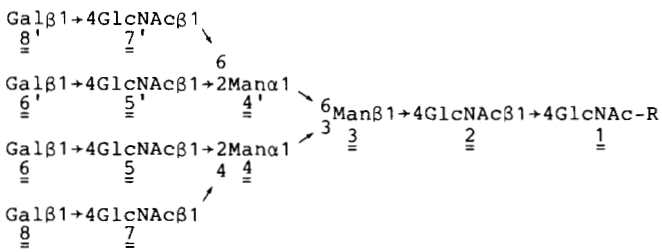


FIG. 1. **The denoting system used in this study to indicate the constituent monosaccharides of the various oligosaccharides and glycopeptides.** The system is illustrated for glycopeptide *GP4*.

summarized in Table V for the glycopeptides *GP2*,[2] *GP3*, *GP4*, and *GP4(Fuc)* as well as the oligosaccharides *OS2*, *OS3*, *OS3′*, and *OS4*, which have residue GlcNAc-**2** at the reducing end. With all these substrates, Gal-**6** (at the Galβ1→4GlcNAcβ1→2Manα1→3 branch) is fully sialylated before a sialic acid residue is attached to any of the other branches. Introduction of a second sialic acid into the two isomeric triantennary oligosaccharides *OS3* and *OS3′*, as well as the triantennary glycopeptide *GP3*, occurs for 90% at Gal-**6′** (at the Galβ1→4GlcNAcβ1→2Manα1→6 branch) and for only 10% at Gal-**8** (in the case of *OS3* and *GP3*) or Gal-**8′** (in *OS3′*). Trisialo derivatives in which all terminal galactoses are sialylated can be obtained with *OS3* and *GP3*, but not with *OS3′*. Of the latter oligosaccharide, the Galβ1→4GlcNAcβ1→6Manα1→6 branch (Gal-**8′**) in particular appears to be resistant to a full sialylation.

With the tetraantennary substrates *OS4* and *GP4*, the second sialic acid is attached either to Gal-**8** (for 65%) or to Gal-**6′** (for 35%), but not to Gal-**8′**. Further sialylation of *OS4*, yielding a trisialo derivative, results in a saturation of Gal-**8** and Gal-**6**, whereas Gal-**8′** remains unsubstituted. Introduction of a 3rd sialic acid residue by the sialyltransferase to *GP3*, however, is for 20% to Gal-**8′** leaving an equivalent amount of Gal-**6′** uncovered. A full sialylation of *OS4* and *GP4* to a tetrasialo compound is not achieved due to the resistance of Gal-**8′**.

To the fucose-containing glycopeptide *GP4(Fuc)*, only 2 sialic acid residues can be attached under the conditions employed. The second sialic acid becomes predominantly linked to Gal-**6′** (for 45%) and Gal-**8** (for 40%). Note that, with *GP4(Fuc)*, the sialylation pattern obtained is not solely due to the branch specificity of the sialyltransferase, since this substrate is a mixture of isomers differing in the branch to which fucose is linked.

With the two pentasaccharide substrates, *OS2(3)* and *OS2(3′)*, which are partial structures of the tetraantennary oligosaccharide *OS4*, the sialyltransferase shows a branch specificity which differs from that seen with *OS4*. In *OS2(3)*, the sialyltransferase prefers Gal-**8** over Gal-**6** for attachment of the 1st sialic acid residue; with *OS2(3′)*, Gal-**8′** is highly preferred over Gal-**6′** for sialylation (Fig. 6).

*Comparative Rates of Sialylation of Branched Oligosaccharides*—The progress curves of sialylation of oligosaccharides *OS2*, *OS3*, *OS3′*, and *OS4* (Fig. 7, *A–D*) show that there are large differences in the rates of incorporation of the 1st, the 2nd, and the 3rd sialic acid residue into these substrates. In all instances, a high concentration of monosialo products is

TABLE V

*The location of sialic acid in mono-, bi-, and trisialo products formed by in vitro α2→6-sialylation of branched acceptors of the N-acetyllactosamine type*

The various acceptor substrates are depicted in a schematical form, with numbers indicating the positions of substitution at the branching points (compare Fig. 1). Data are presented for glycopeptides (*GP*) and oligosaccharides (*OS*).

| Acceptor substrate | Relative sialylation of the branches in the monosialo product | bisialo product | trisialo product | Branching pattern[d] |
|---|---|---|---|---|
| | % | | | |
| *GP2/OS2* | 0 | 100 | - | |
| | 100 | 100 | | |
| *GP3/OS3* | 0 | 90 | 100 | |
| | 100 | 100 | 100 | |
| | 0 | 10 | 100 | |
| *OS3′* | 0 | 10 | | |
| | 0 | 90 | na[a] | |
| | 100 | 100 | | |
| *GP4/OS4* | 0 | 0 | 20[b] 0[c] | |
| | 0 | 35 | 80 100 | |
| | 100 | 100 | 100 100 | |
| | 0 | 65 | 100 100 | |
| *GP4(Fuc)* | 0 | 15 | | |
| | 0 | 45 | | |
| | 100 | 100 | na | |
| | 0 | 40 | | |

[a] na, not available.
[b] For *GP4*.
[c] For *OS4*.
[d] Fucose α1→3-linked to GlcNAc is present on either of the branches marked with an asterisk.

[2] The abbreviations used are: *GP*, glycopeptide; *OS*, oligosaccharide; suffixes *MS*, *BS*, and *TS* indicate mono-, bi-, and trisialyl-derivatives, respectively; structures of the substrates *OS2*, *OS3*, *OS3′*, *OS4*, *GP2*, *GP3*, *GP4*, *GP4(Fuc)*, *OS2(3)*, and *OS2(3′)* are presented in Fig. 2 (Miniprint Section).

TABLE VI

*Kinetic efficiency ($V \cdot K_m^{-1}$) of sialic acid transfer to bi-, tri-, and tetraantennary oligosaccharides*

Kinetic efficiencies ($V/K_m$) were calculated for the sialylations of asialo-, monosialo- and bisialo-oligosaccharides *OS2*, *OS3*, *OS3'*, and *OS4*. Each of the asialo-oligosaccharides was incubated with α2→6-sialyltransferase and CMP-[$^{14}$C]NeuAc as described under "Materials and Methods." At intervals, aliquots were withdrawn from the reaction mixture and subjected to gel filtration on Bio-Gel P-6. The amount of each of the sialylated products was estimated from the radioactivity incorporated. Kinetic efficiencies were estimated from the corresponding progress curves (Fig. 7) and are expressed in Table VI in arbitrary units relative to the efficiency of the reaction: *OS2* → monosialo-*OS2*.

| Acceptor oligosaccharide | Relative kinetic efficiency | | |
|---|---|---|---|
| | Asialo → monosialo | Monosialo → bisialo | Bisialo → trisialo |
| | | % | |
| *OS2* | 100 | 7.9 | |
| *OS3* | 61 | 5.9 | 2 |
| *OS3'* | 35 | 0.8 | <0.1 |
| *OS4* | 23 | 1.2 | <0.3 |

built up before appreciable amounts of bisialo derivative are formed. Our results show that the formation of the monosialo product, which invariably corresponds to the α2→6-sialylation of Gal-**6** (Table V), is 10–40-fold faster than that of the bisialo derivative (Table VI). In turn, trisialo derivatives are formed 3–8 times more slowly than bisialo products (Table VI), whereas the tetrasialo derivative of *OS4* is formed at an extremely low rate (Fig. 7*D*).

Apart from these rate differences, the efficiency of transfer of the first sialic acid decreases with increased branching of the acceptor molecule. In particular, the presence of the Galβ1→4GlcNAcβ1→6Manα1→6 branch (in *OS3'* and *OS4*) (Table V and Fig. 7, *C* and *D*) slows down the rate of incorporation of the first sialic acid (Table VI). This branch also strongly decreases the efficiency of transfer of the 2nd sialic acid residue to *OS3'* and *OS4*. The Galβ1→4GlcNAcβ1→4Manα1→3 branch (present in *OS3* and *OS4*), on the other hand, has much less pronounced effects (Table VI). It decreases the incorporation rate of the first sialic acid by approximately 40%. The rate of incorporation of the second sialic acid into *OS3* is only slightly decreased as compared to *OS2*, whereas the efficiency of transfer of the second sialic acid residue to *OS4* is even higher than that seen with *OS3'*.

## DISCUSSION

In order to clarify the mechanism underlying the *in vivo* production of specific sialylation patterns occurring on the glycans of *N*-glycoproteins, we investigated the branch specificity of bovine colostrum α2→6-sialyltransferase with bi-, tri-, and tetraantennary substrates *in vitro*. Previously, we have shown that this enzyme can differentiate between the branches of bi- and triantennary glycopeptides (19, 20). In this study, we provide evidence that the attachment of α2→6-linked sialic acid to the different *N*-acetyllactosamine units that form the branches of the acceptors, proceeds stepwise in a highly preferred order. This order was deduced from the analysis by 500-MHz $^1$H NMR spectroscopy of partially sialylated products formed *in vitro*. Interestingly, it was found that with all acceptors, except those which lack the Manβ1→4GlcNAc portion of the core, the 1st mol of sialic acid is introduced to Gal-**6** (located at the Galβ1→4GlcNAcβ1→2Manα1→3 branch), regardless of the degree of branching. Increase of branching, however, strongly affects the rate of this sialic acid transfer. It has been proposed that the Manα1→3 branch extends from the core in one fixed spatial orientation in bi-, tri-, as well as tetraantennary structures (29). We have suggested earlier that this orientation favors a specific interaction of the α2→6 sialyltransferase with both a recognition site of the core, notably GlcNAc-**2**, and the substrate site (Gal-**6**) of biantennary acceptors (20). Our present data seem to indicate that additional branches do not interfere with the specificity of the interaction between enzyme and substrate. They might, however, sterically hinder the access of the sialyltransferase to either the substrate site or recognition site of the acceptor.

Additional sialic acid residues can be incorporated by the sialyltransferase into the primary monosialylated products. The rate of this incorporation, however, is always much lower than that of the 1st mol of sialic acid. The conformation of the branch to be sialylated secondly might be such that an interaction with the enzyme is less favored. In addition, a charge repulsion effect of the sialic acid moieties might play a role.

Although with both triantennary oligosaccharides (*OS3* and *OS3'*) the introduction of a 2nd mol of sialic acid occurred predominantly to Gal-**6'**, the rate at which it proceeded largely differed for the two substrates. The Gal-**8'**/GlcNAc-**7'** branch slows down this sialylation reaction dramatically, whereas the Gal-**8**/GlcNAc-**7** branch hardly has an effect on the rate. Similarly, the former branch has a much greater effect on the rate of incorporation of the 1st mol of sialic acid (to Gal-**6**) than the latter branch. The Gal-**8**/GlcNAc-**7** branch thus seems to have only a local effect, mainly due to sterical hindrance as can be expected from the proposed conformation of this branch (30). The Gal-**8'**/GlcNAc-**7'** branch, however, exerts a much further reaching influence. Consequently, one might speculate that this branch has a strong effect on the conformation of the entire acceptor. Such an effect has also been suggested on the basis of NMR data (25). Possibly, the Gal-**8'**/GlcNAc-**7'** branch interferes with the interaction of the sialyltransferase with the recognition site (GlcNAc-**2**) of the core causing a decrease in sialylation rate of Gal-**6** (20). A full understanding of the effects of this branch on sialylation, however, requires more detailed information on its conformation (31). In the sialylation of *OS4*, the effects of the Gal-**8**/GlcNAc-**7** and Gal-**8'**/GlcNAc-**7'** branches occur combined. Because of the strong inhibition by the latter branch of the sialylation of Gal-**6'**, the 2nd mol of sialic acid is for the larger part introduced in this substrate at Gal-**8**.

The importance of the core structure for branch-specific sialylation of *N*-acetyllactosamine type glycans is further illustrated by the results obtained with *OS2(3)* and *OS2(3')*. In these oligosaccharides, lacking Man-**3** and GlcNAc-**2**, the Galβ1→4GlcNAcβ1→2Man branch (containing either Gal-**6** or Gal-**6'**) was no longer the preferred substrate for the α2→6-sialyltransferase: their monosialo products were mostly sialylated at Gal-**8** and Gal-**8'**, respectively. This supports our view (20) that the integrity of the core structure of the acceptor is required for expression of the branch specificity of α2→6-sialyltransferase.

It was possible to transfer a 3rd mol of sialic acid to *OS3* and *OS4 in vitro*. The trisialo derivative of *OS3'*, however, could be obtained in small amounts only. In accord with this result, no sialic acid linked to Gal-**8'** could be detected in *OS4-TS*. Only with tetraantennary glycopeptides, in particular if fucosylated at GlcNAc-**7**, some transfer of sialic acid to Gal-**8'** was observed. Apparently, the extreme resistance of the Gal-**8'**/GlcNAc-**7'** branch to α2→6-sialylation by the colostrum enzyme is diminished in the presence of a peptide moiety. The polypeptide part of $\alpha_1$-acid glycoprotein might

interfere even more with the branch specificity of α2→6-sialyltransferase. Such interaction could explain the observed nearly stoichiometric α2→6-sialylation *in vitro* of asialo-$\alpha_1$-acid glycoprotein (32, 33), as well as the observed α2→6-sialylation of Gal-**8′** in native $\alpha_1$-acid glycoprotein (13).

Sialylation patterns occurring on *N*-linked carbohydrate chains isolated from various sources (Table I, and also the pentaantennary structure in Ref. 34) are consistent with the *in vitro* preference of bovine colostrum α2→6-sialyltransferase for the Galβ1→4GlcNAcβ1→2Manα1→3 branch. This indicates that the specificity of the enzyme may be representative of the properties of Galβ1→4GlcNAc-R α2→6-sialyltransferase from a variety of tissues and species. On the other hand, the α2→6-sialylation of Gal-**8** in *GP3, OS3, GP4,* and *OS4 in vitro* may seem to be in disaccord with the observation that the Galβ1→4GlcNAcβ1→4Manα1→3 branch of triantennary glycans in glycoproteins almost invariably carries sialic acid in α2→3-linkage (Table I). It should, however, be realized that the rate of α2→6-sialylation of Gal-**8** is very low. Attachment of sialic acid in α2→6-linkage to this Gal residue therefore occurs *in vitro* only because no other sialyltransferase competing for Gal-**8** is present. Many tissues, however, contain an α2→3-sialyltransferase (32, 35). The specific sialylation patterns as observed *in vitro* therefore are produced in a competition between the α2→3- and α2→6-sialyltransferase in which the branch specificities of these enzymes play major roles. However, the final structure of the sialylated glycans seems to be a function of the polypeptide moiety also (36–38).

The terminal structures of tetraantennary glycans of the *N*-acetyllactosamine type are thought to be produced in an interplay of at least five different enzymes. In addition to the α2→3- and α2→6-sialyltransferase already mentioned, GDP-Fuc:[Galβ1→4]GlcNAc-R α1→3-fucosyltransferase (39), UDP-Gal:Galβ1→4GlcNAc-R α1→3-galactosyltransferase (40), and UDP-GlcNAc:Galβ1→4GlcNAc-R β1→3-*N*-acetylglucosaminyltransferase (41, 42) can act on the Galβ1→4GlcNAc disaccharide units of tetraantennary glycans. Unless the enzymes are physically separated from one another in some way, competition would determine which structure eventually will be produced.

Competition is observed when the action of one enzyme at a given branch precludes subsequent action of a second enzyme on the same branch. Such competition is not limited to an interaction with one and the same terminal sugar residue. Mutual exclusion has been observed for α2→6-sialylation of the Gal and α1→3-fucosylation of the GlcNAc in the *N*-acetyllactosamine unit (33). This is illustrated by the results we obtained with the fucosylated tetraantennary substrate (which consists of a mixture of three isomers) *GP4(Fuc)*. Free competition, however, does not occur since glycosyltransferases show branch specificity. Each of the enzymes possesses a distinct preference for one or two of the Galβ1→4GlcNAc branches, and has a low or intermediate affinity for the other branches (21, 43, 44). In this way, branch specificity reproducibly leads to the formation of specific carbohydrate structures.

The functional significance of the observed glycosylation patterns remains as yet unknown. Specific NeuAc→Gal linkages play a role in recognition processes such as binding of paramyxo- and influenza viruses to cells (45, 46), but functions for the other terminal sugar structures of *N*-acetyllactosamine type glycans are still to be discovered.

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Supplementary material to:

BRANCH SPECIFICITY OF BOVINE COLOSTRUM CMP-SIALIC ACID:Galβ1→4GlcNAc-R α2→6-SIALYLTRANSFERASE

by: D.H. Joziasse, W.E.C.M. Schiphorst, D.H. Van den Eijnden, J.A.Van Kuik, H. van Halbeek, and J.F.G. Vliegenthart

## MATERIALS AND METHODS

### Materials

The complete structures of the various oligosaccharides and glycopeptides referred to in this study and the denoting system which is used to indicate their constituent monosaccharides are presented in Figs. 1 and 2. The glycopeptides GP2, GP3, GP4 and GP4(Fuc) were donated by Dr. K. Schmid (Boston University, Boston, MA) and correspond to the glycopeptides GPII-6, GPII-5, GPV-4 and GPV-3 obtained from desialylated $\alpha_1$-acid glycoprotein, respectively (22). Glycopeptide GP4(Fuc) is a mixture of monofuco-glycopeptides, with L-fucose α1→3-linked to one of the GlcNAc residues 7', 5' or 7 (numbering refers to Fig. 1). GlcNAc-7' contains 25% of the fucose, GlcNAc-5' 15% and GlcNAc-7 60% (4). The oligosaccharides OS2, OS3, OS3' and OS4 (isolated from the urine of patients with $GM_1$ gangliosidosis) were a gift from Dr. G. Strecker (Université de Lille, Villeneuve d'Ascq, France). The synthetic pentasaccharides OS2(3) and OS2(3'), representing partial structures of OS3 and OS3', respectively, were kindly donated by Dr. J. Lönngren (University of Stockholm, Stockholm, Sweden). CMP-[$^{14}$C]-NeuAc and CMP-[$^3$H]NeuAc (specific radioactivity 1.68 Ci/mol and 18,900 Ci/mol, respectively) were obtained from New England Nuclear (Boston, MA) and diluted with unlabeled CMP-NeuAc (23) to the desired specific activity. Bovine colostrum CMP-NeuAc:Galβ1→4GlcNAc-R α2→6-sialyltransferase was partially purified as described previously (19). Enzyme activity was assayed with asialo-$\alpha_1$-acid glycoprotein as acceptor (19).

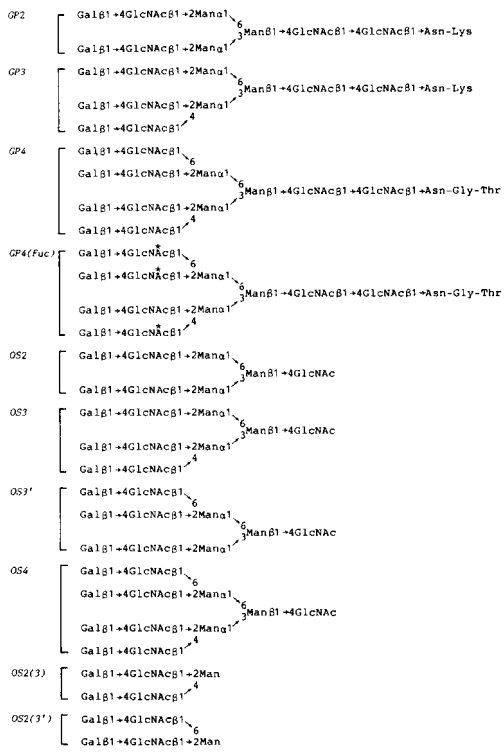


*Fig. 2 - Structures of the various glycopeptides and oligosaccharides used in the present study.* Structures of the branched acceptor substrates referred to in this study are presented in the figure. Glycopeptide GP4(Fuc) is a mixture of monofuco-glycopeptides, in which fucose is present on either of the branches marked with an asterisk.

### Preparation of sialylated products

Oligosaccharides and glycopeptides were sialylated by incubation at 37°C of reaction mixtures containing CMP-[$^3$H]-NeuAc, specific radioactivity 0.312 Ci/mol (2.50 mM), glycerol (20%, v/v), Tris-maleate pH 6.8 (0.2 M), bovine serum albumin (1 mg/mL), and amounts of the various acceptors yielding concentrations of 2.0 mM terminal galactose residues. Oligosaccharides OS3' and OS4 were sialylated at a concentration of 1.5 mM terminal galactose residues. Sialyltransferase concentrations ranged from 7 to 69 mU/mL and incubation times varied from 2 to 24 h in order to obtain sufficient quantities of mono-, bi- and trisialo-products to allow analysis by $^1$H-NMR. The monosialo-product of GP4(Fuc) was isolated and part of it was again incubated with fresh enzyme and CMP-[$^3$H]NeuAc in order to obtain higher yields of bi- and trisialo-product. After incubation the products were separated on a column (1.6 x 200 cm) of Bio-Gel P-6 (200-400 mesh) equilibrated and eluted with 50 mM ammonium acetate at pH 5.2 and 37°C (24). Fractions containing the $^3$H-labeled products were pooled and lyophilized, and each of the products was characterized by 500-MHz $^1$H-NMR.

### High-resolution 500-MHz $^1$H-NMR spectroscopic analysis of the products of sialylation

Prior to $^1$H-NMR spectroscopic analysis, the various substrates and their products of sialylation (30 to 100 nmoles) were repeatedly treated with $D_2O$ at pD 7 and room temperature, and lyophilized. Each sample was then dissolved in 400 µL $D_2O$ (99.996 atom% D, Aldrich, Milwaukee, WI). Further experimental details have been described previously (25).

### Kinetic efficiencies of sialylation of bi-, tri- and tetra-antennary oligosaccharides

The time course of incorporation of sialic acid into branched oligosaccharides was established as follows. A reaction mixture containing 45 nmol oligosaccharide, 225 nmol CMP-[$^{14}$C]-NeuAc (1.68 Ci/mol), 18 µmol Tris-maleate buffer pH 6.8, 90 µg bovine serum albumin and 1.2 mU (for sialylation of the biantennary oligosaccharide OS2), 3.9 mU (triantennary oligosaccharides OS3 and OS3') or 6.6 mU sialyltransferase (tetraantennary oligosaccharide OS4) in a total volume of 90 µL was incubated at 37°C. At intervals aliquots (10 µL) were withdrawn from the reaction mixtures and immediately frozen. Subsequently, the aliquots were subjected to gel chromatography on a column (1.6 x 200 cm) of Bio-Gel P-6 (200-400 mesh) equilibrated and eluted with 50 mM ammonium acetate at pH 5.2 and 37°C, in order to isolate the sialylated products (24). Radioactive compounds were quantified by liquid-scintillation counting and the amounts of mono-, bi- and trisialo-products were calculated. Progress curves were constructed for each of the various sialylated products.

After interpolation using the cubic spline method (computer program E01 BAF of the NAG program library) curves were fitted through the experimental points. From polynomial fits rough estimates were calculated for the kinetic efficiencies (pseudo-first order rate constants) of transfer of the first and the second sialic acid residue, assuming first-order kinetics for the reaction by which monosialo-oligosaccharide is formed from asialo-oligosaccharide (experimental points from 0 to 10 min) and for the disappearance of the monosialo-product (This rate was taken to be equal to the rate of formation of the bi-sialo product).

## RESULTS

### Structures of the products of sialylation as determined by $^1$H-NMR

The sialylated products formed after incubation of the various acceptors with sialyltransferase and CMP-NeuAc could be separated from each other, and from the starting substrates, by gel filtration on Bio-Gel P-6. A typical elution profile showing the separation of the sialylated derivatives of triantennary oligosaccharide OS3 is given in Fig. 3. For each acceptor the mono-, bi- and, if available, trisialo derivatives were subjected separately to 500-MHz $^1$H-NMR spectroscopy.

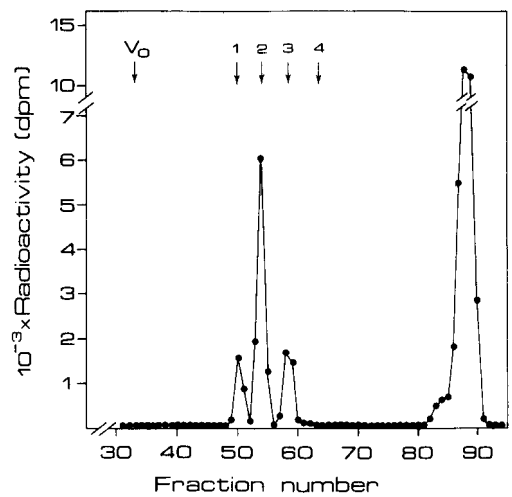


*Fig. 3 - Separation of sialylated oligosaccharides by gel filtration on Bio-Gel P-6.* Oligosaccharide OS3 was incubated with CMP-[$^{14}$C]NeuAc and bovine colostrum α2→6-sialyltransferase as described under "Methods". After 3h at 37°C the mixture was applied to a column (1.6 x 200 cm) of Bio-Gel P-6 (200-400 mesh) equilibrated and eluted at a flow rate 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. OS3-TS; 2. OS3-BS; 3. OS3-MS and 4. the asialo acceptor OS3.

The line of reasoning for determining the position of sialic acid in the sialylated products is illustrated by the $^1$H-NMR spectra of the asialo glycopeptide substrate (GP3), its monosialylated (GP3-MS), bisialylated (GP3-BS) and trisialylated (GP3-TS) derivatives (Fig. 4). The chemical shifts of the structural-reporter groups of the triantennary acceptors GP3, OS3 and OS3' and their sialylated products are listed in Table II.

The $^1$H-NMR data of the asialo glycopeptide GP3 have been discussed extensively (25). The introduction of one NeuAc residue into GP3 gave rise to a single product, GP3-MS; its monosialyl character is confirmed by the intensity ratio of NeuAc reporter group signals and those of other residues being 1:1 (Fig. 4B). This NeuAc residue is present in α2→6-linkage to a Gal residue, as is evident from the typical set of chemical shifts of the NeuAc structural-reporter groups: δH-3ax 1.717; δH-3eq 2.667 and δNAc 2.030 (compare (25)). In GP3-MS NeuAc is attached to Gal-6 exclusively; this can be deduced from the shift increments observed for H-1 of Man-4 (Δδ 0.010 ppm), for H-1 and NAc of

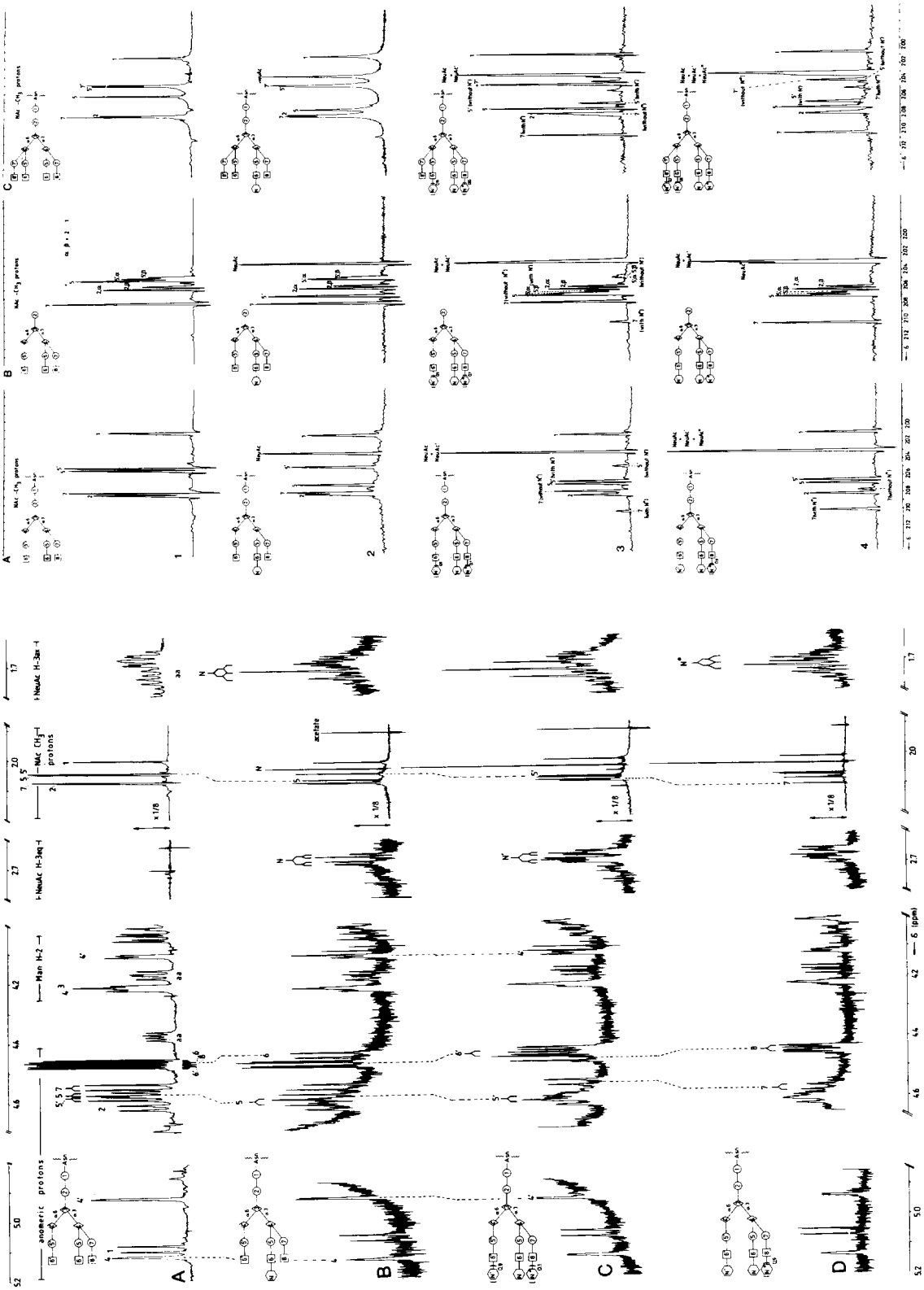

Fig. 4 – *Comparison of the structural-reporter-group regions of the 500-MHz $^1$H-NMR spectra of the triantennary glycopeptides (A) GP3; (B) GP3-BS and (D) GP3-TS. Numbers refer to the corresponding reporter monosaccharides in the structures (cf. Fig. 1). From one trace to another, only the characteristic effects on chemical shifts of reporter-groups due to the NeuAc attachment in question, have been indicated by dashed lines.*



Fig. 5 – *N-Acetyl methyl resonances in the resolution-enhanced 500-MHz $^1$H-NMR spectra of the substrates (trace 1) GP3 (A), OS3 (B) and GP4 (C), their monosialylated (trace 2), bisialylated (trace 3) and trisialylated (trace 4) derivatives. The numbers in the spectra refer to the corresponding reporter monosaccharides in the structures. Assignments belonging to the major components of a mixture have been indicated on top of the spectrum, those of the minor component at the bottom, if appropriate.*

TABLE II

$^1$H Chemical shifts of structural-reporter groups of constituent monosaccharides for various N-acetyllactosamine-type, tri- and tri'-antennary compounds and their in-vitro sialylated derivatives

| Reporter group | Residue[c] | Chemical shift[a] in[b] Anomer of oligosaccharide | | | | | | | | | | Anomer of oligosaccharide | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | GP3 | GP3-MS | GP3-BS1 | GP3-TS | | OS3 | OS3-MS | OS3-BS1 | OS3-BS2 | OS3-TS | | OS3' | OS3'-MS | OS3'-BS1 | OS3'-BS2 |
| H-1 | GlcNAc-1 | 5.092 | 5.057 | 5.057 | 5.060 | | – | – | – | – | – | | – | – | – | – |
| | GlcNAc-2 | 4.614 | 4.613 | 4.614 | 4.615 | α | 5.211 | 5.211 | 5.213 | 5.213 | 5.214 | α | 5.206 | 5.206 | 5.209 | 5.209 |
| | | | | | | β | 4.723 | 4.72 | 4.72 | 4.72 | 4.72 | β | 4.721 | 4.72 | 4.72 | 4.72 |
| | Man-3 | 4.755 | 4.76 | 4.76 | 4.76 | α | 4.767 | 4.76 | 4.76 | 4.76 | 4.76 | α | 4.776 | 4.77 | 4.77 | 4.77 |
| | | | | | | β | 4.757 | | | | | β | 4.770 | | | |
| | Man-4 | 5.120 | 5.130 | 5.130 | 5.133 | α,β | 5.118 | 5.133 | 5.133 | 5.133 | 5.138 | α | 5.133 | 5.143 | 5.136 | 5.136 |
| | | | | | | | | | | | | β | 5.131 | 5.138 | | |
| | Man-4' | 4.924 | 4.923 | 4.939 | 4.936 | α | 4.923 | 4.923 | 4.944 | 4.925 | 4.940 | α | 4.874 | 4.877 | 4.894 | 4.886 |
| | | | | | | β | 4.925 | 4.928 | | | | β | | 4.872 | | |
| | GlcNAc-5 | 4.570 | 4.592 | 4.590 | 4.594 | α,β | 4.569 | 4.593 | 4.592 | 4.592 | 4.596 | α,β | 4.585 | 4.610 | 4.613 | 4.613 |
| | GlcNAc-5' | 4.580 | 4.579 | 4.600 | 4.602 | α,β | 4.584 | 4.583 | 4.603 | 4.579 | 4.603 | α,β | 4.592 | 4.593 | 4.621 | 4.596 |
| | GlcNAc-7 | 4.545 | 4.547 | 4.548 | 4.571 | α,β | 4.546 | 4.549 | 4.551 | 4.570 | 4.572 | | – | – | – | – |
| | GlcNAc-7' | – | – | – | – | | – | – | – | – | – | α,β | 4.555 | 4.557 | 4.556 | 4.576 |
| | Gal-6 | 4.462 | 4.441 | 4.438 | 4.439 | α,β | 4.463 | 4.442 | 4.438 | 4.438 | 4.438 | α,β | 4.469 | 4.445 | 4.442 | 4.442 |
| | Gal-6' | 4.473 | 4.472 | 4.446 | 4.448 | α | 4.471 | 4.469 | 4.444 | 4.469 | 4.443 | α | 4.469 | 4.468 | 4.442 | 4.467 |
| | | | | | | β | 4.473 | 4.472 | 4.447 | | 4.446 | β | 4.471 | | | |
| | Gal-8 | 4.468 | 4.468 | 4.468 | 4.440 | α,β | 4.468 | 4.469 | 4.469 | 4.444 | 4.441 | | – | – | – | – |
| | Gal-8' | – | – | – | – | | – | – | – | – | – | α,β | 4.481 | 4.480 | 4.478 | 4.454 |
| H-2 | Man-3 | 4.209 | 4.213 | 4.217 | 4.220 | α | 4.223 | 4.227 | 4.229 | 4.229 | 4.229 | α | 4.263 | 4.268 | 4.269 | 4.269 |
| | | | | | | β | 4.212 | 4.216 | 4.218 | 4.218 | 4.218 | β | 4.252 | 4.258 | 4.259 | 4.259 |
| | Man-4 | 4.218 | 4.221 | 4.222 | 4.225 | α,β | 4.218 | 4.221 | 4.222 | 4.222 | 4.223 | α | 4.201 | 4.207 | 4.208 | 4.208 |
| | | | | | | | | | | | | β | 4.198 | | | |
| | Man-4' | 4.108 | 4.109 | 4.115 | 4.116 | α,β | 4.114 | 4.113 | 4.119 | 4.119 | 4.118 | α,β | 4.099 | 4.098 | 4.102 | 4.102 |
| H-3ax | NeuAc | – | 1.717 | 1.717 | 1.717 | α,β | – | 1.719 | 1.718 | 1.718 | 1.718[d] | α,β | – | 1.719 | 1.718 | 1.718 |
| | NeuAc' | – | – | 1.717 | 1.717 | α,β | – | – | 1.718 | – | 1.721[d] | α,β | – | – | 1.718 | – |
| | NeuAc* | – | – | – | 1.706 | α,β | – | – | – | 1.705 | 1.706 | | – | – | – | – |
| | NeuAc*' | – | – | – | – | | – | – | – | – | – | α,β | – | – | – | 1.711 |
| H-3eq | NeuAc | – | 2.667 | 2.668 | 2.670 | α,β | – | 2.668 | 2.668 | 2.668 | 2.669 | α,β | – | 2.670 | 2.672 | 2.672 |
| | NeuAc' | – | – | 2.673 | 2.674 | α,β | – | – | 2.672 | – | 2.672 | α,β | – | – | 2.672 | – |
| | NeuAc* | – | – | – | 2.670 | α,β | – | – | – | 2.668 | 2.669 | | – | – | – | – |
| | NeuAc*' | – | – | – | – | | – | – | – | – | – | α,β | – | – | – | 2.672 |
| NAc | GlcNAc-1 | 2.003 | 2.008 | 2.008 | 2.008 | | – | – | – | – | – | | – | – | – | – |
| | GlcNAc-2 | 2.078 | 2.079 | 2.081 | 2.082 | α | 2.060 | 2.061 | 2.063 | 2.063 | 2.063 | α | 2.060 | 2.059 | 2.063 | 2.063 |
| | | | | | | β | 2.057 | 2.058 | 2.060 | 2.060 | 2.060 | β | 2.057 | 2.057 | 2.060 | 2.060 |
| | GlcNAc-5 | 2.048 | 2.067 | 2.068 | 2.069 | α,β | 2.050 | 2.070 | 2.070 | 2.070 | 2.071 | α,β | 2.057 | 2.074 | 2.074 | 2.074 |
| | GlcNAc-5' | 2.045 | 2.046 | 2.064 | 2.065 | α | 2.048 | 2.049 | 2.068 | 2.050 | 2.068 | α | 2.043 | 2.045 | 2.069 | 2.053 |
| | | | | | | β | 2.046 | 2.048 | 2.066 | 2.047 | 2.066 | β | 2.046 | 2.047 | 2.065 | |
| | GlcNAc-7 | 2.075 | 2.076 | 2.077 | 2.101 | α,β | 2.078 | 2.079 | 2.079 | 2.103 | 2.103 | | – | – | – | – |
| | GlcNAc-7' | – | – | – | – | | – | – | – | – | – | α | 2.039 | 2.041 | 2.041 | 2.057 |
| | | | | | | | | | | | | β | 2.040 | | | |
| | NeuAc | – | 2.030 | 2.030 | 2.030 | α,β | – | 2.031 | 2.030 | 2.030 | 2.029[e] | α,β | – | 2.031 | 2.031 | 2.031 |
| | NeuAc' | – | – | 2.030 | 2.030 | α,β | – | – | 2.030 | – | 2.029[e] | α,β | – | – | 2.031 | – |
| | NeuAc* | – | – | – | 2.030 | α,β | – | – | – | 2.030 | 2.031[e] | | – | – | – | – |
| | NeuAc*' | – | – | – | – | | – | – | – | – | – | α,β | – | – | – | 2.031 |

(a) Chemical shifts were acquired at 500 MHz for neutral solutions in $D_2O$ at 27°C. Values are in ppm relative to DSS (using internal acetone at δ 2.225).

(b) In the table-heading, structures are represented by short-hand symbolic notation. ● = GlcNAc; ◆ = Man; ■ = Gal; ○ = NeuAc.

(c) For numbering of monosaccharide residues, see Table I. NeuAc denotes the sialic acid linked to Gal-6. NeuAc' the one linked to Gal-6', NeuAc* the one linked to Gal-8 and NeuAc*' the one linked to Gal-8', respectively.

(d) Assignments may have to be interchanged.

(e)

TABLE III

$^1$H Chemical shifts of structural-reporter groups of constituent monosaccharides for various N-acetyllactosamine-type, tetra-antennary compounds and their in-vitro sialylated analogues

| Reporter group | Residue[c] | Chemical shift[a] in[b] Anomer of oligosaccharide | | | | | Anomer of oligosaccharide | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | GP4 | GP4-MS | GP4-BS1 | GP4-BS2 | GP4-TS | | OS4 | OS4-MS | OS4-BS1 | OS4-BS2 | OS4-TS | GP4(Fuc) | GP4(Fuc)-MS |
| H-1 | GlcNAc-1 | 5.053 | 5.050 | 5.059 | 5.059 | 5.055 | | – | – | – | – | – | 5.067 | 5.052 |
| | GlcNAc-2 | 4.614 | 4.611 | 4.611 | 4.611 | 4.614 | α | 5.205 | 5.204 | 5.204 | 5.204 | 5.207 | 4.611 | 4.613 |
| | | | | | | | β | 4.719 | 4.72 | 4.72 | 4.72 | 4.72 | | |
| | Man-3 | 4.757 | 4.76 | 4.76 | 4.76 | 4.76 | α | 4.768 | 4.76 | 4.76 | 4.76 | 4.76 | 4.758 | 4.76 |
| | | | | | | | β | 4.761 | | | | | | |
| | Man-4 | 5.129 | 5.135 | 5.136 | 5.136 | 5.136 | α | 5.131 | 5.138 | 5.14[f] | 5.14[f] | 5.137 | 5.124 | 5.136 |
| | | | | | | | β | 5.129 | | | | | | |
| | Man-4' | 4.868 | 4.870 | 4.870 | 4.884 | 4.880 | α,β | 4.870 | 4.873 | 4.873 | 4.889 | 4.885 | 4.866 | 4.871 |
| | GlcNAc-5 | 4.573 | 4.593 | 4.598 | 4.598 | 4.598 | α,β | 4.572 | 4.591 | 4.597 | 4.597 | 4.60 | 4.570 | 4.596 |
| | GlcNAc-5' | 4.596 | 4.593 | 4.598 | 4.611 | 4.614 | α,β | 4.592 | 4.596 | 4.59 | 4.610 | 4.602 | 4.594 | 4.595 |
| | | | | | | | | | | | | | 4.61 | F 4.61 |
| | GlcNAc-7 | 4.547 | 4.547 | 4.569 | 4.549 | 4.570 | α,β | 4.547 | 4.551 | 4.571 | 4.551 | 4.576 | 4.545 | |
| | | | | | | | | | | | | | 4.558 | F 4.555 |
| | GlcNAc-7' | 4.553 | 4.554 | 4.556 | 4.556 | 4.556 | α,β | 4.554 | 4.558 | 4.557 | 4.557 | 4.559 | 4.550 | 4.55 |
| | | | | | | | | | | | | | 4.563 | F 4.56 |
| | Gal-6 | 4.465 | 4.438 | 4.437 | 4.437 | 4.438 | α,β | 4.464 | 4.440 | 4.437 | 4.437 | 4.44[g] | 4.462 | 4.441 |
| | Gal-6' | 4.472 | 4.469 | 4.469 | 4.441 | 4.442 | α | 4.469 | 4.468 | 4.467 | 4.442 | 4.44[g] | 4.470 | 4.471 |
| | | | | | | | β | 4.471 | | | | | 4.462 | F n.d. |
| | Gal-8 | 4.470 | 4.469 | 4.441 | 4.469 | 4.438 | α,β | 4.469 | 4.468 | 4.442 | 4.467 | 4.44[g] | 4.468 | 4.471 |
| | | | | | | | | | | | | | 4.447 | F 4.452 |
| | Gal-8' | 4.481 | 4.480 | 4.482 | 4.482 | 4.481 | α,β | 4.480 | 4.481 | 4.482 | 4.482 | 4.481 | 4.479 | 4.482 |
| | | | | | | | | | | | | | 4.473 | F 4.471 |
| H-2 | Man-3 | 4.210 | 4.218 | 4.223 | 4.223 | 4.223 | α | 4.214 | 4.236 | 4.235 | 4.235 | 4.230 | 4.209 | 4.223 |
| | | | | | | | β | 4.203 | 4.219 | 4.223 | | | | |
| | Man-4 | 4.224 | 4.229 | 4.233 | 4.233 | 4.231 | α | 4.228 | 4.230 | 4.235 | 4.235 | 4.230 | 4.222 | 4.230 |
| | | | | | | | β | 4.224 | | | | | | |
| | Man-4' | 4.092 | 4.092 | 4.096 | 4.096 | 4.094 | α,β | 4.099 | 4.099 | 4.100 | 4.100 | 4.106 | 4.092 | 4.096 |
| H-3ax | NeuAc | – | 1.717 | 1.719 | 1.719 | 1.719 | α,β | – | 1.718 | 1.720 | 1.720 | 1.720 | – | 1.719 |
| | NeuAc' | – | – | – | 1.717 | 1.717 | α,β | – | – | – | 1.718 | 1.718 | – | – |
| | NeuAc* | – | – | 1.706 | – | 1.707 | α,β | – | – | 1.706 | – | 1.706 | – | – |
| H-3eq | NeuAc | – | 2.669 | 2.670 | 2.670 | 2.673 | α,β | – | 2.671 | 2.672 | 2.672 | 2.671 | – | 2.670 |
| | NeuAc' | – | – | – | 2.670 | 2.673 | α,β | – | – | – | 2.672 | 2.671 | – | – |
| | NeuAc* | – | – | 2.670 | – | 2.673 | α,β | – | – | 2.672 | – | 2.671 | – | – |
| NAc | GlcNAc-1 | 2.008 | 2.006 | 2.006 | 2.006 | 2.006 | | – | – | – | – | – | 2.006 | 2.006 |
| | GlcNAc-2 | 2.078 | 2.077[e] | 2.077 | 2.077 | 2.079 | α | 2.059 | 2.059 | 2.059 | 2.062 | 2.062 | 2.076 | 2.076 |
| | | | | | | | β | 2.056 | 2.056 | 2.056 | 2.059 | 2.060 | | |
| | GlcNAc-5 | 2.054 | 2.070 | 2.071 | 2.071 | 2.072 | α,β | 2.054 | 2.073 | 2.073 | 2.073 | 2.074 | 2.052 | 2.071 |
| | GlcNAc-5' | 2.042[d] | 2.042 | 2.042 | 2.065 | 2.065 | α | 2.041 | 2.043 | 2.043 | 2.067 | 2.067 | 2.040 | 2.041 |
| | | | | | | | β | 2.045 | 2.046 | 2.046 | | | 2.033 | F 2.035 |
| | GlcNAc-7 | 2.079 | 2.078[e] | 2.103 | 2.077 | 2.103 | α,β | 2.079 | 2.080 | 2.105 | 2.080 | 2.105 | 2.077 | 2.079 |
| | | | | | | | | | | | | | 2.067 | F 2.069 |
| | GlcNAc-7' | 2.041[d] | 2.042 | 2.042 | 2.042 | 2.039 | α | 2.038 | 2.040 | 2.043 | 2.043 | 2.040 | 2.038 | 2.041 |
| | | | | | | | β | 2.040 | | | | | 2.029 | F 2.031 |
| | NeuAc | – | 2.030 | 2.030 | 2.030 | 2.031 | α,β | – | 2.031 | 2.032 | 2.032 | 2.032 | – | 2.031 |
| | NeuAc' | – | – | – | 2.030 | 2.031 | α,β | – | – | – | 2.032 | 2.032 | – | – |
| | NeuAc* | – | – | 2.030 | – | 2.031 | α,β | – | – | 2.028 | – | 2.029 | – | – |
| H-1 | Fuc | – | – | – | – | – | | – | – | – | – | – | 5.112 | 5.115 |
| | | | | | | | | | | | | | 5.124 | 5.118 |
| H-5 | Fuc | – | – | – | – | – | | – | – | – | – | – | 4.832 | 4.835 |
| $CH_3$ | Fuc | – | – | – | – | – | | – | – | – | – | – | 1.177 | 1.178 |

(a) Chemical shifts were acquired at 500 MHz for neutral solutions in $D_2O$ at 27°C. Values are in ppm relative to DSS (using internal acetone at δ 2.225).

(b) In the table-heading, structures are represented by short-hand symbolic notation ● =GlcNAc; ◆ = Man; ■ = Gal; ○ = NeuAc; □ = Fuc.
Oligosaccharides ending in GlcNAc-2 occur, under the applied conditions, as an anomeric mixture in the ratio α/β = 2/1. If for a certain proton, Δδ α-β ≤ 0.001, only an average δ-value has been listed.

(c) For numbering of monosaccharide residues, see Table I.
NeuAc denotes the sialic acid linked to Gal-6. NeuAc' the one linked to Gal-6', NeuAc* the one linked to Gal-8 and NeuAc*' the one linked to Gal-8', respectively.

(d) Assignments may have to be interchanged.

(e)

(f) The H-1 signals of Man-4 in the spectrum of OS4-BS1 and -BS2 was split into two, slightly separated signals.

(g) The H-1 signals of Gal-6, -6' and 8 were hardly separated from each other.

(F) Chemical shift in fucosylated branch.

GlcNAc-5 (Δδ 0.022 ppm and Δδ 0.019 ppm, respectively), and for H-1 of Gal-6 (Δδ 0.021), compared to GP3 [3].

The bisialylated product (GP3-BS) of the incubation of GP3 with CMP-NeuAc in the presence of sialyltransferase appeared to be a mixture of two isomers differing in the branch to which the secondly introduced NeuAc residue was attached. This can be seen from the occurrence of two sets of structural-reporter groups, in the intensity ratio 9:1 (Fig. 4C). The higher-intensity signals belong to the major component GP3-BS1 having NeuAc' in α2→6-linkage to Gal-6'; this could be inferred from the shift effects observed going from GP3-MS to GP3-BS1 (Fig. 4B+C). The relevant structural-reporter groups are H-1 and H-2 of Man-4' (Δδ 0.016 ppm and Δδ 0.006 ppm, respectively), H-1 and NAc of GlcNAc-5' (Δδ 0.021 ppm and Δδ 0.018 ppm, respectively), H-1 of Gal-6' (Δδ -0.026 ppm) and NAc of GlcNAc-2 (Δδ 0.002 ppm) (Table II). A set of lower-intensity signals reveal the attachment of NeuAc in α2→6-linkage to Gal-8 in the minor (10%) component GP3-BS2. Especially the NAc signal of GlcNAc-7' (δ 2.101) is characteristic for NeuAc attached to Gal-8 (25). Concomitantly, an equally-low-intensity NAc signal at δ 2.047 ppm revealed the presence of non-sialylated Gal-6' linked to GlcNAc-5' in GP3-BS2. This is corroborated by the presence of small signals for H-1 of Man-4' at δ 4.923, and for H-1 of Gal-6' at δ 4.472 (Fig. 4C)[4]. In the spectrum of the completely sialylated product GP3-TS, the afore-mentioned shift effects for reporter groups of the three branches were found together, indicating that all three Gal residues bear an α2→6-linked NeuAc residue. The NMR-features of GP3-TS have been described before (25,27). It should be noted that the chemical shift of H-3ax of NeuAc*, i.e. NeuAc linked to Gal-8, (δ 1.706) in conjunction with that of the NAc protons of GlcNAc-7 (δ 2.101) is typical of α2→6-sialylation of the β1→4-linked N-acetyllactosamine branch 7-8 (25).

The 500-MHz $^1$H-NMR characteristics of the triantennary oligosaccharide ending in GlcNAc-2, OS3, have been published (25). It should be noted that this compound, as usual for N-acetyllactosamine-type oligosaccharides ending in GlcNAc-2 (compare OS3' and OS4) occurs, under the applied measuring conditions, as a mixture of two anomers, containing GlcNAc-2 in the α- and β-pyranose form, in the ratio of 2:1 (cf. refs. 20,25). 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 anomerization as is reflected by doubling of each of these signals in the anomeric intensity ratio (see for example, the NAc region of the OS3 spectrum (Fig. 5B1)).

The monosialylated (OS3-MS), bisialylated (OS3-BS) and trisialylated (OS3-TS) products are the oligosaccharide analogues of GP3-MS, GP3-BS and GP3-TS, respectively, lacking the GlcNAc (β1→N)Asn-Lys part. In OS3-MS the NeuAc residue is α2→6-linked to Gal-6 exclusively. OS3-BS again is a mixture of two isomers in a ratio of 9:1, which carry NeuAc residues at Gal-6 and Gal-6' (OS3-BS1) and Gal-6 and Gal-8 (OS3-BS2), respectively (see Fig. 5B3). The rationale for localizing the NeuAc residues in certain branches is analogous to that applied for GP3-BS (see above) and OS-MS (20). In OS3-TS (Fig. 5B4) all three branches are terminated by NeuAc α2→6-linked to Gal. The NMR features of the latter compound have been described (25,27). The shift effects due to sialylation described above for the glycopeptides are found again for the corresponding steps in the oligosaccharide series (Table II).

The 500-MHz $^1$H-NMR spectrum of the so called tri'-antennary structure OS3', that is an oligosaccharide with GlcNAc-2 in the reducing position, having the third Galβ1→4GlcNAc branch attached to Man-4' in β1→6 bond, has been published (25). The branch-selectivity of the sialyltransferase towards this substrate is demonstrated by the structure of the monosialyl product OS3'-MS. This oligosaccharide possesses NeuAc solely attached to Gal-6; this conclusion is based on the chemical shift effects observed in the step from OS3' to OS3'-MS, which are comparable to those discussed for similar steps (GP3 to GP3-MS; OS3 to OS3-MS). Analogous to the situation with OS3-BS, OS3'-BS consists of a mixture of two isomers, OS3'-BS1 (with NeuAc residues linked to Gal-6 and Gal-6') and OS3'-BS2 (with NeuAc residues linked to Gal-6 and Gal-8'), in a ratio of 9:1. The shift effects observed upon comparison of the data of the major component OS3'-BS1 with those of OS3, are essentially the same as described for the step from GP3 to GP3-BS1. The minor component, OS3'-BS2 can be recognized from its set of lower-intensity structural-reporter group signals. The most pronounced effect of the attachment of NeuAc to Gal-8' (NeuAc*') is the change in chemical shift for NAc of GlcNAc-7' (Δδ 0.016 ppm) together with those for H-1 of GlcNAc-7' (Δδ 0.02 ppm) and for H-1 of Gal-8' (Δδ -0.024 ppm). Notably, the H-3ax of NeuAc*' has a unique chemical shift (δ 1.711), which distinguishes this residue from all other NeuAcα2→6 residues in tri-, tri'- or tetraantennary glycans.

The $^1$H-NMR features of the tetra-antennary substrates GP4, OS4 and GP4(Fuc) have been described in detail (25). Relevant NMR parameters for the 3 structures and their sialylated derivatives are listed in Table III. The branch location of NeuAc residues in the latter products could be deduced by the same reasoning as described for the sialylated triantennary structures. The sequence in which the NeuAc residues are attached to the various branches is readily deduced from the NAc methyl proton regions of the $^1$H-NMR spectra of the substrate GP4, and of its products of sialylation (GP4-MS, GP4-BS and GP4-TS), as illustrated in Fig. 5C.

The chemical shifts of the structural-reporter groups of the pentasaccharides OS2(3) and OS2(3') -which are partial structures of tri- and tetraantennary oligosaccharides- have been described by Bock et al. (28) who acquired their data at 400 MHz. The corresponding values for the substrates, refined at 500 MHz, and those for their sialylated derivatives are compiled in Table IV. The NAc CH$_3$-regions of the NMR spectra of OS2(3) and OS2(3'), OS2(3)-MS, OS2(3)-BS, OS2(3')-MS and OS2(3')-BS are depicted in Fig. 6. From these spectra it can be deduced that OS2(3)-MS is a mixture of two isomers in a ratio of 3:1, carrying sialic acid α2-6-linked to Gal-8 and Gal-6, respectively. The product OS2(3')-MS is a mixture of two compounds in a ratio of 9:1, in which a sialic acid residue is attached to Gal-8' or Gal-6', respectively.

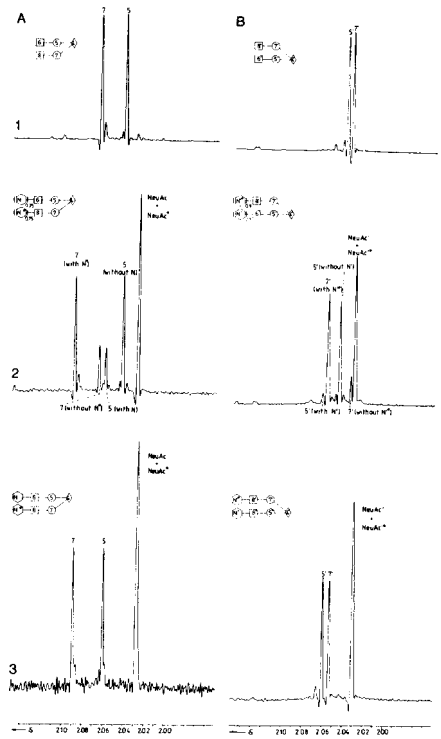


*Fig. 6 - N-Acetyl methyl resonances in the resolution-enhanced 500-MHz $^1$H-NMR spectra of the substrates (trace 1)* OS2(3) *(A) and* OS2(3') *(B), their monosialylated (trace 2) and bisialylated (trace 3) derivatives.* The numbers in the spectra refer to the corresponding GlcNAc residues in the structures. Assignments belonging to the major component of a mixture have been indicated on top of the spectrum, those of the minor component at the bottom, if appropriate.

TABLE IV

$^1$H Chemical shifts of structural-reporter groups of constituent monosaccharides for two N-acetyllactosamine-type pentasaccharides and their in-vitro sialylated analogues

| Reporter group | Residue[c] | Chemical shift[a] in[b] OS2(3) | OS2(3)-MS1 | OS2(3)-MS2 | OS2(3)-BS | OS2(3') | OS2(3')-MS1 | OS2(3')-MS2 | OS2(3')-BS |
|---|---|---|---|---|---|---|---|---|---|
| H-1 | Man-4 | 5.174 | 5.174 | 5.190 | 5.190 | - | - | - | - |
| | Man-4' | - | - | - | - | 5.158 | 5.170 | 5.175 | 5.186 |
| | GlcNAc-5 | 4.589 | 4.593 | 4.629 | 4.631 | - | - | - | - |
| | GlcNAc-5' | - | - | - | - | 4.611 | 4.616 | 4.640 | 4.643 |
| | GlcNAc-2 | 4.560 | 4.563 | 4.543 | 4.564 | - | - | - | - |
| | GlcNAc-2' | - | - | - | - | 4.600 | 4.620 | 4.604 | 4.622 |
| | Gal-6 | 4.465 | 4.466 | 4.444 | 4.446 | - | - | - | - |
| | Gal-6' | - | - | - | - | 4.466 | 4.466 | 4.446 | 4.445 |
| | Gal-8 | 4.468 | 4.440 | 4.469 | 4.438 | - | - | - | - |
| | Gal-8' | - | - | - | - | 4.472 | 4.446 | 4.472 | 4.445 |
| H-2 | Man-4 | 4.066 | 4.068 | 4.068 | 4.072 | - | - | - | - |
| | Man-4' | - | - | - | - | 4.026 | 4.030 | 4.030 | 4.040 |
| H-3ax | NeuAc | - | - | 1.717 | 1.719 | - | - | - | - |
| | NeuAc' | - | - | - | - | - | - | 1.720 | 1.722 |
| | NeuAc* | - | 1.705 | - | 1.705 | - | - | - | - |
| | NeuAc*' | - | - | - | - | - | 1.713 | - | 1.714 |
| H-3eq | NeuAc | - | - | 2.668 | 2.668 | - | - | - | - |
| | NeuAc' | - | 2.668 | - | - | - | - | 2.669 | 2.671 |
| | NeuAc* | - | - | - | 2.668 | - | - | - | - |
| | NeuAc*' | - | - | - | - | - | 2.669 | - | 2.671 |
| NAc | GlcNAc-5 | 2.041 | 2.043 | 2.060 | 2.060 | - | - | - | - |
| | GlcNAc-5' | - | - | - | - | 2.039 | 2.044 | 2.060 | 2.060 |
| | GlcNAc-7 | 2.095 | 2.090 | 2.066 | 2.090 | - | - | - | - |
| | GlcNAc-7' | - | - | - | - | 2.034 | 2.056 | 2.033 | 2.053 |
| | NeuAc | - | - | 2.028 | 2.028 | - | - | - | - |
| | NeuAc' | - | - | - | - | - | - | 2.029 | 2.030 |
| | NeuAc* | - | 2.028 | - | 2.028 | - | - | - | - |
| | NeuAc*' | - | - | - | - | - | 2.029 | - | 2.030 |

(a) Chemical shifts were acquired at 500 MHz for neutral solutions in $D_2O$ at 27°C. Values are in ppm relative to DSS (using internal acetone at δ 2.225).

(b) In the table-heading, structures are represented by short-hand symbolic notation. ● = GlcNAc; ♦ = Man; ■ = Gal; ○ = NeuAc.
It should be noted that for these oligosaccharides only the α-anomer of the reducing Man residue could be detected. Hence, no anomerization effects on chemical shifts were observed.

(c) For numbering of monosaccharide residues, see Table I. NeuAc denotes the sialic acid linked to Gal-6, NeuAc' the one linked to Gal-6', NeuAc* the one linked to Gal-8 and NeuAc*' the one linked to Gal-8', respectively.

3. On the basis of the present data the previously assigned chemical shift values for H-1 of Gal-6 and Gal-8 (26) had to be interchanged. The correct assignments for these protons are now mentioned throughout this paper for substrates GP3, OS3, GP4, OS4, and GP4(Fuc) (Tables II and III).

4. Note that this result is at variance with our tentative conclusion (19) that the second NeuAc residue was almost exclusively attached to Gal-8. Integration of the 360-MHz spectrum of a sialylated triantennary structure, which, at the time, had not been subfractionated by gel filtration on Bio-Gel, suggested that more than one NeuAc residue per mole was present. Attachment of NeuAc to Gal-6' could be excluded since no chemical shift effect was observed on Man-4' H-1. Therefore attachment of NeuAc to Gal-8 was proposed. In the light of the present results it is most likely that the integration has been misleading in suggesting the incorporation of nearly two, instead of one NeuAc residue. Today the effect on the N-acetyl of GlcNAc-7 is a very sensitive marker for α2→6-sialylation of Gal-8.

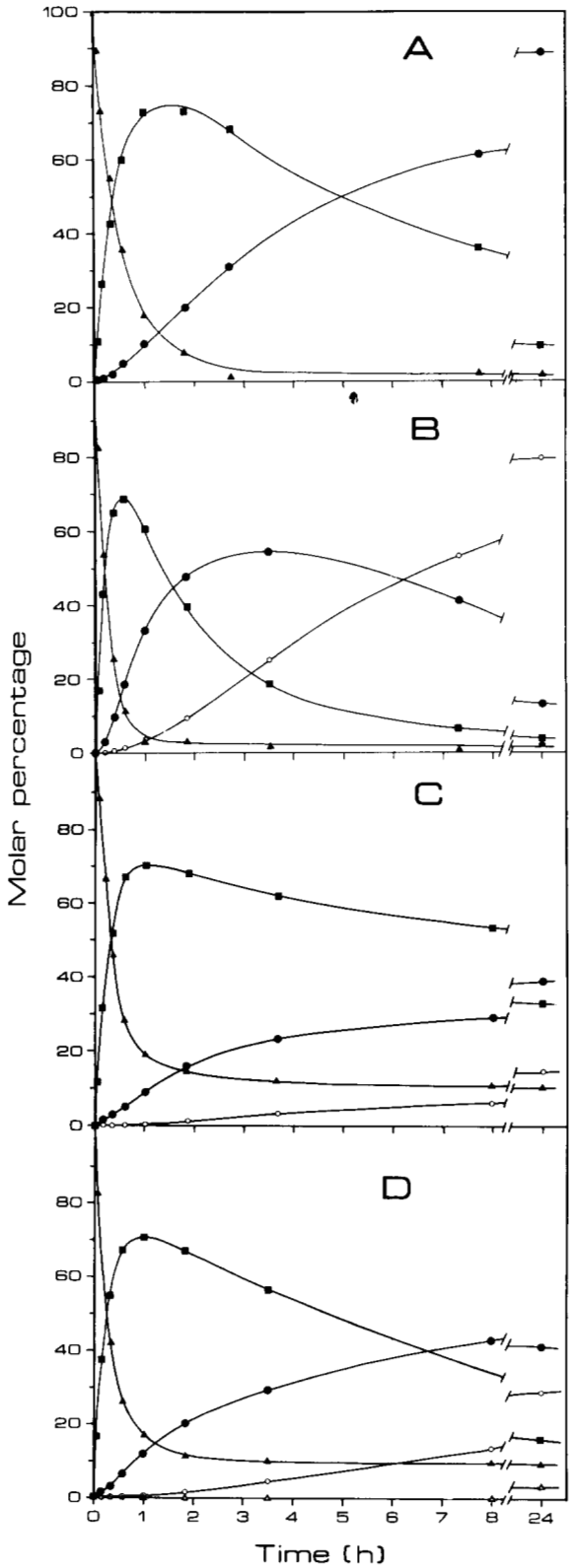


Fig. 7 - *Sialylation* in vitro *of branched oligosaccharides of the* N-*acetyl-lactosamine type.* Oligosaccharides OS2 (A), OS3 (B), OS3' (C) and OS4 (D) were sialylated using bovine colostrum α2→6-sialyltransferase and CMP-[$^{14}$C]-NeuAc. At intervals samples were taken from the reaction mixtures and treated as described in the legend to Fig. 3. Labeled products were quantified by liquid-scintillation counting and progress curves were constructed for each of the various sialylated species. Key: ▲, asialo-; ■, monosialo-; ●, bi-sialo-; ○, trisialo- and △, tetrasialo-oligosaccharide.