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^{13}C-N.M.R.-SPECTRAL STUDY OF THE MODE OF BINDING OF Mn^{2+} AND Gd^{3+} TO *N*-ACETYL-α-NEURAMINIC ACID

MARSHA E. DAMAN AND KILIAN DILL
Department of Chemistry, Clemson University, Clemson, SC 29631 (U.S.A.)
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ABSTRACT

Natural-abundance, ^{13}C-n.m.r. spectroscopy was used to study the binding of Gd^{3+} and Mn^{2+} to *N*-acetyl-2-*O*-methyl-α-neuraminic acid (**2**) and to methyl *N*-acetyl-2-*O*-methyl-α-neuraminate (**3**). The results showed that Gd^{3+} and Mn^{2+} bind in the region of the glycerol-1-yl side-chain and the 5-acetamido group of compound **3**. When the α-NeuAc derivative contains a carboxylate anion, as in compound **2**, multiple, metal-ion-binding sites occur, involving the head (the carboxyl end) and the tail (the glycerol-1-yl and 5-acetamido groups) of the molecule.

INTRODUCTION

In 1971, Long and Mouat[1] found that intact erythrocytes, as well as ghost-cell membranes, bind calcium ions (Ca^{2+}), and also trivalent metal-ions. Furthermore, their work showed that *N*-acetylneuraminic acid (NeuAc) plays a role in the metal-ion-binding phenomenon.

As a result of their work, several studies have appeared in the literature concerning the binding of Ca^{2+} to free NeuAc[2–4], modified NeuAc[4,5], and polysaccharides containing NeuAc[6]. In the majority of these studies, NeuAc was present in the β-pyranose* form[3–5]; strong calcium-ion binding was observed in these cases. The few studies that were conducted with α-NeuAc (**1**) indicated that Ca^{2+} binding may occur, but it is weak[4,6]. Therefore, the mode of interaction of Ca^{2+} with α-NeuAc could not be established.

In Nature, NeuAc is present only in the α-pyranose* form[7,8]. More specifically, glycophorin, the major sialoglycoprotein of the red-cell membrane contains NeuAc only in the α-pyranose form[9–11]. Recent, preliminary, ^{13}C-n.m.r.-spectral studies of α-NeuAc-containing glycopeptide fragments of glycophorin A indicated that Ca^{2+} does interact with the NeuAc residues[12]. Although glycophorin serves a variety of functions in the red-cell membranes[13], the specific, functional significance of Ca^{2+} binding is unknown[12].

It would appear, then, that it is extremely relevant to understand the inter-

*Editor's note: herein, the anomeric designator is used in relation to the D configuration of C-8.

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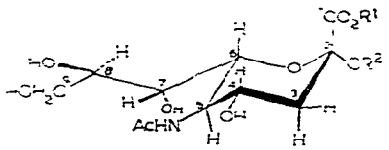
action of metal ions with α-NeuAc. Although the metal ion–α-NeuAc interaction alone is weak, the metals bind to α-NeuAc on the red-cell surface and to glycophorin A. This phenomenon may be due to the interaction of the metal ion with several sugars (α-NeuAc playing a major role), as proposed for the metal-ion-binding by brain gangliosides containing α-NeuAc[14].

To investigate the little-understood, metal ion–α-NeuAc interaction, we decided to use ^{13}C-n.m.r. spectroscopy to investigate the binding of paramagnetic relaxation-reagents (Mn^{2+} and Gd^{3+}) to derivatives of α-NeuAc. Paramagnetic relaxation-reagents were chosen because only a trace is needed in order to permit observation of specific line-broadening, if binding occurs[15]. The derivatives of α-NeuAc that we chose to investigate were 2-*O*-methyl-α-NeuAc (**2**) and its methyl ester (**3**). Compound **2** mimics α-NeuAc in glycoproteins, and study of compound **3** should determine whether the carboxyl group is necessary for metal-ion binding, as has been shown for metal-ion binding in β-NeuAc[3,4].



1 $R^1 = R^2 = H$
2 $R^1 = H$, $R^2 = Me$
3 $R^1 = R^2 = Me$

Our studies provided some startling results. We found that Mn^{2+} and Gd^{3+} bind to α-NeuAc, and that (*i*) their binding sites are slightly different, (*ii*) the carboxyl group is not necessary for metal-ion binding, and (*iii*) with a free carboxyl group, multiple metal-ion-binding sites exist.

EXPERIMENTAL

Materials. — *N*-Acetylneuraminic acid (Type VI) was purchased from Sigma Chemical Co., St. Louis, MO. Gadolinium oxide (99.9%) was obtained from Alfa Products, Danvers, MA, and reagent-grade manganous chloride, tetrahydrate, from Baker and Adamson. All chemicals used in the subsequent syntheses were of at least reagent-grade quality.

Methods. — 2-*O*-Methyl-α-NeuAc methyl ester (**3**) was prepared by the method of Meindl and Tuppy[16]. The final product was separated from a trace of impurity by using diethyl ether–methanol[17]; the overall yield of compound **3** (starting with NeuAc) was 13.8%. 2-*O*-Methyl-α-NeuAc (**2**) was prepared by using the methods of Kuhn *et al.*[18] and Yu and Ledeen[17]. The final product was separated from a trace of impurity by extraction of the solid with small amounts of a solution containing equal portions of diethyl ether, ethyl acetate, and methanol; the overall yield of compound **2** (starting with NeuAc) was 8.4%. During the course of the syntheses, the reaction products at each step were monitored by ^{13}C-n.m.r. spectroscopy. The

chemical shifts of the final products were in excellent agreement with those published by other research groups[19,20], although we disagree on some assignments (see later).

Manganous chloride tetrahydrate was converted into manganous chloride under vacuum. Gadolinium oxide was converted into gadolinium chloride by treatment with HCl, the excess of HCl being removed by a repeated cycle of heating the sample to drive off the excess HCl and water, and addition of more water. Stock solutions of $GdCl_3$ and $MnCl_2$ (~0.6M) were prepared at near neutral pH (~6.0–7.0).

Samples for n.m.r. spectroscopy were prepared by dissolving an approximate amount of the NeuAc derivative in de-ionized, distilled water, the exact concentration of NeuAc derivative in the sample then being determined by the assay method of Warren[21]. The pH of the samples was maintained at ~6.0–7.0. Addition of Gd^{3+} and Mn^{2+} to the NeuAc samples was made in μL quantities (total additions ranged from 80–150 μL), using an Eppendorf digital pipet.

Carbon-13 n.m.r. spectra were recorded with a JEOL-FX90Q instrument operating at 22.5 MHz (21.0 kG) in the F.t. mode. Samples (~1.0 mL) were contained in 10-mm tubes, with a 5-mm tube containing D_2O inserted concentrically to serve as a field-frequency lock, and the probe temperature was maintained at ~25–30° for all samples. For ^{13}C excitation, 90° radio-frequency pulses of 18 μs were used, and the carrier frequency was set ~90 p.p.m. downfield from Me_4Si. A spectral window of 5.0 kHz was used for all samples. Fully proton-decoupled spectra were obtained when the noise-modulated, ^1H irradiation, having a band-width of 1.0 kHz, was centered ~4 p.p.m. downfield from Me_4Si. Spectra with ^{13}C–^1H coupling were obtained by using a proton-decoupling technique the reverse of that used for n.O.e. measurements[22].

Chemical shifts are given relative to a trace of internal 1,4-dioxane (added only when chemical shifts were determined), whose chemical shift was taken to be 67.86 p.p.m. downfield from Me_4Si.

RESULTS AND DISCUSSION

Figs. 1A and 2A show the proton-decoupled, natural-abundance, ^{13}C-n.m.r. spectrum of compound **3**, and Figs. 3A and 4A, the spectrum of compound **2**. Table I gives the chemical shifts of the resonances, and their assignments to specific carbon atoms.

The chemical shifts of compounds **2** and **3** agree with the literature values published for them[19,20], and with the chemical shifts for the α-NeuAc found in some trisaccharides[23]. The assignments of most of the resonances in our spectra to specific carbon atoms of compounds **2** and **3** are based on previous literature assignments[19,20] (of compounds **2** and **3**), as well as assignments made for resonances in the ^{13}C spectra of trisaccharides containing α-NeuAc[23].

We disagree with two assignments presented in the literature. We have assigned the resonances occurring at 53.3 and 52.9 p.p.m. in the spectrum of compound **2** (Figs. 3 and 4) to carbon atoms *αp5* and $-CH_3$ (R^2) respectively; this is in agreement

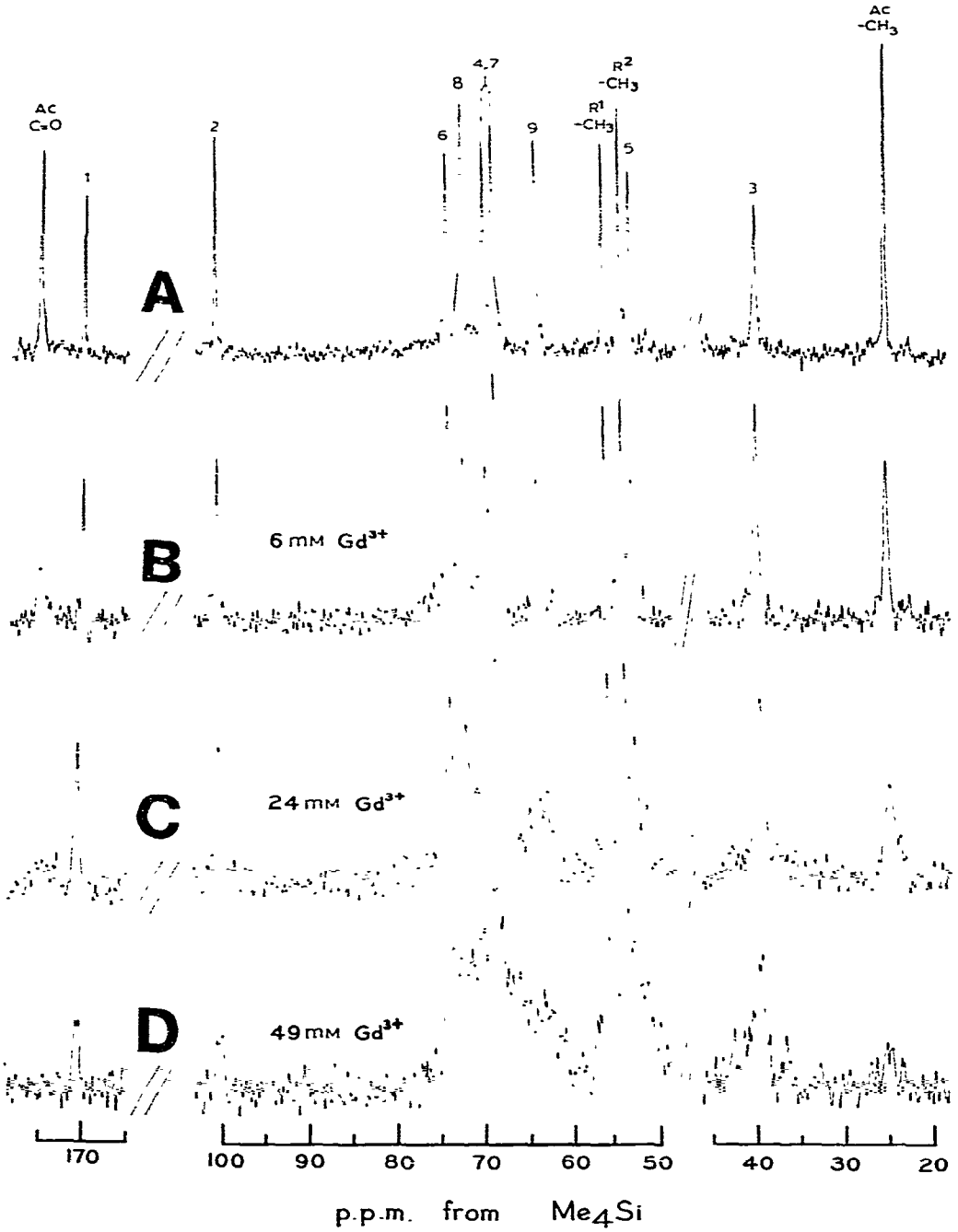


Fig. 1. The effect of Gd^{3+} on the ^{13}C resonances of the proton-decoupled, natural-abundance, ^{13}C-n.m.r. spectrum of 2-*O*-methyl-α-NeuAc methyl ester. [All spectra were recorded with a 3-s, recycle time. The concentration of compound 3 was 82 mM in H_2O, pH 6.9. The vertical gain of the spectra of solutions containing large proportions of paramagnetic relaxation-reagent was increased slightly, so that broadening effects could be clearly observed. (A) Sample contained no Gd^{3+}, and required 5,000 accumulations. A line-broadening factor of 1.2 Hz was used during processing. (B) Sample contained 6 mM Gd^{3+}, and required 5,000 accumulations. A line-broadening factor of 3 Hz was applied during processing. (C) Sample contained 24 mM Gd^{3+}, and required 10,000 accumulations. A line-broadening factor of 5 Hz was applied during processing. (D) Sample contained 49 mM Gd^{3+}, and required 40,000 accumulations. A line-broadening factor of 5 Hz was applied during processing.]

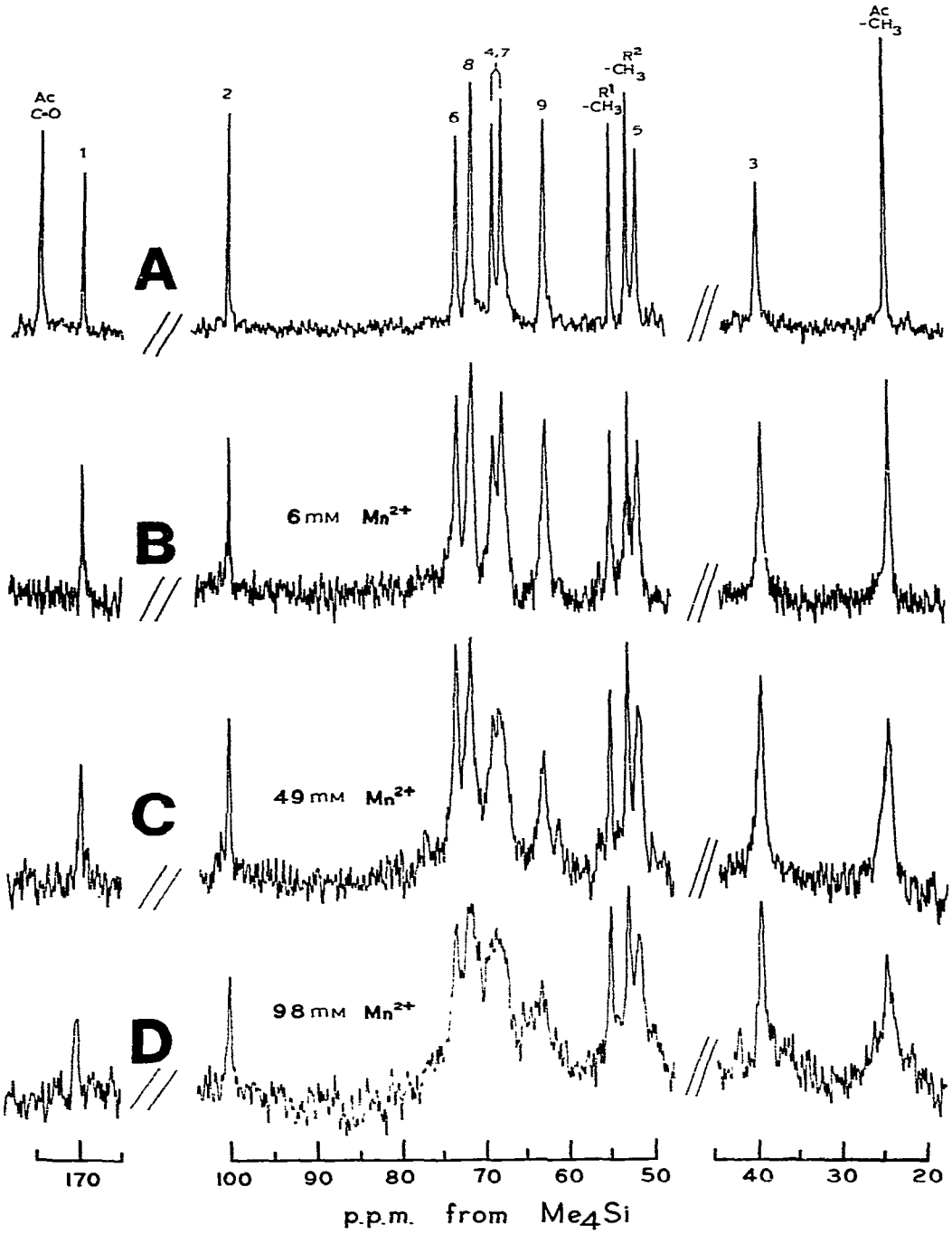


Fig. 2. The effect of Mn^{2+} on the ^{13}C resonances of the proton-decoupled, natural-abundance, ^{13}C-n.m.r. spectrum of 2-*O*-methyl-α-NeuAc methyl ester. [All spectra were recorded with a 3-s, recycle time. The concentration of compound 3 for spectra 2B, 2C, and 2D was 99 mM in H_2O, pH 6.9. The vertical gain of the spectra of solutions containing large proportions of paramagnetic relaxation-reagent was increased slightly, so that broadening effects could be clearly observed. (A) Same as in Fig. 1A. (B) Sample contained 6 mM Mn^{2+}, and required 5,000 accumulations. A line-broadening factor of 1.2 Hz was applied during processing. (C) Sample contained 49 mM Mn^{2+}, and required 10,000 accumulations. A line-broadening factor of 2.0 Hz was applied during processing. (D) Sample contained 98 mM Mn^{2+}, and required 10,000 accumulations. A line-broadening factor of 5.0 Hz was used during processing.]

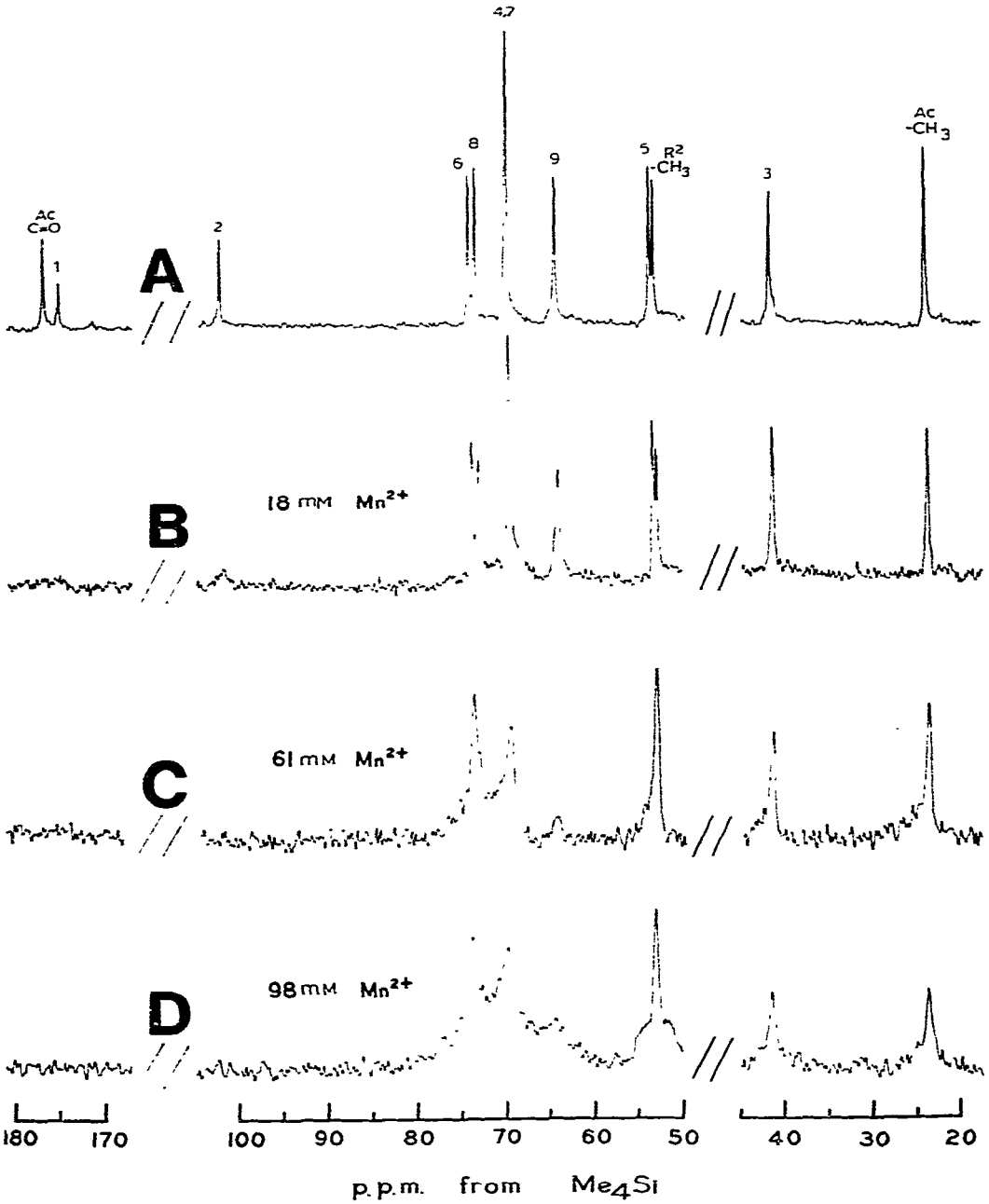


Fig. 3. The effect of Mn^{2+} on the ^{13}C resonances of the proton-decoupled, natural-abundance, ^{13}C-n.m.r. spectrum of 2-*O*-methyl-α-NeuAc. [All spectra were recorded with a 3-s, recycle time. The concentration of compound 2 was 198 mM in H_2O, pH 7.2. The vertical gain of the spectra of solutions containing large proportions of paramagnetic relaxation-reagent was increased slightly, so that broadening effects could be clearly observed. (A) Sample contained no Gd^{3+}, and required 5,000 accumulations. A line-broadening factor of 1.2 Hz was applied during processing. (B) Sample contained 18 mM Mn^{2+}, and required 5,000 accumulations. A line-broadening factor of 1.6 Hz was applied during processing. (C) Sample contained 61 mM Mn^{2+}, and required 10,000 accumulations. A line-broadening factor of 3.8 Hz was applied during processing. (D) Sample contained 98 mM Mn^{2+}, and required 15,000 accumulations. A line-broadening factor of 4.5 Hz was applied during processing.]

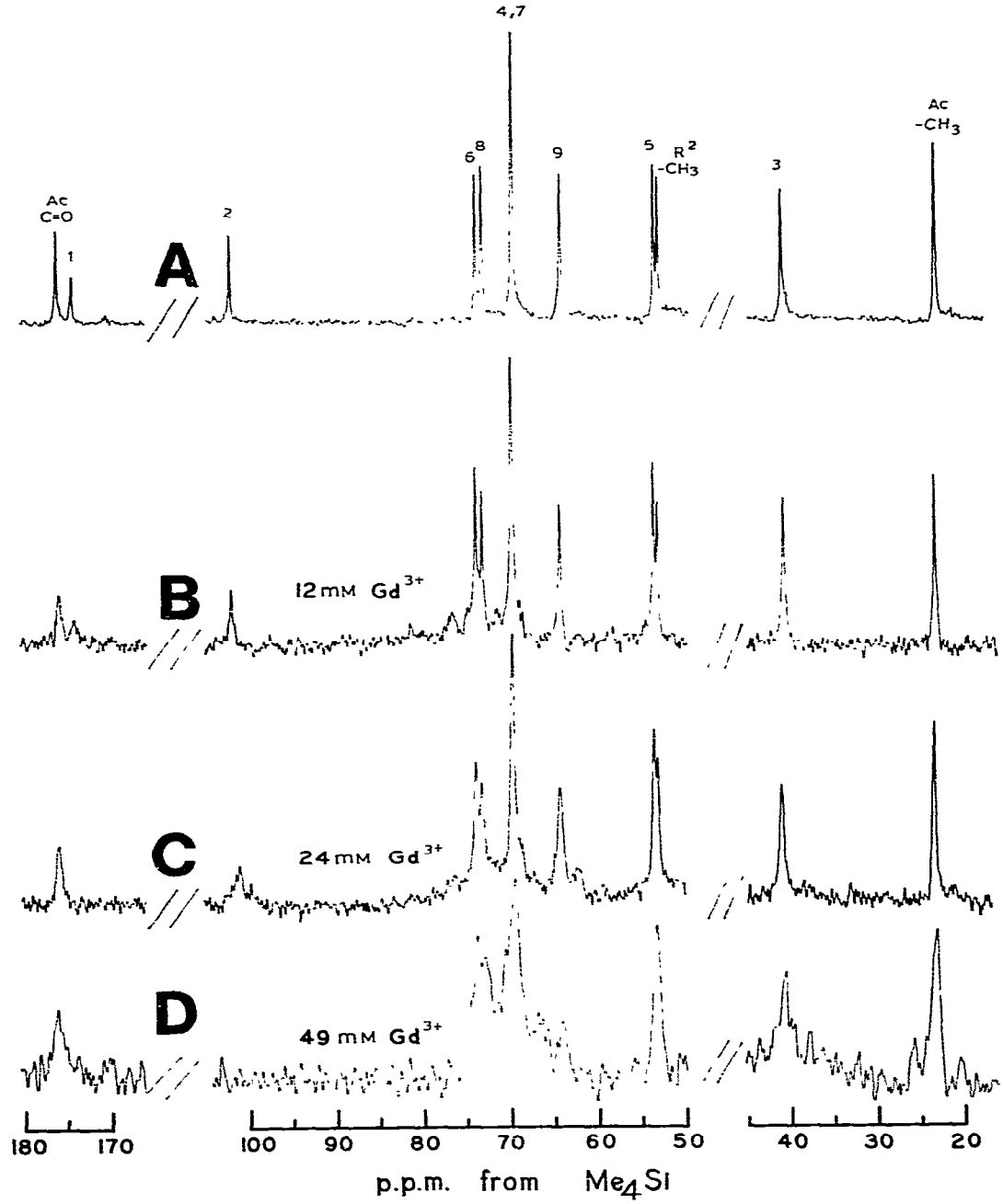


Fig. 4. The effect of Gd^{3+} on the ^{13}C resonances of the proton-decoupled, natural-abundance, ^{13}C-n.m.r. spectrum of 2-*O*-methyl-α-NeuAc. [All spectra were recorded with a 3-s, recycle time. The concentration of compound 2 for spectra 4B, 4C, and 4D was 188 mM in H_2O, pH 6.8. The vertical gain of the spectra of solutions containing large proportions of paramagnetic relaxation-reagent was increased slightly, so that broadening effects could be clearly observed. (A) Same as in Fig. 3A. (B) Sample contained 12 mM Gd^{3+}, and required 5,000 accumulations. A line-broadening factor of 1.4 Hz was applied during processing. (C) Sample contained 24 mM Gd^{3+}, and required 15,000 accumulations. A line-broadening factor of 3.0 Hz was applied during processing. (D) Sample contained 49 mM Gd^{3+}, and required 15,000 accumulations. A line-broadening factor of 5.0 Hz was applied during processing.]

TABLE I

^{13}C-N.M.R. CHEMICAL-SHIFT DATA[a] FOR 2-*O*-METHYL-α-NeuAc (2) AND 2-*O*-METHYL-α-NeuAc METHYL ESTER (3)

Carbon atom	2[b]	3[c]
*αp*1	174.7	171.1
*αp*2	102.0	100.5
*αp*3	41.4	40.2
*αp*4	69.7[d]	69.8 or 68.4
*αp*5	53.3	52.9
*αp*6	73.9	73.9
*αp*7	69.7[d]	69.8 or 68.4
*αp*8	73.1	72.2
*αp*9	64.1	64.0
$-CH_3(R^1)$		55.3
$-CH_3(R^2)$	52.9	53.4
$-CH_3$(Ac)	23.5	24.2
C=O (Ac)	176.4	176.2

[a]Chemical shifts were determined from samples at neutral pH (~7.0) containing a trace of 1,4-dioxane. Estimated precision for the chemical shifts is ±0.05 p.p.m. Spectra (3,000 accumulations) were recorded using a 5.0-kHz window and a recycle time of 3 s. [b]59 mM sample in H_2O. [c]80 mM sample in H_2O. [d]Overlap of resonances, see Figs. 3 and 4.

with the literature assignments. The value of $^1J_{CH}$ observed for these resonances was 145 Hz; *αp*5 showed a doublet, and the methyl group, a quartet. We have reversed these assignments in the spectrum of compound 3 (Figs. 1 and 2). The resonance located at 52.9 p.p.m. (*αp*5) showed a doublet ($^1J_{CH}$ 145 Hz), whereas that at 53.4 p.p.m. ($-CH_3$, R^2) exhibited a quartet ($^1J_{CH}$ 150 Hz) in the coupled spectrum*.

In view of the metal-binding studies, using β-NeuAc, which indicated that the carboxyl group was essential for metal complexation[3,4], we decided to study, first, the binding of metal ions to compound 3, which has the carboxyl group methyl-esterified. Two metals were chosen for our studies: Mn^{2+} and Gd^{3+}; both are paramagnetic relaxation-reagents[15] (also line-broadening probes) and would be expected to broaden, specifically, the carbon resonances near the binding site. Gadolinium was chosen because it (*a*) is a trivalent metal-ion that has been used in ^{13}C-n.m.r.-spectral studies of proteins to assign resonances to specific carbon atoms[24,25], and (*b*) mimics the trivalent lanthanum ion used in the original, red-cell membrane–metal-ion-binding studies[1]. Manganous ion was chosen because it is a divalent metal ion used to mimic Mg^{2+} in n.m.r.-spectral studies of biological systems[26].

The effects of the addition of Gd^{3+} and Mn^{2+} on the ^{13}C resonances of the proton-decoupled, natural-abundance, ^{13}C-n.m.r. spectrum of compound 3 in aqueous

*Only three of the resonances of the quartet were discernible, as the downfield wing of the quartet was buried under other carbon resonances.

s' 'ution are shown in Figs. 1 and 2. The results were quite surprising; although the carboxyl group is esterified, metal-ion binding occurs with compound 3. As regards Fig. 1, gradual additions of Gd^{3+} result in specific line-broadening of resonances attributable to the carbon atoms of the carbonyl and methyl groups of the acetamido group, $\alpha p8$, $\alpha p9$, $\alpha p7$, and, to some extent, $\alpha p5$. We tentatively assigned resonances at 69.8 and 68.4 p.p.m. to $\alpha p7$ and $\alpha p4$, respectively; these assignments were based on the Gd^{3+}–compound 3 binding-structure that we shall propose (see later). All other resonances in our spectra are eventually broadened as a result of the general broadening phenomena that are due to the large proportion of paramagnetic species in solution. The broadening of the ^{13}C resonances in Fig. 1 may be rationalized from one metal-ion-binding structure, and this structure must have Gd^{3+} at the tail of the molecule enclosed by the glycerol-1-yl side-chain ($\alpha p9$, $\alpha p8$, and $\alpha p7$) and the entire acetamido group (see the formulas). The metal binding must occur far from the head of the molecule (the carbonyl end), because the resonances attributable to $\alpha p3$, $\alpha p2$, and the methyl groups are not broadened.

The Mn^{2+} binds to compound 3 in the same general area as Gd^{3+}, but there appear to be two possible metal-binding sites near the tail of the molecule. Fig. 3 shows the broadening effect of Mn^{2+} on the ^{13}C resonances of the proton-decoupled, natural-abundance, ^{13}C-n.m.r. spectrum of compound 3. The ^{13}C resonances that appear to broaden with the addition of Mn^{2+} may be attributed to carbon atoms of the carbonyl group of the acetamido group, $\alpha p4$, $\alpha p7$, $\alpha p9$, and, to some extent, $\alpha p5$ and the methyl group of the acetamido group. The results may be rationalized by two neighboring, metal-ion-binding sites in compound 3. Both of these sites occur at the tail of the molecule. One metal-binding site involves the oxygen atoms of $\alpha p7$, $\alpha p9$, and the carbonyl group of the acetamido group. The other structure involves the oxygen atoms of the carbonyl group of the acetamido group and $\alpha p4$.

Figs. 3 and 4 show the effects of Mn^{2+} and Gd^{3+}, respectively, on the ^{13}C resonances of the proton-decoupled, natural-abundance, ^{13}C-n.m.r. spectrum of compound 2. In comparison to compound 3, the metal binding of compound 2 (having a free carboxyl group) shows vastly different results. As regards the effects of Mn^{2+} on the ^{13}C resonances (see Fig. 3), it was found that, on addition of Mn^{2+}, resonances attributable to the carbonyl carbon atoms (acetamido group and $\alpha p1$) and $\alpha p2$ (the anomeric carbon atom) immediately disappeared (see Fig. 3B). Subsequent additions of Mn^{2+} resulted in eventual broadening of resonances attributable to carbon atoms $\alpha p8$, $\alpha p9$, $\alpha p4$, and/or $\alpha p7$, and, to some extent, $\alpha p3$ and the methyl group of the acetamido group. These results can only be rationalized on the basis of multiple metal-ion-binding sites, one near the head of the molecule, and at least one other near the tail of the molecule.

The binding of Gd^{3+} to compound 2 shows a slightly different result in comparison to that of Mn^{2+}. In Fig. 4, the resonances pertaining to the carbonyl carbon atoms (acetamido group and $\alpha p1$) and $\alpha p2$ (the anomeric carbon atom) appear to broaden after the addition of Gd^{3+} (see Fig. 4B). Subsequent additions of Gd^{3+} broaden the resonances of carbon atoms $\alpha p9$, $\alpha p3$ and, to some extent, $\alpha p8$. Although

many other resonances have already broadened beyond observation (see Fig. 4D), the resonance of the carbonyl carbon atom of the acetamido group remains observable. There are two possible explanations for these results. One is that two metal-ion-binding sites exist: one, near the head of the molecule, involving the carboxyl group, and one near the tail, involving the glycerol-1-yl side-chain. In this case, the binding constants must favor the site near the head. Another explanation is that the primary site for binding of Gd^{3+} involves not only the carboxyl group but also a reorientation of the glycerol-1-yl side-chain, such that the oxygen atoms of $\alpha p8$ and $\alpha p9$ are involved in metal binding above the pyranoid ring. The oxygen atom of the carbonyl group of the acetyl group may be involved by a distant interaction, or a very weak, secondary binding-site for Gd^{3+} may exist near the acetamido group. Our results favor the latter explanation, in view of the literature evidence suggesting that europium binds similarly to α-NeuAc (also involving other carbohydrates in the binding structure) of brain gangliosides[14].

In conclusion, our results are the first to show definitely that Mn^{2+} and Gd^{3+} bind to α-NeuAc. Moreover, they clearly indicate that multiple, metal-ion-binding sites may exist on this molecule. These results may aid future metal-ion-binding studies of oligosaccharides and glycopeptides containing α-NeuAc.

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