(Jones et al., 1980; Littledike et al., 1982) have shown previously that renal 24-hydroxylase of rat is stimulated by prior treatment of the animal with large doses of vitamin D_3, and the studies here avoided the influence of the 24-hydroxylase by utilizing 24(*R*),25-$(OH)_2D_3$ as the substrate.

The functional significance of the pathway culminating in 24,25,26,27-tetranor-23-$(OH)D_3$ is still in question. Though the final product isolated thus far is probably inactive due to its abbreviated side chain, it will be interesting to determine whether its precursors, 24-oxo-25-$(OH)D_3$ or 24-oxo-23,25-$(OH)_2D_3$, have biological activity. Perhaps these intermediates of the pathway represent oxidations of the side chain of the vitamin D molecule in preparation for cleavage between carbons 23 and 24.

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Registry No. 24(*R*),25-$(OH)_2D_3$, 55721-11-4; 24,25,26,27-tetranor-23-$(OH)D_3$, 88200-28-6; 24-oxo-25-$(OH)D_3$, 74886-61-6; vitamin D_3, 67-97-0.

References

Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911–917.
Jones, G. (1980) *J. Chromatogr. 221*, 27–37.
Jones, G. (1983) *J. Chromatogr. 276*, 69–75.
Jones, G., Vieth, R., Cifuentes, R. F., & Radde, I. C. (1980) *Am. Soc. Bone Min. Res.*, 36A.
Jones, G., Kung, M., & Kano, K. (1983) *J. Biol. Chem. 258*, 12920–12928.
Kano, K., & Jones, G. (1984) *Endocrinology (Baltimore) 114*, 330–336.
Kung, M., Jones, G., Kooh, S. W., & Fraser, D. (1983) *Can. Fed. Biol. Soc.*, 43.
Littledike, E. T., & Horst, R. L. (1982) *Endocrinology (Baltimore) 111*, 2008–2013.
Mayer, E., Reddy, G. S., Chandraratna, R. A. S., Okamura, W. H., Kruse, J. R., Popjak, G., Bishop, J. E., & Norman, A. W. (1983) *Biochemistry 22*, 1798–1805.
Reddy, G. S., Jones, G., Kooh, S. W., & Fraser, D. (1982) *Am. J. Physiol. 243*, E265–E271.
Reddy, G. S., Jones, G., Kooh, S. W., Fraser, D., & DeLuca, H. F. (1983) *Am. J. Physiol. 245*, E359–E364.
Rosenthal, A. M., Jones, G., Kooh, S. W., & Fraser, D. (1980) *Am. J. Physiol. 239*, E12–E20.
Takasaki, Y., Horiuchi, N., & Suda, T. (1978) *Biochem. Biophys. Res. Commun. 85*, 601–607.
Takasaki, Y., Suda, T., Yamada, S., Takayama, H., & Nishii, Y. (1981) *Biochemistry 20*, 1681–1686.
Takasaki, Y., Suda, T., Yamada, S., Ohmori, M., Takayama, H., & Nishii, Y. (1983) *J. Biol. Chem. 257*, 3732–3738.
Wichmann, J. K., Schnoes, H. K., & DeLuca, H. F. (1981) *Biochemistry 20*, 7385–7391.
Yamada, S., Nakayama, K., & Takayama, H. (1981a) *Chem. Pharm. Bull. 29*, 2893–2896.
Yamada, S., Ohmori, M., Takayama, H., Suda, T., & Takasaki, Y. (1981b) *Chem. Pharm. Bull. 29*, 1187–1188.
Yamada, S., Ohmori, M., Takayama, H., Takasaki, Y., & Suda, T. (1983) *J. Biol. Chem. 258*, 457–463.

Structural and Conformational Analysis of Sialyloligosaccharides Using Carbon-13 Nuclear Magnetic Resonance Spectroscopy†

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ABSTRACT: The analysis of the carbon-13 chemical shift data of NeuAcα(2→3)Galβ(1→4)Glc and NeuAcα(2→3)Galβ-(1→4)GlcNAc and their respective NeuAcα(2→6) isomers established distinct and different conformations of the sialic acid residue, depending on the type of anomeric linkage [α-(2→3) vs. α(2→6)]. Interactions between the NeuAc residue and the Glc or GlcNAc residue are particularly strong in the case of the α(2→6) isomers. Similar effects are observed for the larger oligosaccharides [$II^3(NeuAc)_2Lac$ and $IV^6NeuAcLcOse_4$] and even in intact glycoproteins and polysaccharides. It is proposed that the NeuAcα(2→3) isomers assume an extended conformation with the sialic residue at the end (terminal) of the oligosaccharide chain or branch. The NeuAcα(2→6) isomers are assumed to be folded back toward the inner core sugar residues.

Carbon-13 nuclear magnetic resonance (^{13}C NMR) spectroscopy has recently emerged as a complementary method to 500-MHz ^1H NMR spectroscopy for structural elucidation of naturally occurring carbohydrate side chains of oligosaccharides (Shashkov et al., 1979; Berman, 1983; Jaques et al., 1980; Nunez et al., 1981; Messer et al., 1982), glycopeptides (Prohaska et al., 1981; Berman & Allerhand, 1981; Daman & Dill, 1983; Dijkstra et al., 1983), glycolipids (Sillerud et al., 1982; Sillerud & Yu, 1983), and glycoproteins (Dill & Allerhand, 1979; Berman et al., 1980; Berman et al., 1981; Barrett-Bee et al., 1982; Goux et al., 1982). It is well documented that glycosidases show a considerable degree of specificity toward the kind of sugar linkages encountered in a particular structure (Kobata, 1979); however, the degree of specificity thus observed is also strongly dependent on the overall carbohydrate structure (Berman & Allerhand, 1981; Kobata, 1979). This may be due to some defined structural features, as in the case of various endo-β-*N*-acetylglucosaminidases (Kobata, 1979), or it may be, to a certain degree, related to a well-defined conformational change. Such conformational effects may be the major reason that most sialidases show substrate specificity toward the $x = 3$ or $x = 6$ linkages in NeuAcα(2→x)Galβ(1→4)Glc[NAc]β1→R^1

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Table I: Chemical Shifts (ppm) and Assignments of NeuAcα(2→8)NeuAc(2→3)Galβ(1→4)Glc [II^3(NeuAc)$_2$Lac][a]

sugar	carbon 1	2	3	4	5	6	7	8	9	10	11
NeuAcα(2→8)	174.77[b]	101.85[b]	41.77[b]	69.76[b]	53.58	73.94	69.45[b]	73.02	63.90	176.27	23.33[b]
NeuAcα(2→3)	174.61[b]	101.48[b]	41.03[b]	70.59[b]	53.05	75.26	69.21[b]	79.44	62.84	176.27	23.61[b]
Galβ(1→4)	103.99	70.64	76.50[c]	68.77	76.74[c]	62.39					
Glcα	93.13	72.48	72.63	79.44	71.44	61.20					
Glcβ	97.08	75.14	75.57	79.34	76.13	61.34					

[a] In D_2O, pH 6.5, T = 30 °C, 11 500 scans, 3-s recycle. [b] Assignment interchanged down any column. [c] Assignment interchanged along any row.

Table II: Chemical Shifts (ppm) and Assignments of NeuAcα(2→6)Galβ(1→4)GlcNAcβ(1→3)Galβ(1→4)Glc [IV^{6}NeuAcLcOse$_4$][a]

sugar	carbon 1	2	3	4	5	6	7	8	9	10	11
NeuAcα(2→6)	174.77	101.45	41.38	69.72	53.21	73.86	69.72	73.03	64.01	176.21	23.55
6-Galβ(1→4)	104.75	72.07	73.77	69.51	75.01	64.66					
GlcNAcβ(1→3)[c]	104.23	56.29	73.55	81.77	75.68[b]	61.50					
3-Galβ(1→4)	103.85	71.30	83.30	69.87	76.20[b]	62.29					
Glcα	93.13	72.46	72.72	79.79	71.43	61.29					
Glcβ	97.05	75.12	75.61	79.67	76.11	61.41					

[a] In D_2O, pH 6.1, T = 30 °C, 22 000 scans, 2-s recycle. [b] Assignment interchanged down any column. [c] CH_3 = 23.61; CO = 174.77.

(Schauer, 1982; Friebolin et al., 1981; Corfield et al., 1982; Keilich et al., 1982).

Long-range chemical shift effects were previously observed in the ^{1}H NMR spectra of glycopeptides and oligosaccharides that carry the NeuAcα(2→6) linkages vs. the NeuAcα(2→3) linkages (Montreuil & Vliegenthart, 1977; Vliegenthart, 1979; Dorland et al., 1978). These effects extend as far as the H1 of the mannose residue, which is three sugar residues removed from the NeuAc attachment point to the Gal residue (Vliegenthart, 1979). In contrast, the α(2→3) linkages do not have substantial long-range effects. Thus the long-range chemical shift changes observed for α(2→6) linkages (Montreuil & Vliegenthart, 1977) must be of conformational origin. In principle, ^{13}C NMR spectroscopy can be used to detect similar effects[2] with some advantages over ^{1}H NMR. The minimal ^{13}C NMR chemical shift overlap allows monitoring of all the ring carbons of a given sugar for such conformational effects. Finally, most important of all is the large ^{13}C NMR chemical shift range (Dill & Allerhand, 1979; Berman et al., 1981), which makes it possible to observe these effects in intact glycoproteins. Indeed, Jennings et al. (1981) have established the utility of ^{13}C NMR as a sensitive probe for conformational changes in polysaccharides that possesses these types of structures.

In this work we report the ^{13}C NMR chemical shift changes observed upon sialic acid substitution (Berman, 1983) in some sialylglycans and compare them with similar substitutions in other types of sugars (Messer et al., 1982; Dijkstra et al., 1983; Srivastava et al., 1980; Rana et al., 1982; Roy & Jennings, 1983; McCleary et al., 1982; Abbas et al., 1983).[2]

Experimental Procedures

II^3(NeuAc)$_2$Lac from cow colostrum and IV^{6}NeuAcLcOse$_4$ from human milk were gifts from Drs. H. Friebolin, University of Heidelberg, and H. V. Nicolai, University of Bonn, and NeuAcα(2→6)Lac was kindly given by Dr. B. Binsch, University of Heidelberg.

^{13}C NMR. Spectra were run at 60.5 MHz, with 10-mm tubes and in D_2O with traces of dioxane as an internal standard (67.86 ppm). Data processing was done as described before (Berman, 1983).

Results and Discussion

Tables I and II give chemical shifts and the assignments for NeuAcα(2→8)NeuAcα(2→3)Galβ(1→4)Glc [II^3(NeuAc$_2$)Lac] and NeuAcα(2→6)Galβ(1→4)GlcNAcβ(1→3)-Galβ(1→4)Glc [IV^{6}NeuAcLcOse$_4$]. The assignments are based on comparison with previous literature (Berman, 1983; Prohaska et al., 1981; Daman & Dill, 1983; Harris & Thornton, 1978; Bhattacharjee et al., 1975; Eschenfelder et al., 1975; Collins et al., 1981), the use of partially relaxed ^{13}C spectra,[2] and on comparison of deuterium isotope chemical shifts (Berman, 1983). The assignments for a synthetic NeuAcα(2→6)Galβ(1→4)Glc [NeuAcα(2→6)Lac] are given in column 3 of Table IV.

In Table III, we compare the chemical shift substitution patterns observed for the sialyllactosamine isomers in order to establish the contribution of the *N*-acetylamino group. Table IV lists the range of ^{13}C substituent chemical shifts (SCS) observed upon C6 substitution of a given sugar, in a variety of oligosaccharides, by another hexose (Messer et al., 1982; Dijkstra et al., 1983; Srivastava et al., 1980; Rana et al., 1982; Roy & Jennings, 1983; McCleary et al., 1982; Abbas et al., 1983) and by sialic acid (Berman, 1983). We shall deal first with the assignments for the above three compounds and then with the interpretation of the results in Tables III and IV.

Assignments. (A) II^3(NeuAc)$_2$Lac. The assignments (Table I) were based on deuterium isotope effects, which helped to distinguish between free hydroxyl-bearing carbons ($\Delta\delta \sim$ 0.1–0.20 ppm) and O-substituted carbons ($\Delta\delta <$ 0.06 ppm), and pH-induced chemical shift changes due to the free carboxyl grouping on the sialic acid residues. The assignment for the lactose part of the molecule [i.e., →3Galβ(1→4)Glc] was made by comparison to other lactose derivatives (Berman, 1983). The chemical shifts observed for the above sugar moiety compared very well (a.d. = 0.04 ppm) with those found for NeuAcα(2→3)lactose (Berman, 1983) with the exception of C4 of the α- and β-D anomers of the Glc residues ($\Delta\delta$ = −0.22 and 0.19 ppm, respectively). The assignments of the

[1] Abbreviations: II^3(NeuAc)$_2$Lac, NeuAcα(2→8)NeuAcα(2→6)-Galβ(1→4)Glc; IV^{6}NeuAcLcOse$_4$, NeuAcα(2→6)Galβ(1→4)-GlcNAcβ(1→3)Galβ(1→4)Glc; NeuAcα(2→6)Lac, NeuAcα(2→6)-Galβ(1→4)Glc; NeuAcα(2→6)lactosamine, NeuAcα(2→6)Galβ(1→-4)GlcNAc; NeuAcα(2→3)Lac, NeuAcα(2→3)Galβ(1→4)Glc; NeuAcα(2→3)lactosamine, NeuAcα(2→3)Galβ(1→4)GlcNAc; Galβ-(1→3)Lac, Galβ(1→3)Galβ(1→4)Glc; a.d., average deviation; SCS, substituent chemical shift. All hexoses are in the D-pyranoside form.

[2] See Allerhand & Berman (1984).

Table III: ^{13}C NMR Chemical Shift (ppm) Comparison for NeuAcα(2→3) and NeuAcα(2→6) Isomers of Lactose [Galβ(1→4)Glc] and Lactosamine [Galβ(1→4)GlcNAc][a]

	lactose			lactosamine		
sugar	2→3	2→6	Δδ (ppm)	2→3	2→6	Δδ (ppm)
NeuAc						
1	175.08	174.76	0.32	175.13	174.81	0.32
2	101.09	101.60	−0.51	101.12	101.49	−0.37
3	40.93	41.41	−0.48	40.95	41.39	−0.44
4	69.59	69.71[b]	−0.12	69.71	69.71[b]	0.00
5	52.98	53.10	−0.12	53.00	53.19	−0.19
6	74.15	73.83[b]	0.67	74.18	73.85[b]	0.33
7	69.41	69.71[b]	−0.30	69.43	69.71[b]	−0.28
8	73.04	73.10	−0.06	73.04	73.04	0.00
9	63.89	63.96	−0.07	63.92	63.97	−0.05
10	176.29	176.22	0.07	176.32	176.24	0.08
11	23.32	23.36	−0.04	23.33	23.33	0.00
Gal						
1	103.92	104.52	−0.60	103.92	104.78	−0.86
2	70.64[b]	72.10	−1.46	70.67	72.06	−1.39
3	76.43[b]	73.69[b]	2.74	76.47	73.76[b]	2.71
4	68.77[b]	69.83	−1.06	68.78	69.53[b]	−0.75
5	76.78	75.04	1.74	76.80	75.00	1.80
6	62.29	64.86	−2.57	62.27	64.67	−2.40
Glcα or GlcNAcα						
1	93.10	93.12	−0.02	91.88	91.88	0.00
2	72.46	72.38	0.08	55.02	54.76	0.26
3	72.65	72.93	−0.28	70.53	70.67	−0.14
4	79.66	81.04	−1.38	80.02	82.22	−2.20
5	71.37	71.27	0.10	71.54	71.32	0.22
6	61.25	61.44	−0.19	61.27	61.54	−0.27
CO				176.02	175.73	0.29
CH_3				23.19	23.33	−0.14
Glcβ or GlcNAcβ						
1	97.05	96.96	0.09	96.21	96.03	0.18
2	75.10	75.04	0.06	57.51	57.32	0.19
3	75.61	75.93	−0.32	73.76	73.76	0.00
4	79.53	80.94	−1.41	79.59	81.98	−2.39
5	76.07	75.93	0.14	76.15	75.87	0.28
6	61.39	61.59	−0.20	61.39	61.70	0.31
CO				176.02	175.73	0.29
CH_3				23.46	23.59	−0.13

[a] The chemical shifts are given relative to dioxane as internal standard (67.86 ppm) in D_2O solutions at neutral pH. The data of the NeuAcα-(2→3) lactose isomer and the two lactosamine isomers were taken from Berman (1983) with minor assignments corrections. [b] Assignments may be interchanged.

Table IV: Observed Substituent Chemical Shifts (SCS) (ppm) of Oligosaccharides upon C6 Substitution[a]

		carbon							carbon						
X	Y	1	2	3	4	5	6	Z	1	2	3	4	5	6	ref
Manα(1→6)	Manα(1→6)	0.20	0.04	0.38	−0.32	−1.84	4.37	Manβ(1→4)	0.14	0.05	0.04	0.04	0.04	0.21	*b*
Galβ(1→6)	Galβ(1→6)	−0.04	−0.05	0.15	−0.07	−1.42	7.64	Galβ-OMe	−0.12	−0.14	−0.15	0.19	−0.05	−0.08	*c*
Fucα(1→6)	GlcNAcβ	0.17	−0.12	−0.10	−0.06	−2.22	7.27								*d*
Glcβ(1→6)	GalNAcβ	0.10	0.00	−0.10	−0.30	−0.90	7.80								*e*
GlcNAcβ(1→6)	GalNAcα	−0.04	−0.04	−0.19	−0.08	−2.00	8.03								*f*
Manα(1→6)	Manβ(1→4)	*0.57*	−0.02	−0.05	0.27	−1.74	5.35	GlcNAcβ(1→4)	0.12	−0.11	0.02	*1.01*	−0.13	0.04	*g*
Galα(1→6)	Manβ(1→4)	−0.15	−0.15	0.00	−0.10	−1.90	5.25	Manβ	−0.05	−0.15	0.00	*0.55*	−0.15	0.05	*h*
NeuAcα(2→6)	Galβ(1→4)	*0.57*	−0.25	−0.11	−0.37	−1.65	*2.34*	GlcNAcβ	−0.15	−0.2	*−1.24*	*2.21*	−0.27	0.25	*i*
NeuAcα(2→6)	Galβ(1→4)	*0.35*	−0.18	−0.26	−0.03	−1.54	*2.57*	Glcβ	−0.04	−0.06	0.28	*1.31*	−0.12	0.19	*i*

[a] $\Delta\delta Ci = \delta C(XYZ)i - \delta C(YZ)i$; the italicized numbers are discussed in the text. [b] See footnote 2. [c] Srivastava et al. (1980). [d] Rana et al. (1982). [e] Roy & Jennings (1983). [f] Abbas et al. (1983). [g] Dijkstra et al. (1983); chemical shift values were corrected for different solvents (D_2O vs. H_2O and dioxane vs. acetone). [h] McCleary et al. (1982). [i] This work.

di-NeuAc unit were assisted by comparison with the ^{13}C chemical shifts observed for colominic acid (Bhattacharjee et al., 1975) and the gangliosides G_{D1b} and G_{T1b} (Sillerud et al., 1982), but it was not possible to make one-on-one assignments for most of the corresponding resonances in the di-NeuAc unit. The assignments presented in Table I are the most likely ones in view of our results.

(*B*) *IV*^{6}*NeuAcLcOse*$_4$. The assignments (Table II) were made by a similar approach to that outlined for II^3(NeuAc)$_2$Lac. The assignments for both the nonreducing and the reducing parts of the molecule were assisted by direct comparison with the assignments made for NeuAcα(2→6)Galβ-(1→4)Glcβ (Berman, 1983) and Galβ(1→3)Galβ(1→4)Glc (Messer et al., 1982; Collins et al., 1981). Good chemical shift correlation was found for the NeuAcα(2→6)Galβ(1→4)→ and the →β(1→4)Glc parts of the molecule (a.d. = 0.03 ppm), with the exception of C4 of both the α- and β-D anomers of Glc. The middle part of the molecule [i.e., →β(1→4)-GlcNAcβ(1→3)Galβ(1→4)→] was assigned by adding the appropriate chemical shift increments, due to C1 glycosidation, to the GlcNAcβ anomer of NeuAcα(2→6)lactosamine (Berman, 1983) and by comparing the chemical shifts of the →β(1→3)Galβ(1→4) part of the molecule with the appropriate part of Galβ(1→3)lactose (Messer et al., 1982; Collins

et al., 1981) (a.d. = 0.10 ppm).[3]

(*C*) *NeuAcα(2→6)Lac.* Assignments (Table III, column 3) were essentially based on those reported for the corresponding lactosamine derivative (Table III, last column); the agreement for the NeuAcα(2→6)Galβ(1→4) part of the molecule was good (a.d. = 0.05 ppm) with the exception of C2 of NeuAc ($\Delta\delta$ = 0.11 ppm) and C1, C4, and C6 of the Gal residue ($\Delta\delta$ = −0.26, 0.30, and 0.19 ppm, respectively). The α- and β-D anomers of Glc were assigned by comparison with the NeuAcα(2→3)lactose isomer (Berman, 1983). The chemical shifts and assignments reported here for the synthetic NeuAcα(2→6)lactose do not agree in most parts with those reported for the same compound isolated from colostrum (Jacques et al., 1980). It is possible that Jaques et al. (1980) have unknowingly recorded the ^{13}C spectrum of NeuAcα-(2→6)lactosamine rather than NeuAcα(2→6)lactose. This conclusion is reinforced by the results reported by Veh et al. (1981) that the two major sialyloligosaccharides obtained from bovine colostrum are in fact NeuAcα(2→3)lactose and NeuAcα(2→6)lactosamine. It may be noted that the chemical shifts reported by Jaques et al. (1980) are in better agreement with the values reported for the NeuAcα(2→6)lactosamine isomer isolated from urine (Berman, 1983) than for those observed for the lactose derivative. The two small peaks observed in the spectrum of the "NeuAcα(2→6)lactose" [Figure 2 in Jaques et al. (1980)] with chemical shifts between 58 and 54 ppm probably correspond to the C2 of the β- and α-D anomers of the reducing GlcNAc (Berman, 1983).

Substituent Chemical Shift Effects (*SCS Effects*). The unusual chemical shift increments observed upon sialylation of a variety of sialyloligosaccharides have been reported for both ^{13}C NMR (Berman, 1983; Prohaska et al., 1981; Sillerud et al., 1982; Harris & Thornton, 1978) and ^{1}H NMR (Vliegenthart et al., 1981; Montreuil & Vliegenthart, 1977; Vliegenthart, 1979; Dorland et al., 1978). It was pointed out that long-range SCS's are observed mainly for the α(2→6) rather than for the α(2→3) linkages (Berman, 1983; Vliegenthart et al., 1981; Jennings et al., 1981).

Table III compares the chemical shifts of the carbons of the corresponding sialyl isomers of lactose and lactosamine. While the absolute range of chemical shift difference is unusually large, up to 2.39 ppm, it is essentially confined to particular carbons in the structure. If we ignore the chemical shift differences observed for C1, C2, C3, C6, and C7 of the corresponding NeuAc residues, we obtain an average deviation of ~0.08 ppm for sialyllactose and of ~0.05 ppm for sialyllactosamine. Similarly, if we neglect the large differences observed for the C4 of the reducing-end residues, we get a.d. = ~0.15 ppm for both α and β anomers of sialyllactose and ~0.18 ppm for the two anomers of sialyllactosamine.

The large chemical shift differences observed for C1, C2, and C3 of the NeuAc residues between the respective isomers may be attributed to through-bond effects due to two different types of linkages (i.e., 2→3 vs. 2→6), rather than through-space (conformational) effects. The larger and unexpected chemical shift differences observed for C6 and C7 between the isomers must reflect different NeuAc glycerol side chain conformations in relation to the rest of the molecule and thus indicate different conformations for the NeuAc residue for the two respective isomers (Veluraja & Rao, 1980).

When the chemical shift differences were compare for the reducing sugar residues (i.e., Glc and GlcNAc) these were, on the average, twice as large in magnitude for the GlcNAc anomers, probably reflecting the fact that the overall conformation of the α(2→6) isomer differs to a greater extent from the α(2→3) isomer conformation in the case of the sialyllactosamines. However, it is not likely that the *N*-acetylamino group of the GlcNAc residues is strongly involved in intramolecular hydrogen bonding to the sialic residue, since the chemical shifts of the NeuAc residue in the respective isomers for sialyllactose and sialyllactosamine are very similar indeed (a.d. = <0.04 ppm). Proximal interactions between the Gal residue and the *N*-acetyl group as a result of a NeuAc substitution at C6 of the Gal residue are the most probable cause for the observed increase in the magnitude of the chemical shift differences upon the introduction of the *N*-acetyl group. This notion is reinforced by the fact that larger chemical shift deviations (a.d. = ~0.15 ppm) between the Gal residues of the respective α(2→6) isomers of sialyllactose and sialyllactosamine are observed. For C1, C4, and C6 of the Gal residue, $\Delta\delta$ values are >0.19 ppm.

The most profound anomalous effect is the unexpected large downfield shift of the C4 resonances of both Glc and GlcNAc upon the C6 substitution of the Gal residue by NeuAc. It may be noted that this downfield shift is retained even if the GlcNAc residue is not at the reducing end, as in the case of IV^6 NeuAcLcOse$_4$. Thus, it may be argued that the additional degree of freedom about the C6–C5 bond of the Gal residue may allow the NeuAc residue in the case of the α(2→6) isomers to assume such a conformation that will bring some parts of the NeuAc residue in close proximity to the glycosidic bond between the Gal and the Glc or GlcNAc residues. As will be shown later, such a conformation is not unique for the case of NeuAc substitution at C6 alone. The conformation of the NeuAcα(2→6) residue about the lactose or lactosamine moiety remains the same in oligosaccharides larger than the trisaccharides discussed so far. When a direct comparison is made between the respective chemical shifts of sialyllactosamine (Table III, column 6) and IV^6NeuAcLcOse$_4$ (Table II, rows 1 and 2), the average chemical shift deviation observed for the NeuAcα(2→6)Gal grouping is <0.02 ppm. Similarly, the average deviation does not increase considerably in more complex carbohydrate chains like those of the N-linked triantennary oligosaccharide structures of calf fetuin.[4] Thus, the sialic acid residue, in the α(2→6) linkage assumes a conformation in which the residue is "folded over" toward the two inner sugar residues, while in the α(2→3) linkage the sialic acid residue is probably "extending away" from the two inner residues.

We compared the substituent chemical shifts computed for a variety of C6-substituted oligosaccharides with those observed for the sialyl analogues. The results are shown in Table IV. The table was constructed in the following manner: the chemical shifts of the parent oligosaccharide were tabulated for the nonreducing terminal hexose (Y in Table IV) and for the hexose to which Y is linked (Z in Table IV). These chemical shifts were substracted from the corresponding chemical shifts observed for the same Y and Z residues upon C6 substitution of Y by another sugar residue (X in Table IV). Care was taken to derive the listed SCS values from spectra that were run under the same conditions.

The range of SCS values observed for C6 substitution by sugar residues other than NeuAc indicates that, in all cases, the observed SCS values for the Y sugar carbons follow an expected pattern: a large downfield shift for Y-C6 (~4.4–8.0 ppm) and relatively large upfield shifts for Y-C5 (~0.9–2.2 ppm). Other carbons show small SCS values (<±0.38 ppm)

[3] A factor of 0.5 ppm was added to chemical shifts given in Collins et al. (1981) due to a different referencing system.

[4] E. Berman, unpublished results.

with the noticeable exception of Y-C1 of Manβ(1→4) substituted by Manα(1→6) (0.57 ppm). When the substituent residue is NeuAcα(2→6), the corresponding SCS values are very similar in magnitude and direction for Y-C2–Y-C5 and somewhat larger for Y-C1. The most noticeable exception is observed for Y-C6, for which the computed SCS values are considerably smaller. Similar small SCS values were observed for the C3 of hexoses substituted by NeuAc (Berman, 1983; Sillerud & Yu, 1983).

The SCS values for the Z residues were expected to be negligible and unusually large observed SCS values are most likely to arise from conformational (through-space) effects. Indeed, small SCS values are observed for all the Z carbons with the exception of Z-C4 of the last four entries in Table IV and Z-C3 of sialyllactosamine. We note that these large Z-C4 SCS values correlate very well with the corresponding large Y-C1 SCS values. The magnitude is larger for Z = hexosamine vs. hexose. It is also larger for NeuAc substitution vs. hexose substitution. The last four entries in Table IV follow the sugar sequence of Xα→Yβ→Zβ with Y and Z residues substituted at C6 and C4, respectively. Thus, this common trisaccharide sequence may have a unique conformation that depends entirely on the relative arrangement of the sugar residues.

In Table IV we also observed a large SCS value for Z-C3 of sialyllactosamine (upfield shift of −1.24 ppm), which reflects the fact that Z (=GlcNAcβ) has an *N*-acetylamino group that can be involved in some intramolecular interactions with the Z-C3 hydroxyl group in the sialyllactosamine β-anomer compared with the lactosamine β-anomer. This is supported by the fact that the SCS for Z-C3 of the respective β-anomer of the α(2→3) isomer is also large (−1.24 ppm) (Berman, 1983), and both are considerably larger than the corresponding SCS observed for the Z-C3 of the sialyllactoses. Although the nature of such intramolecular interactions is not clear, they may be triggered by the change in conformation due to substitution by NeuAc, since they are not observed in the case of X = Manα(1→6) and Z = GlcAcβ(1→4).

Somewhat different intramolecular hydrogen-bonding interactions were advanced by Jennings et al. (1981) to explain the correlation between observed long-range ^{13}C chemical shift effects and the conformation of the NeuAcα(2→6)Galβ(1→4)GlcNAcβ(1→4) unit in polysaccharides. They observed downfield shifts of 0.8 and 0.7 ppm for C4 of the GlcNAc and C1 of the Gal residues upon chemical modification of the C1 carboxylate group of the NeuAc residue. They proposed the existence of intramolecular hydrogen bonding between the C1 carboxylate group of the NeuAc and the C3 hydroxyl group of the GlcNAc [Figure 6 in Jennings et al. (1981)]. Such a postulate cannot be excluded for the oligosaccharides studied in the present work. The unusual ^{13}C SCS shifts observed for the Z-C3 of the sialyllactosamines may be a reflection of such conformational arrangment. However, the absence of the corresponding SCS effect on C1 of NeuAc makes it difficult to ascertain such an interaction. Also, the existence of a large SCS value for Z-C3 in the case of the NeuAcα(2→3) isomer cannot be explained by similar intramolecular hydrogen bonding due to the restricted degree of freedom of the NeuAcα(2→3) residue about the glycosidic bond.

Conclusions

It is imperative to state that no detailed conformation of the NeuAc residue, with respect to the rest of the oligosaccharide chain, could be extracted from the data analysis presented so far. However, the direction and nature of the chemical shifts analyzed in this work are sufficient to give an overall view of the type of conformation to be considered.

On the basis of chemical shift comparisons and SCS values, it was concluded that the sialo residue in the α(2→3) isomer forms a linear extension of the oligosaccharide as indicated by "normal" SCS values in the case of Glc and GlcNAc. The α(2→6) isomer is in contrast folded over the oligosaccharide chains in close proximity to the Glc and GlcNAc residues. It is also clear that the *N*-acetylamino group on the glucose pyranoside ring plays some role in the conformation although it may not be involved in direct interaction with the NeuAc residue.

The conformational difference between NeuAc residues linked α(2→3) vs. α(2→6) is probably also manifested in the large differences encountered by enzymatic hydrolysis of these residues (Schauer, 1982; Friebolin, et al., 1981; Cornfield et al., 1982; Keilich et al., 1982). Thus, for sialyllactose, the α(2→3) isomer is hydrolyzed faster (~500 times) than the corresponding α(2→6) isomer, when both are treated with sialidase from Newcastle disease virus (Corfield et al., 1982). The same enzyme cleaves the α(2→6) isomer of sialyllactosamine faster (~10 times) than the corresponding α-(2→6) isomer of sialyllactose. This correlation between the unusual chemical shift patterns observed for sialyloligosaccharides and the observed specificity of some sialidases underscores the important role played by the conformation of the carbohydrate side chains in biochemical processes. Similar correlations between unusual long-range ^{13}C chemical shifts and the immunospecificity of polysaccharides determinants were also reported (Jennings et al., 1981).

The unique chemical shift effects observed for small sialyloligosaccharides (Berman, 1983) are also observed in the case of intact glycoproteins such as α_1-acid glycoprotein[5] and calf fetuin.[4] Thus, we may conclude that, at intermediate pH values, the sialic acid conformation is determined largely by the first two sugar residues to which it is linked. This study implies that once the sialic acid residue detailed conformation is determined for smaller and simpler oligosaccharides, it can be assumed to be the same for the much larger glycoproteins.

References

Abbas, S. A., Barlow, J. J., & Matta, K. L. (1983) *Carbohydr. Res. 113*, 63–70.

Allerhand, A., & Berman, E. (1984) *J. Am. Chem. Soc.* (in press).

Barrett-Bee, K., Bedford, G., & Loftus, P. (1982) *Biosci. Rep. 2*, 257–263.

Berman, E. (1983) *Carbohydr. Res. 118*, 9–20.

Berman, E., & Allerhand, A. (1981) *J. Biol. Chem. 256*, 6657–6662.

Berman, E., Allerhand, A., & De Vries, A. L. (1980) *J. Biol. Chem. 255*, 4407–4410.

Berman, E., Walters, D. E., & Allerhand, A. (1981) *J. Biol. Chem. 256*, 3853–3857.

Bhattacharjee, A. K., Jennings, H. J., Kenny, C. P., Martin, A., & Smith, I. C. P. (1975) *J. Biol. Chem. 250*, 1926–1932.

Collins, J. G., Bradbury, J. H., Trifonoff, E., & Messer, M. (1981) *Carbohydr. Res. 92*, 136–140.

Corfield, A. P., Wember, M., Schauer, R., & Rott, R. (1982) *Eur. J. Biochem. 124*, 521–525.

Daman, M. E., & Dill, K. (1983) *Carbohydr. Res. 111*, 205–214.

Dijkstra, B. W., Vliegenthart, J. F. G., Strecker, G., & Montreuil, J. (1983) *Eur. J. Biochem. 130*, 111–115.

[5] E. Berman and A. Allerhand, unpublished results.

Dill, K., & Allerhand, A. (1979) *J. Biol. Chem. 254*, 4524–4531.
Dorland, L., Haverkamp, J., Vliegenthart, J. F. G., Strecker, G., Michalski, J.-C., Fournet, B., Spik, G., & Montreuil, J. (1978) *Eur. J. Biochem. 87*, 323–329.
Eschenfelder, V., Brossmer, R., & Friebolin, H. (1975) *Tetrahedron Lett.*, 3069–3072.
Friebolin, H., Baumann, W., Keilich, G., Ziegler, D., Brossmer, R., & Von Nicolai, H. (1981) *Hoppe-Seyler's Z. Physiol. Chem. 362*, 1455–1463.
Goux, W. J., Perry, C., & James, T. L. (1982) *J. Biol. Chem. 257*, 1829–1835.
Harris, P. L., & Thornton, E. R. (1978) *J. Am. Chem. Soc. 100*, 6738–6745.
Jaques, L. W., Glant, S., & Weltner, W., Jr. (1980) *Carbohydr. Res. 80*, 207–211.
Jennings, H. J., Lugowski, C., & Kasper, D. L. (1981) *Biochemistry 20*, 4511–4518.
Keilich, G., Ziegler, D., Brossmer, R., Friebolin, H., Baumann, W., Kurz, D., Wajda, R., Weibhaar, G., & Von Nicolai, H. (1982) *Fresenius' Z. Anal. Chem. 311*, 393–394.
Kobata, A. (1979) *Anal. Biochem. 100*, 1–14.
McCleary, B. V. Taravel, F. R., & Cheetham, N. W. H. (1982) *Carbohydr. Res. 104*. 285–297.
Messer, M., Trifonoff, E., Collins, J. G., & Bradbury, J. H. (1982) *Carbohydr. Res. 102*, 316–320.
Montreuil, J., & Vliegenthart, J. F. G. (1979) *Proceedings of the International Symposium on Glycoconjugates, 4th*, Woodshole, MA, pp 35–78, Academic Press, New York.
Nunez, H. A., Matsuura, F., & Sweeley, C. C. (1981) *Arch. Biochem. Biophys. 212*, 638–643.
Prohaska, R., Koerner, T. A. W., Jr., Armitage, I. M., & Furthmayr, H. (1981) *J. Biol. Chem. 256*, 5781–5791.
Rana, S. S., Barlow, J. J., & Matta, K. L. (1982) *Carbohydr. Res. 101*, 245–253.
Roy, R., & Jennings, H. J. (1983) *Carbohydr. Res. 112*, 63–72.
Schauer, R. (1982) *Adv. Carbohydr. Chem. Biochem. 40*, 131–234.
Shashkov, A. S., Arbatsky, N. P., Derevitskaya, V. A., & Kochetkov, N. K. (1979) *Carbohydr. Res. 72*, 218–221.
Sillerud, L. O., & Yu, R. K. (1983) *Carbohydr. Res. 113*, 173–188.
Sillerud, L. O., Yu, R. K., & Schafer, D. E. (1982) *Biochemistry 21*, 1260–1271.
Srivastava, V. K., Sondheimer, S. J., & Schuerch, C. (1980) *Carbohydr. Res. 86*, 203–214.
Veh, R. W., Michalski, J.-C., Corfield, A. P., Sander-Wewer, M., Gies, D., & Schauer, R. (1981) *J. Chromatogr. 212*, 313–322.
Veluraja, K., & Rao, V. S. R. (1980) *Biochim. Biophys. Acta 630*, 442–446.
Vliegenthart, J. F. G. (1979) *Adv. Exp. Med. Biol. 125*, 77–91.
Vliegenthart, J. F. G., Van Halbeek, H., & Dorland, L. (1981) *Pure Appl. Chem. 53*, 45–77.

Neoglycoproteins: In Vitro Introduction of Glycosyl Units at Glutamines in β-Casein Using Transglutaminase†

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ABSTRACT: Exploring different methods for preparing neoglycoproteins with a specific number of oligosaccharides in specific positions, we have used guinea pig liver transglutaminase to incorporate glycosyl units into glutamine residues in β-casein. In order to prevent ε-(γ-glutamyl)lysine cross-link formation, the lysine residues of β-casein were first blocked either by amidination with ethyl acetimidate or by acylation with succinic anhydride. The glycosyl donor substrates prepared for this work were maltotriose reductively aminated with cadaverine, *N*-(Glc-Glc-glucitol-1)-cadaverine, and an asparaginyl nonasaccharide from ovalbumin modified with a 6-aminohexanoyl group at the α-amino group. The transglutaminase-catalyzed incorporation of these two donors into the β-casein derivatives was monitored in comparison to the incorporation of the commonly used transglutaminase substrate dansylcadaverine under conditions of optimal incorporation (multiple additions of enzyme, large excess of donor, and long incubation time). For both dansylcadaverine and Glc-Glc-Glc(OH)-cadaverine, 5 and 8 mol of donor were incorporated per mol of amidinated and succinylated β-casein, respectively. Competition experiments showed that the two donor substrates are incorporated into the same glutamine sites. Partial sequencing of the glycosylated β-casein permitted the identification of glutamine residues 56, 79, 167, 175, and 194 as the primary sites of incorporation in amidinated casein with residues 54 and 182 as possible sites for partial glycosylation. The results are consistent with a specific glycosylation of only selected glutamines in this transglutaminase-catalyzed process. The bulkier nonasaccharide derivative was also found to be a glycosyl donor in the transglutaminase reaction, but in this case the incorporation was lower (a maximum of 4 mol/mol) than for the other donor substrates, and multiple distinct bands were observed upon sodium dodecyl sulfate gel electrophoresis of the glycosylated product.

Lectin–sugar interactions are important in various biological recognition and communication processes such as receptor-mediated endocytosis and cell–cell interactions (Barondes, 1981). The specificity determinants of these interactions are not well understood, but recent studies, in particular those on the binding of glycoconjugates by hepatic lectins and the subsequent internalization of the glycoproteins, strongly suggest that the total process in terms of both binding and the subsequent biological consequences of binding is complex (Ashwell & Harford, 1982). These studies suggest that the efficacy of binding and clearance of glycoproteins depend not only on the

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