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N.M.R. SPECTROSCOPY AND CALCIUM BINDING OF SIALIC ACIDS: *N*-GLYCOLYLNEURAMINIC ACID AND PERIODATE-OXIDIZED *N*-ACETYLNEURAMINIC ACID*

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ABSTRACT

N.m.r. spectroscopy (^{1}H- and ^{13}C-) of *N*-glycolylneuraminic acid, and of its interaction product with Ca^{2+} at pH 7, indicated that a 1:1 complex is formed, with a formation constant of 193 M^{-1} [compared to 121 M^{-1} for *N*-acetylneuraminic acid (**1**)]. From analysis of electric-field shifts, an approximate position of the Ca^{2+} ion in the complex is inferred, with the hydroxyl group of the *N*-glycolyl group providing the additional binding. Compound **1** was oxidized with sodium periodate, and ^{13}C-n.m.r. spectroscopy was applied in an attempt to identify the aldehyde formed, and to demonstrate that the loss of the glycerol-1-yl side-chain (carbon atoms 8 and 9) decreases its Ca^{2+} ion-binding capacity.

INTRODUCTION

We have recently been concerned with the anomers of *N*-acetylneuraminic acid[1,2] (**1**), and with its favored complexing with Ca^{2+} ion, first observed by Behr and Lehn[3]. Although there is much evidence of participation of Ca^{2+} in cell-surface events[4–6], it is not clear whether the relatively weak binding to this sialic acid has biological significance.

It is, however, clear that sodium periodate ($NaIO_4$) induces, for several types of lymphocytes[7], blastogenesis which has been identified with the presence of this cell-surface sialic acid[8–11]. The mild conditions that provide optimal stimulation of lymphocytes by IO_4^- favor the oxidation of 1,2-glycols to aldehydes. The only neighboring hydroxyl groups in **1** occur on C-7, C-8, and C-9 of the D-*erythro*-glycerol-1-yl side-chain, so that oxidation leads to the splitting off of C-8 and C-9, and the retention of C-7 in an aldehyde group.

However, further oxidation can apparently occur, and other products are formed[12,13]. Our interest then lay, not only in confirming, by periodate oxidation, that complexing with Ca^{2+} involves the glycerol-1-yl side-chain, but also in attempting to identify the oxidation products.

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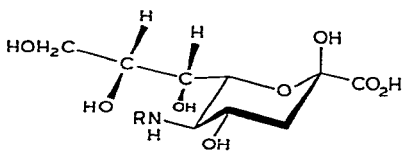
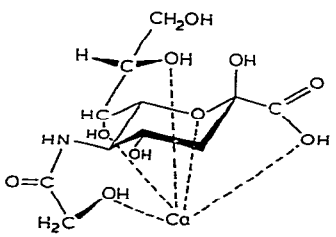
In man, *N*-glycolylneuraminic acid (**2**) is less prevalent than *N*-acetylneuraminic acid, and is presumably formed from the latter by the action of the cytoplasmic enzyme *N*-acetylneuraminate monooxygenase[14]. However, in humans, it has been found in the carbohydrate moiety of secreted "light chain" synthesized by murine myeloma[15], and it has recently been identified as the antigen attached to gangliosides that cause "serum sickness" in humans[16]. It is the dominant sialic acid in porcine submaxillary glands, which were the source of the *N*-glycolylneuraminic acid used in this research. The n.m.r. spectra of this acid, and the effect of the additional, glycolyl hydroxyl group on the calcium-binding constant, are of interest here[2].



1 R = $COCH_3$
2 R = $COCH_2OH$

RESULTS AND DISCUSSION

N.m.r. spectra (^{13}C- *and* ^{1}H-) *of* N-*glycolylneuraminic acid* (**2**). — The assignment of the carbon resonances was straightforward, because of the similarity between **1** and **2**; the spectrum of **1** had previously been assigned[2,17,18]. The difference between the two molecules is that the CH_3 group in the side chain on C-5 of **1** is replaced by CH_2OH in **2**. [See **3**, where *N*-glycolylneuraminate ion, with Ca^{2+}, is shown in its $^{2}C_5$(L) conformation.] Therefore, the methyl resonance of **1** should be absent for



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2, but there should be an added, downfield resonance in the spectrum of the latter. Table I, columns 2 and 3, compares the ^{13}C chemical-shifts of the two neuraminate ions at pD ~7. For *N*-glycolylneuraminate, the resonance of C-11 is, as expected, shifted far downfield. The replacement of CH_3 by CH_2OH has a very small effect (<0.5 p.p.m.) on the shifts of the resonances of carbon atoms 2 to 10. The C-1 chemical-shift of the *N*-glycolylneuraminate is 0.87 p.p.m. downfield from that of the *N*-acetylneuraminate ion.

TABLE I

^{13}C AND ^{1}H CHEMICAL-SHIFTS FOR GcNeu$^-$ AND AcNeu$^-$ AT pD ~7, RELATIVE TO EXTERNAL Me_4Si[a] AND INTERNAL TSP-d_4[b] REFERENCE-STANDARDS

Proton, or carbon atom	*^{13}C chemical-shifts*		*^{1}H chemical-shifts*	
	AcNeu$^-$	*GcNeu$^-$*	*AcNeu$^-$*	*GcNeu$^-$*
1	177.87[c]	178.08		
2	97.61	97.65		
3*a*	40.63	40.61	1.887	1.852
3*e*			2.331	2.239
4	68.51	68.18	4.117	
5	53.50	53.18	3.968	3.997
6	71.45	71.20	4.108	4.101
7	69.82	69.63	3.597	3.511
8	71.59	71.62	3.789	3.770
9	64.55	64.47	3.656	3.619
9′			3.880	3.848
10	175.98[c]	176.85		
11	23.34	62.25	2.067	4.149

[a]Tetramethylsilane. [b]Sodium salt of 2,2,3,3-tetradeuterio-4,4-dimethyl-4-silapentanoic acid. [c]The assignments in ref. 2 mistakenly reversed carbon atoms 1 and 10.

The spectra are all attributed to the β anomer of **2**, as, at the concentrations of these experiments, no evidence of the α anomer was found.

Table I, columns 4 and 5, shows the assignments and chemical-shift values for the ^{1}H-n.m.r. spectra of the *N*-acetyl- and *N*-glycolyl-neuraminate ions. The only major difference is, as expected, the large, downfield shift of the protons attached to C-11 on going from the *N*-acetyl- to the *N*-glycolyl-neuraminate. Fig. 1 shows the approximate, first-order, spin–spin splitting-patterns for all of the protons of *N*-

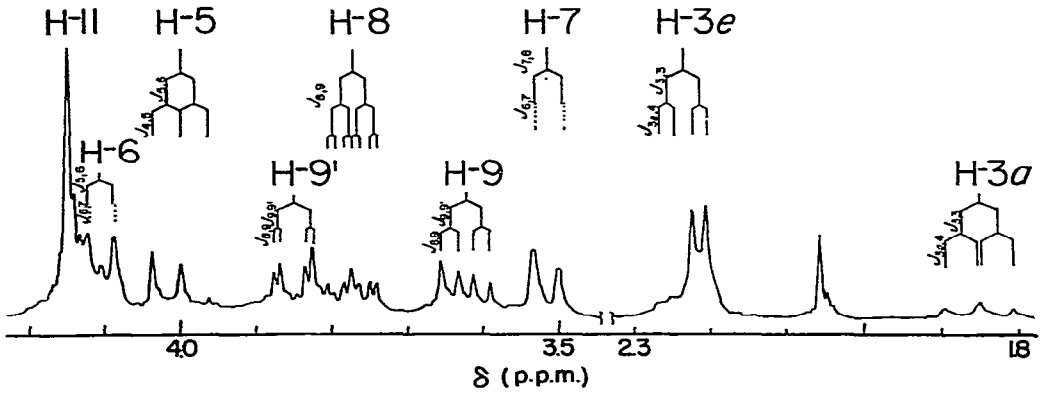


Fig. 1. ^{1}H-N.m.r. spectrum (270 MHz) of GcNeu$^-$ in D_2O, with assignments and splitting patterns indicated.

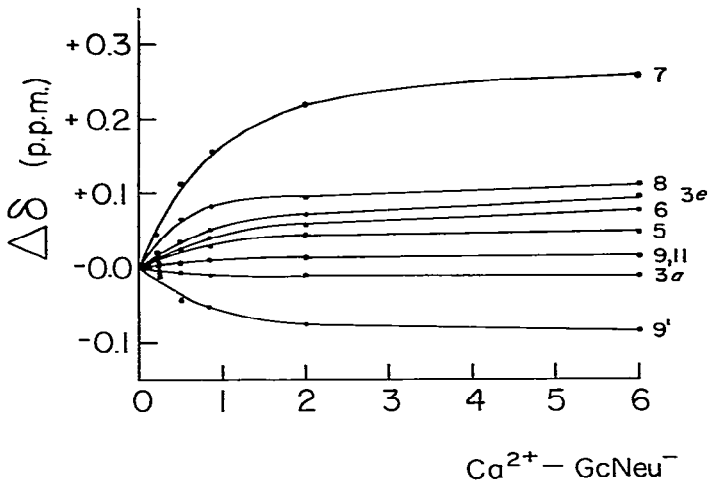


Fig. 2. ^{1}H Chemical-shifts (p.p.m.) of GcNeu$^-$ induced by Ca^{2+} at 25° and pD ~7 (relative to the shifts of GcNeu$^-$ in D_2O in the uncomplexed state).

glycolylneuraminate ion (except H-4, as the large, H-11 resonance obliterates the fine structure of that resonance). The dotted lines in Fig. 2 indicate unresolved couplings that broaden the resonances, but do not split them. Computer simulation verified that the center of gravity gave chemical-shift values within ±0.01 p.p.m. of the computer values. The assignments for *N*-glycolylneuraminate were made from the similar, ^{1}H-n.m.r. spectrum of *N*-acetylneuraminate ion, previously assigned[1]. [The spectrum (Fig. 2) clearly shows saturation effects, as may be seen by comparison of the areas of H-3*e* and H-3*a*, but the assignments are not in doubt. The unassigned

TABLE II

^{1}H–^{1}H COUPLING-CONSTANTS (Hz)[a] OF *N*-GLYCOLYLNEURAMINATE ION

Coupling constant	*Initial* [Ca^{2+}]:[*GcNeu*$^-$]					
	0.00	*0.22*	*0.50*	*0.86*	*2.00*	*6.00*
$^2J_{3a,3e}$	−12.6	−12.8	−12.8	—	−12.6	−12.2
$^3J_{3a,4}$	11.4	11.6	11.6	—	11.4	11.8
$^3J_{3e,4}$	4.6	4.4	4.6	—	4.6	4.6
$^3J_{4,5}$	10.2	10.0	10.4	10.0	10.0	10.0
$^3J_{5,6}$	10.2	10.2	9.8	10.0	10.0	10.0
$^3J_{6,7}$[b]	1.0	1.0	1.0	1.0	1.0	1.0
$^3J_{7,8}$	9.0	9.0	8.6	8.0	7.8	7.2
$^3J_{8,9}$	6.2	6.0	6.4	6.8	6.4	6.6
$^3J_{8,9'}$	2.8	2.8	3.4	—	3.4	3.8
$^2J_{9,9'}$	−11.4	−11.6	−11.8	−12.0	−12.0	−12.0

[a]Accurate to within ±0.2 Hz. [b]Estimated from line widths.

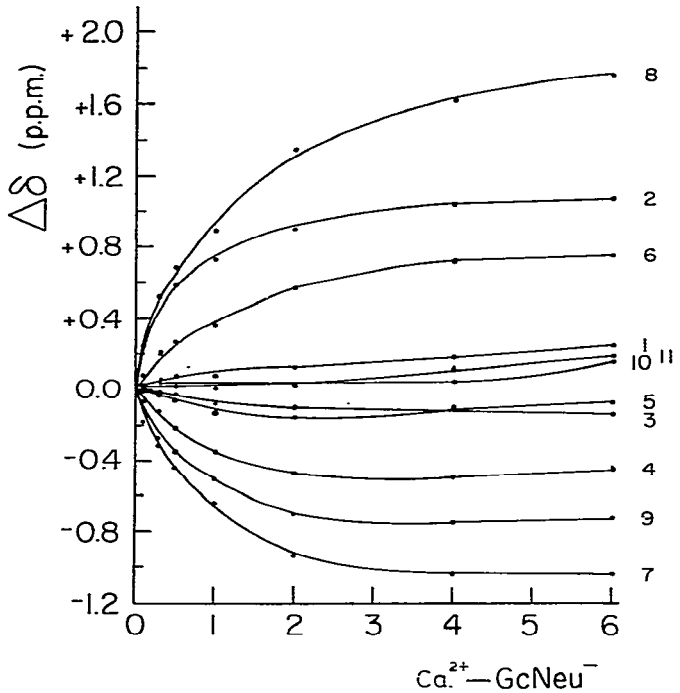


Fig. 3. ^{13}C Chemical-shifts (p.p.m.) of $GcNeu^-$ induced by Ca^{2+} at 25° and pD ~7 (relative to the shifts of $GcNeu^-$ in D_2O in the uncomplexed state).

resonance at 2.05 p.p.m. is residual *N*-acetylneuraminate.] Table II, column 2, shows the first-order, calculated coupling-constants for *N*-glycolylneuraminate ion. Computer simulation verified first-order, coupling-constant values to within ±0.2 Hz, which is the approximate limit of error for the measurements. Although $[\delta(\text{H-4}) - \delta(\text{H-5})]/J_{4,5}$ is ~3.5, which indicates some second-order effects in the spectrum, the values of J and δ obtained by computer iteration for H-4 and H-5 are correct within the accuracy of the experiment.

Calcium-induced, ^1H and ^{13}C chemical-shifts of N-*glycolylneuraminate ion.* — Fig. 2 shows the induced, proton shifts measured when the ratio of Ca^{2+}: *N*-glycolylneuraminate was varied from 0:1 to 6:1. The concentration of the sugar ranged from 0.05M to 125mM in these measurements, made at pD 7. The spin–spin coupling-constants as a function of the ratio are presented in Table II. The fact that $J_{7,8}$, $J_{8,9}$, and $J_{8,9'}$ showed significant change as calcium was added indicates, for the glycerol-1-yl side-chain, a change of orientation that enhances the interaction with the cation. The relative constancy of the ring coupling-constants indicates that the conformation does not change as calcium is added.

It has been shown by several workers[19–23] that ^{13}C-resonances are more drastically shifted by an electric charge than proton resonances. Fig. 3 shows the calcium-induced, ^{13}C-shifts as the ratio of Ca^{2+}: *N*-glycolylneuraminate is varied

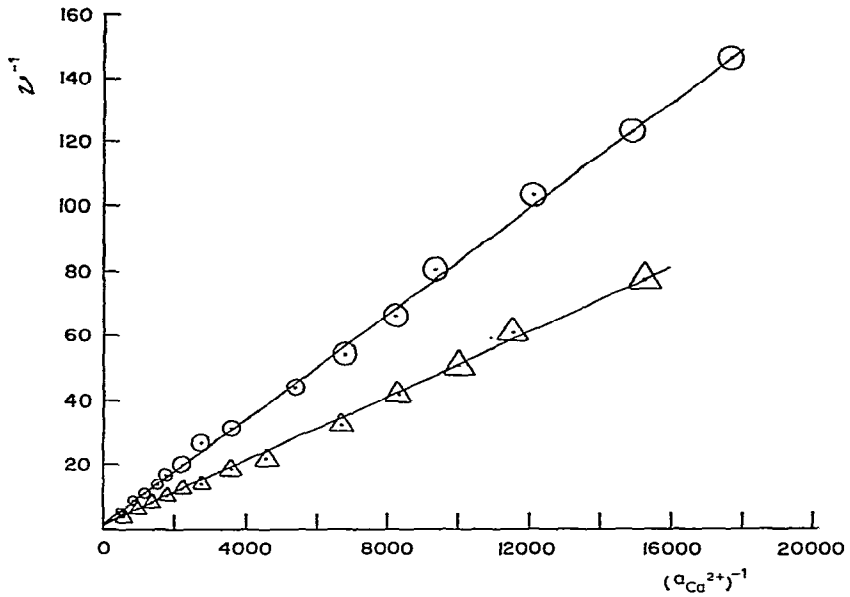


Fig. 4. Plot of ν^{-1} (total carbohydrate:bound Ca^{2+}) *vs.* $(a_{Ca})^{-1}$, where a_{Ca} is the activity of free Ca^{2+} for both the AcNeu$^-$ complex and the GcNeu$^-$ complex. (The circles indicate approximate limits of error for the AcNeu$^-$ system, and the triangles, approximate limits of error for the GcNeu$^-$ system.)

at pD 7. The concentration of the neuraminate was held at 153mM during these measurements.

Calcium N-*glycolylneuraminate complex.* — By use of a calcium-ion-selective electrode to measure the free calcium in the solutions, and the iterative procedure described previously[2], a plot of v^{-1} (total carbohydrate:bound calcium) *versus* $(a_{Ca})^{-1}$ could be made[24], as shown in Fig. 4. The data for both *N*-acetyl- and *N*-glycolyl-neuraminate ions yield least-squares, straight lines having intercepts approximately equal to unity (1.02 for *N*-acetyl- and 0.80 for *N*-glycolyl-neuraminic acid), indicating a 1:1 complex for both. From the good linearity over the entire range in Fig. 4, it is clear that only a 1:1 complex is formed, even when the ratio of carbohydrate:Ca^{2+} is high. The slopes indicate a formation constant, K_f, of 193 M^{-1} for the calcium complex with *N*-glycolylneuraminate, *versus* 121 M^{-1} for *N*-acetylneuraminate.

Knowing that only a 1:1 complex is formed, the calcium-induced chemical-shifts of the complex can be calculated from the measured data in Figs. 3 and 4 by a procedure similar to that of Prestegard and Chan[25] with $K_f = 193$ M^{-1}. From theories of electric-field shifts, this $\Delta\delta$(complex) may then be used to find the approximate position of the Ca^{2+} ion relative to the *N*-glycolylneuraminic acid.

Position of the Ca^{2+} ion: calculation of electric-field shift. — The signs of the ^{1}H-shifts in Fig. 3 and column 2 of Table III determine the general position of the calcium ion relative to the protons of the ring and side-chain. The direction of all

TABLE III

CALCIUM-INDUCED CHEMICAL-SHIFTS IN THE Ca^{2+}–GcNeu$^-$ COMPLEX. POSITION OF Ca^{2+} IN COMPLEX, DERIVED FROM ELECTRIC-FIELD-SHIFT THEORY

Proton, or carbon nucleus (i)	*Δδ(complex)*[a] *(p.p.m.) Found* ^1H	^{13}C	*r(Ca²⁺–i) (nm)*	*θ (degrees)*[b] *(calc.)* ^1H	*θ (degrees)*[c] *(calc.)* ^{13}C
1		+0.17			
2		+1.21	0.51[d]		
3*a*	−0.015	−0.09	0.70	87	
3*e*	+0.088		0.70	76	68
4	—	−0.47	0.75	—	50
5	+0.054	−0.13	0.75	(100)[e]	(107)[e]
6	+0.071	+0.55	0.70	79	86
7	+0.278	−0.96	0.72	35[f]	30
8	+0.163	+1.45	0.53	86	90
9	+0.017	−0.74	0.80	86[g]	
9′	−0.099		0.70	73[g]	51[g]

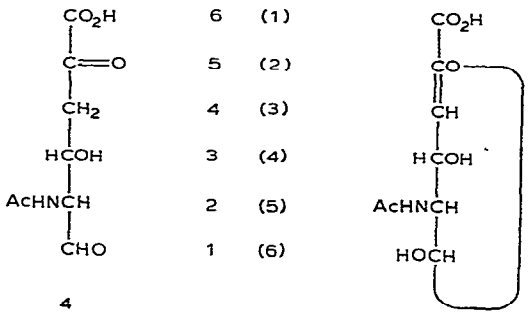
[a]Chemical shift of ^1H_i or $^{13}C_i$ in GcNeu (Gc = glycolyl) in the complex, relative to that in the uncomplexed state at pH ~7. [b]Angle between C–H bond direction and line joining Ca^{2+} and center of bond, derived by using Buckingham's theory[26]. [c]Angle between symmetry axis of carbon atom and line joining Ca^{2+} and carbon nucleus, derived by using Batchelor's theory[27]. [d]C-2 is assumed to be a quaternary carbon atom, and there is no uniform field-shift. The value of r was obtained from the field-gradient term[27]. [e]cos θ is here calculated to be negative, and is not in agreement with the model (see text). [f]Calculated on the assumption that Ca^{2+}–H-7 is the same as for AcNeu. [g]For a comparison of these angles, the average of the two $\theta(^1H)$ angles should be used.

of the induced shifts is the same as for *N*-acetylneuraminate ion, indicating that the geometries in the two complexes are very similar[2]. A detailed description of the application of Buckingham's electric-field-shift theory[26] to these shifts may be found in an earlier paper[2]. The major difference in this complex is that H-7 is less deshielded than in the *N*-acetylneuraminate complex. An examination of a molecular (CPK) model revealed that, as the extra hydroxyl group in the glycolyl chain participates in the binding, Ca^{2+} should be positioned slightly more towards the center of the ring system. This slight perturbation decreases the Ca^{2+}–C-7–H-7 angle to give the aforementioned results. The induced shifts remaining are very much the same as for AcNeu, so it is presumed that the distance from the Ca^{2+} to the protons is approximately the same for both of these complexes. By knowing the distances from Ca^{2+} to the protons, listed in column 4 of Table III, the angles (θ) can be calculated; they are given in column 5.

The application of electric-field-shift theory to induced, ^{13}C chemical-shifts was given in most explicit form by Batchelor[27], and has been applied to calcium ion binding to *N*-acetylneuraminic acid[2]. A modified version has recently been applied to calcium binding with D-glucuronic and D-galacturonic acid[23]. Here, we have made crude calculations in order to ascertain the general location of the Ca^{2+} ion relative

to *N*-glycolylneuraminate ion. If the distances given in column 4, Table III, and the experimental shifts in column 3 are used in the theory, the angle $\theta(^{13}C)$ (between the symmetry axis at the particular carbon atom and the line joining the charge and that carbon atom) can be derived. These angles, compiled in column 6 of Table III, are in reasonable agreement. As was found with *N*-acetylneuraminate[2], C-5 is "anomalous", because the $\Delta\delta$ shift has the wrong sign (indicated by $\theta > 90°$), as was true for the proton spectra. As the ring-proton coupling-constants are invariant as calcium is added, there is no conformational change, but a change in the orientation of the glycerol-1-yl side-chain occurs when the complex is formed. The O-7 atom interacts strongly with the calcium ion *via* its filled *p*-orbital, and, in doing so, the O-7–H bond is oriented for maximum attraction. As may best be seen from a CPK model, the other lobe of the filled *p*-orbital is simultaneously pushed toward H-5. The proximity of this negative charge is considered to be the reason for the downfield shift of H-5 and the upfield shift for C-5. The Ca^{2+} ion offers only a weak counter-effect, being at a much greater distance than this negative charge.

Periodate oxidation of N-*acetylneuraminic acid* (**1**). — Even though the C-8 and C-9 resonances are conspicuously absent from the ^{13}C spectrum, indicating that periodic oxidation had occurred, the spectrum was not "clean", in that it contained extra, unaccounted-for, peaks (based on the product expected). The presence of several products was also indicated by paper chromatography, which showed three spots on the chromatogram. This formation of extra products in periodate oxidation is not unusual[12], and has been discussed specifically for **1** by Paerels and Schut[13]. They suggested that oxidation can continue farther, with the elimination of C-7 (as formaldehyde), and the formation of a "3-deoxy-2-hexulosuronic acid" (**4**) (numbered as in **1**), namely, 2-acetamido-2,4-dideoxy-L-*threo*-5-hexulosuronic acid, which may tautomerize to the cyclic form (**5**) containing a carbon–carbon double-bond. However, this "pre-chromogen" (ref. 13) was judged to be unstable, as there



was a gradual decrease of the extinction curve by ~20% during a period of ~30 h. Our oxidation product was also unstable. An attempt to record the ^{13}C-n.m.r. spectrum at room temperature was thwarted, because the sample noticeably began to decompose after several hours. All spectra of the oxidation products were therefore

TABLE IV

^{13}C CHEMICAL-SHIFTS[a] (p.p.m.) OF PERIODATE-OXIDIZED *N*-ACETYLNEURAMINIC ACID AT 0° AND OF *N*-ACETYLNEURAMINIC ACID AT 25°, RELATIVE TO EXTERNAL Me_4Si

Carbon No.	*Periodate-oxidized AcNeu*	*AcNeu*
11	23.32	23.27
3	31.15	40.00
5	53.89	53.26
4	75.88	67.88
6	80.58	71.34
2	105.64	96.46
1	176.18	174.49
10	176.18	176.06
7	209.14	69.43
O-C=*C*-H[b]	103.43	
O-*C*=C-H[b]	168.36	

[a]See text for further explanation. [b]Also listed are extra resonances, possibly from the pre-chromogen[13].

recorded at 0°, a temperature at which little decomposition was observed during 7 h.

Although, in the ^{13}C-n.m.r. spectrum, there was evidence of some shoulders on peaks assigned to oxidized *N*-acetylneuraminic acid, two distinct, extra lines did appear (see Table IV) that suggested that a compound containing alkene carbon atoms, such as those in **4**, was present in the oxidation products. Off-resonance decoupling indicated that the carbon atom producing the extra peak at 103.43 p.p.m. has one hydrogen atom attached, and that the carbon atom producing the resonance at 168.36 p.p.m. has no attached hydrogen atoms. The 168.36-p.p.m. resonance correlates well with other signals due to doubly-bonded carbon atoms attached to oxygen that have been observed[28] at 161.6 and 166.0 p.p.m. These signals are, then, assignable to the alkene carbon atoms in **5**.

An attempt was made to synthesize methyl (methyl 4,5-dideoxy-L-*threo*-hex-4-enosid)uronate ["methyl (methyl $\Delta^{4,5}$-α-D-galactosid)uronate"], a compound closely related to the prechromogen[13], by the methods of Jones and Stacey[29] and Heim and Neukom[30]. Methyl (methyl α-D-galactosid)uronate was prepared, and identified by its ^{13}C-n.m.r. spectrum, but attempts to prepare the alkene from it were unsuccessful. Hence, identification of **5** as a product of the periodate oxidation was inconclusive.

The Ca^{2+} ions were added incrementally, to a ratio of Ca^{2+} to the periodate-oxidized acid of 1:1, and ^{13}C shifts were recorded at 0°. At the highest concentration of calcium, only very slight, induced shifts were observed (<0.20 p.p.m.), compared with maximum shifts of 1.13 p.p.m. for C-8 in the *N*-acetylneuraminate ion. This lack of interaction with calcium is not surprising, because, after periodate oxidation, the glycerol-1-yl side-chain is no longer present, leaving a product somewhat similar

to hexoses, for which no complexing with calcium has been observed[2]. This further agrees with the hypothesis of Behr and Lehn[3], later supported[2], that the glycerol-1-yl side-chain is intimately involved in the complexing of Ca^{2+} with sialic acid.

EXPERIMENTAL

N-Glycolylneuraminic acid. — The mucin of porcine submaxillary glands was purified by a combination of the procedures of Tettamanti and Pigman[31] and Pigman *et al.*[32]. The procedure described by the former workers[31] was carried out as far as the step where 10% cetyltrimethylammonium bromide is added to achieve complete formation of clot. The subsequent dissolution of the clot and fractional precipitation with ethanol were performed as described[32] by the latter workers. The 45–60%-ethanol precipitate was solubilized in distilled water by homogenization in an Omni-mixer at high speed, and then dialyzed extensively against distilled water, and kept as a frozen, aqueous solution.

The *N*-glycolylneuraminic acid in the mucin solution was released by mild hydrolysis with acid. Mucin (~2 g) in 150 mL of solution was heated at 80° with 15mM H_2SO_4 for 90 min, cooled, the pH brought to 6.5 with $Ba(OH)_2$, and the $BaSO_4$ removed by centrifugation. The hydrolyzate was then placed on a column of Dowex-50 (H^+) (200–400 mesh), and the acidic effluent was collected, and absorbed on a column of Dowex-1 (formate) (200–400 mesh). The column was washed with distilled water, and the *N*-glycolylneuraminic acid was eluted with 0.3M formic acid. The pooled, direct-Ehrlich-positive material was lyophilized to dryness, and the product (~130 mg), mostly **2**, was weighed.

Periodate oxidation of **1**. — *N*-Acetylneuraminic acid (Sigma, Type VI; 250 mg) was dissolved in periodic acid (500 mg, 200 mL), and oxidized for 15 min at 0° in the dark. After adding ethylene glycol (25 mL) and mixing, the solution was allowed to stand for 10 min, the pH adjusted to 5 with concentrated NH_4OH, and the solution placed on a column of Dowex-1 (Cl^-) resin (50–100 mesh). The resin was washed with 3 column-volumes of distilled water, and eluted with 0.1M NaCl. The first 200 mL of eluate was negative to resorcinol, whereas the next 450 mL contained resorcinol-positive material; the latter eluate was lyophilized to ~50 mL, desalted by passage through a column of Dowex-50 (H^+) resin, and lyophilized.

The product, a yellow syrup, was obtained in 64% yield (as estimated by the periodate–resorcinol test). An aliquot of the product was chromatographed on Whatman No. 1 filter paper (with **1** as the reference standard), developed in the descending manner with 6:4:3 1-butanol–pyridine–water. A yellow color moved very fast, almost with the solvent front. After color development with the resorcinol dip-reagent, two other spots were noticeable, namely, a deep-blue spot of R_F value ~3 times that of **1**, and a faster-running, pink spot. The slower-moving band is attributed to the aldehyde formed by cleavage between C-7 and C-8 in the glycerol-1-yl side-chain[33].

Preparation of samples for n.m.r. spectroscopy. — Deuterium oxide was pur-

chased from Sigma Chemical Co. (St. Louis, MO), and calcium chloride dihydrate was reagent grade from Mallinckrodt, Inc. (St. Louis, MO).

The ^{13}C-n.m.r. spectra were recorded with a Varian XL-100 n.m.r. spectrometer as described previously[2]. Samples of both acids (**1** and **2**) in D_2O (~50 mg/mL) were prepared in 12-mm, n.m.r. tubes, with Me_4Si as the external reference-standard in a coaxially mounted, 5-mm, n.m.r. tube. The *N*-glycolylneuraminate samples were prepared at pD ~7 by using KOH to neutralize the acidic solutions. Owing to the instability of the sample, the periodate-oxidized *N*-acetylneuraminic acid samples were run at 0° in a nitrogen atmosphere.

The ^1H-n.m.r. spectra of **2** were recorded with a 270-MHz, Bruker instrument at Florida State University, as previously described[1]. The sodium salt of 2,2,3,3-tetradeuterio-4,4-dimethyl-4-silapentanoic acid (TSP-d_4) was used as the internal reference-standard. From 32 to 256 scans were employed for each sample.

The formation constant for the complexing of calcium ion with **2** was determined by use of an Orion 801A digital mV/pH-meter with accompanying electrodes, as described previously[2]. The ratio of Ca^{2+} to **2** ranged from 1:40 to 1:1.

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