^{13}C NMR CHEMICAL SHIFT TITRATION OF METAL ION-CARBOHYDRATE COMPLEXES. AN UNEXPECTED DICHOTOMY FOR Ca^{+2} BINDING BETWEEN ANOMERIC DERIVATIVES OF *N*-ACETYLNEURAMINIC ACID (1)

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Summary: The complexation of Ca^{+2} with *N*-Acetylneuraminic acid, its methyl ester and its α and β methyl glycosides was studied by titrating the ^{13}C NMR chemical shifts with added $CaCl_2$. These studies demonstrate that the strong Ca^{+2} binding previously observed in the β anomer is not shared by the biochemically relevant α anomer. The strong β complex uses three oxygen atoms as binding sites for Ca^{+2} but these oxygens are not involved in the weak binding of the α anomer.

We wish to report the first ^{13}C NMR[b] chemical shift titration of a metal-carbohydrate complex, showing how this technique provides valuable information on structural specificity and binding sites for Ca^{+2} in derivatives of *N*-acetylneuraminic acid. In the α-ketosidically linked form, NeuNAc occurs as a constituent of glycolipids and glycoproteins of neuronal and other cell surfaces and has been suggested as a receptor for intercellular Ca^{+2} (2).

These studies show that (*1*) the β anomeric form of NeuNAc forms a stable Ca^{+2} complex as was previously shown (2b), but the biologically significant α anomer does not. (*2*) The carboxylate group does not participate in the strong β complex, yet the negative charge is required. (*3*) The main binding sites for Ca^{+2} in the β complex are three oxygen atoms (the anomeric oxygen at C-2, the pyranose ring oxygen, and the hydroxyl

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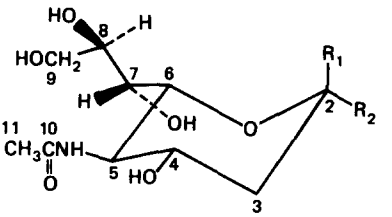
[b] Abbreviations: NMR, nuclear magnetic resonance; NeuNAc, *N*-Acetylneuraminic acid; T_1, spin-lattice relaxation time; NT_{1DD}, dipole-dipole mediated T_1 multiplied by number of attached hydrogens, *N*.



1a, R_1 = OH R_2 = COOH
1b, R_1 = OH R_2 = $COOCH_3$
1c, R_1 = OCH_3 R_2 = COOH
1d, R_1 = COOH R_2 = OCH_3

Figure 1. Structural configuration of NeuNAc (1a), its methyl ester (1b), and the β (1c) and α (1d) methyl glycosides.

oxygen at C-8), but these sites are not involved in the weak binding of the α anomeric form.

The use of ^1H NMR to study the complexes of Ca^{+2} with simple aldoses in aqueous solution is well established (3). The complexity of the mono and polysaccharides associated with the cell membrane, however, precludes the study of their ion binding capacities by ^1H NMR. Behr and Lehn have measured the stability constants of metal ion complexes with NeuNAc and gangliosides using ion-selective electrodes; their preliminary ^{13}C NMR studies suggested that Ca^{+2} complexation in NeuNAc, and by deduction in gangliosides, involves a specific complexation with hydroxyl groups in NeuNAc (2b,c). Our ^{13}C chemical shift titration indicates the stereochemical requirements of this complex.

Materials and Methods: NeuNAc (1a), its methyl ester (1b), and its β (1c) and α (1d) methyl glycosides (Figure 1) were prepared as previously reported (1); free acids were converted to their sodium salts by titrating with NaOH solution to pH 7.8 ± 0.2, followed by lyophilization and reconstitution in D_2O. ^{13}C NMR were measured on a JEOL PFT-100 at 28.0 ± 0.5°. Chemical shifts were measured relative to the methyl carbon resonance of external acetone. We have titrated the ^{13}C chemical shift changes (Δδ) of 0.50 M solutions of 1a - d by the incremental addition of $CaCl_2$ to a total of 4.0 M. Dissociation constants, K_{diss}, for the Ca^{+2} complexes with 1c and 1d were determined by titrating 0.50 M solutions of the methyl glycoside sodium salts with standard $CaCl_2$ solutions; free Ca^{+2} concentration was measured with a Radiometer Ca^{+2} Selectrode, and the titration vessel was thermostated at 26.5 ± 1.0°.

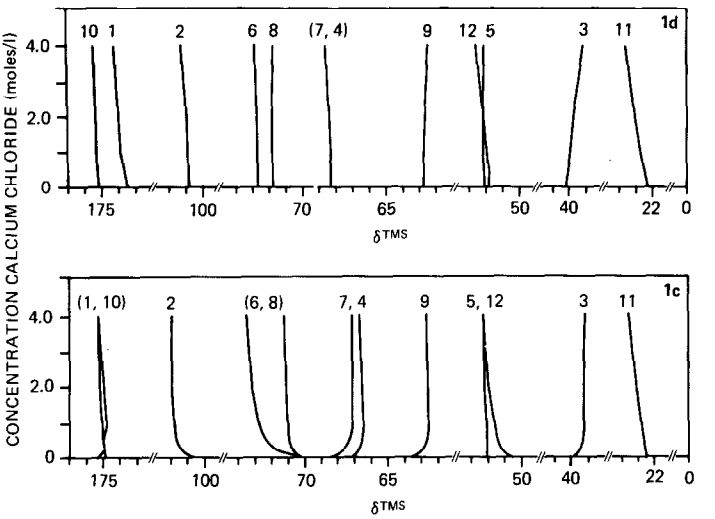


Figure 2. ^{13}C NMR titration curves for the α (1d) and β (1c) methyl glycosides of NeuNAc. Experimental points were determined at 0.13, 0.25, 0.38, 0.50, 1.0, 2.0, and 4.0 M added $CaCl_2$. Parentheses indicate that assignments can be interchanged; however, the substance of our arguments is not affected.

Results and Discussion: ^{13}C NMR chemical shift titration curves for the methyl glycosides (1c and 1d) appear in Figure 2; Δδ values for all substances at 1.0 M $CaCl_2$ are presented in Table I. The conformation and stereochemistry of the neuraminic acids has been confirmed by ^{1}H and ^{13}C NMR and our chemical shifts and assignments are in substantial agreement with the latter (4).

These experiments can be very reasonably interpreted as follows. Downfield shifts observed at C-2, C-6, C-8, and C-12 (methoxy methyl) in the β glycoside (1c) result from direct participation of the attached oxygens in the solvation sphere of Ca^{+2} (Figure 3). Donation of electron density from oxygen polarizes the C-O bond, effecting a net deshielding at carbon. Titratable upfield shifts at C-7 and C-9 are steric effects from the metal ion since these carbons become γ gauche to Ca^{+2} upon complexation.[c] The

[c] T_1 measurements on the Ca^{+2} complex indicate that C-9 (NT_{1DD} = 300 msec) is freely rotating in comparison to C-3 through C-8 (avg NT_{1DD} = 200 msec), precluding direct participation of the C-9 hydroxyl. The upfield shifts could contain a contribution from σ inductive effects since they are adjacent to the methinyl carbons shifted downfield by Ca^{+2}; for a recent discussion see Ref. 5.

Table I. ^{13}C NMR Titration of 0.50 M D_2O Solutions of 1a-d; Δδ values at 1.0 M $CaCl_2$[a]

C	1a	1b	1c	1d
1	0.2[b]	0.5[c]	-0.2[b]	0.4[b]
2	1.0	0.2	1.2	0.1
3	-0.1	0.1	-0.6	-0.2
4	-0.6	-0.1	-0.7	0.0
5	-0.1	0.0	0.0	0.1
6	0.7	0.2	0.8	0.0
7	-1.0	0.0	-1.4	0.0
8	1.7	0.5	2.5	0.0
9	-0.8	-0.2	-1.1	-0.1
10	-0.3	0.2	0.0	0.1
11	0.4	0.5	0.3	0.5
12		0.6[d]	1.0[e]	0.2[e]
Equilibrium constants[f]				
K_{diss}	13 mM[g]	---	16 mM	>398 mM

[a] In certain cases (see Figure 2) overlap prior to or during titration allows an exchange of Δδ values without affecting conclusions. [b] Carboxylate carbon. [c] Ester carboxyl carbon. [d] Ester methyl carbon. [e] Ketoside methoxy carbon. [f] Determined at 26.5 ± 1.0°. [g] Determined by Behr and Lehn, Ref. 2b.

upfield shift at C-3 observed in 1c but not 1a is probably the result of reorientation of the methoxy group which participates directly in the complex, the effect of the hydrogen in 1a being negligible. The small upfield shift at C-4 is in accordance with the predicted δ effect (6). It is interesting to note that the carboxylate carbon does not titrate with complexation, although it is clearly required for strong binding.[d] The methyl ester (1b) which retains the same stereochemistry, but lacks the negative charge, shows only very small Δδ values under the same conditions. Therefore, it is suggested that the carboxylate acts at a distance, its effect mediated by a strong hydrogen bond with a Ca^{+2} water of solvation.[e]

[d] We have observed a 2 ppm downfield shift of the carboxylate carbon in the acetate-Ca^{+2} complex.

[e] A similar effect in the uronic acids has been noted by Perlin using paramagnetic relaxation reagents, Ref. 7.

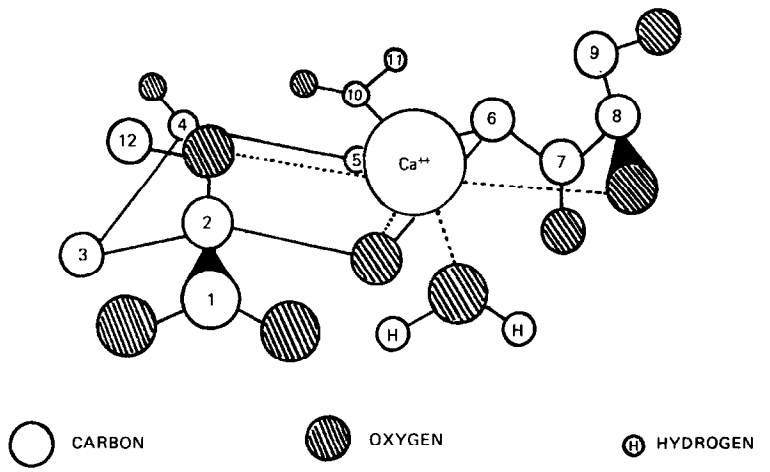


Figure 3. Schematic representation of β-glycoside (1c)-Ca^{+2} complex showing oxygen binding sites and H_2O of solvation in close association with the carboxylate. Van der Waals contact of interacting oxygens with Ca^{+2} was confirmed with space-filling models.

The high stability of the Ca^{+2} -NeuNAc complex is therefore the result of a unique *oxygen cage* formed by the three oxygens and indirectly the carboxylate anion.

When Ca^{+2} interactions with the α glycoside (1d) are studied, dramatic differences are observed. Not only is K_{diss} for the Ca^{+2} complex more than an order of magnitude larger (Table I), but interactions similar to those found in the β anomers are ruled out since the chemical shift patterns are markedly different. The downfield shifts at C-1 and C-12 and the upfield shift at C-3 are consistent with a weak interaction with the carboxylate and the glycosidic oxygen. Since, however, low affinity, non-titrating downfield shifts are observed for several carbons in the other NeuNAc derivatives, these shifts in the α glycoside cannot be ascribed to a primary complexation with complete certainty.[f] Clearly the strict stereo-

[f] Interesting small chemical shifts occur at methyl carbons in all substances and at several other positions. Although certain carbons remain essentially unaffected, these shifts appear to be related to medium effects since they occur in 1a and 1c after full complexation. It is possible that the Van der Waals structure of the solvent is perturbed by Ca^{+2}, effecting these shifts. For a recent discussion see Ref. 8.

chemical requirements that accounted for the high stability of the complexes with la and lc cannot be met by the biochemically relevant α anomer.[g]

Thus the Ca^{+2} complexes of gangliosides, which are very strong (2c), cannot result from a specific, strong interaction with the α-ketosidically linked NeuNAc. The receptor site may utilize the negative charge, but additional specificity must be provided by a more complex interaction of Ca^{+2} with the carbohydrate macrostructure of the glycolipids. Therefore, further ^{13}C NMR studies of these complex carbohydrates and their interactions are in progress.

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[g] An attempt to model the stereochemical effect of the carboxylate configuration by studying the Ca^{+2} complexes of the *cis* and *trans* 4-*t*-butylcyclohexanecarboxylic acids was unsuccessful due to their extreme insolubility.