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Chemical behaviour of cytidine 5′-monophospho-*N*-acetyl-β-D-neuraminic acid under neutral and alkaline conditions

Jean-Marie BEAU, Roland SCHAUER, Johan HAVERKAMP, Johannis P. KAMERLING, Lambertus DORLAND, and Johannes F. G. VLIEGENTHART

Biochemisches Institut, Christian-Albrechts-Universität, Kiel; and Department of Bio-Organic Chemistry, University of Utrecht

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The chemical behaviour of CMP-*N*-acetylneuraminic acid under neutral and different alkaline conditions has been investigated. The products formed were isolated by ion-exchange chromatography and gel filtration and analysed by colorimetric methods, thin-layer chromatography, combined gas-liquid chromatography/mass spectrometry and/or 360-MHz ^{1}H-NMR spectroscopy. A maximum stability of CMP-*N*-acetylneuraminic acid was observed at pH 8–11. In the tested pH range of 6–13, CMP and *N*-acetylneuraminic acid were formed in variable amounts as decomposition products. 2-Deoxy-2,3-dehydro-*N*-acetylneuraminic acid was produced at pH > 7; the amount of this substance increased with increasing pH. In anhydrous triethylamine its yield was 50%. A new neuraminic acid derivative, *N*-acetyl-β-D-neuraminic acid 2-phosphate, could be isolated from the mixture of alkaline decomposition products of CMP-*N*-acetylneuraminic acid. The yield of this compound was maximum 22% in anhydrous triethylamine.

Because 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid was formed under simulated physiological conditions, it is assumed that this compound, which occurs in tissues and fluids of man and animals, is derived from CMP-*N*-acetylneuraminic acid non-enzymically also under conditions *in vivo*.

Cytidine-5′-monophospho-*N*-acetyl-β-D-neuraminic acid (CMP-Neu5Ac), being the *N*-acetylneuraminic acid (Neu5Ac) donor for the sialylation of glycoconjugates [1], appears to play an important role in the regulation of sialic acid metabolism. Although the acid-catalysed hydrolysis of CMP-Neu5Ac to Neu5Ac and CMP is well known [2], the chemical behaviour of the sugar nucleotide under neutral and alkaline conditions has received little attention. In this paper the various products formed under the latter conditions will be presented and discussed.

MATERIALS AND METHODS

Materials

Neu5Ac was isolated from the urine of a patient with sialuria [3, 4] and freed from Neu5Ac2en by cellulose chromatography [5]. Neu5Ac2en was prepared as described [6]. The synthesis and purification of CMP-Neu5Ac using an enzyme preparation from frog liver has been reported elsewhere [7]. Before lyophilization, the CMP-Neu5Ac eluate from the Dowex column was diluted with 3 vol. water. The CMP-Neu5Ac preparation obtained was chromatographically pure. Cytosine, cytidine and CMP were purchased from Boehringer Mannheim GmbH.

Colorimetric analyses

Sialic acids were determined by the orcinol/Fe^{3+}/HCl (Bial) assay [8], the periodic acid/thiobarbituric acid (Warren) assay [9] and the $NaBH_4$/periodic acid/thiobarbituric acid ($NaBH_4$/Warren) assay [10]. The latter method is especially suitable for the differentiation between free Neu5Ac and glycosidically bound derivatives, e.g. CMP-Neu5Ac and Neu5Ac2*P*. Phosphate was analysed by the method of Eibl and Lands [11] before and after treatment with 1 M sulfuric acid for 1 h at 100 °C.

Chromatography and mass spectrometry

Thin-layer chromatography was performed on 0.2-mm silicagel 60 F_{254} aluminium sheets and on 0.1-mm cellulose plastic sheets (E. Merck, Darmstadt). The silicagel sheets were developed with ethyl acetate/acetic acid/water (5:2:2, by vol.; solvent I) or with 1-propanol/acetone/water (5:3:2, by vol.; solvent II). For the cellulose sheets 1-propanol/1-butanol/0.1 M HCl (2:1:1, by vol.; solvent III), acetone/water/1-propanol (3:2:1, by vol., solvent IV) or 95% ethanol/1 M ammonium acetate, pH 7.3 (7:3, by vol., solvent V) were used. Cytosine derivatives and Neu5Ac2en were visualized under ultraviolet light. Sialic acids were stained with the orcinol/Fe^{3+}/HCl spray reagent [12] on cellulose sheets, or with 50% H_2SO_4 in methanol (10 min at 120 °C) on silica gel sheets.

Gas-liquid chromatography and combined gas-liquid chromatography/mass spectrometry of the trimethylsilylated methyl esters of Neu5Ac and Neu5Ac2en using 3.8% SE-30 as stationary phase were carried out as described before [13].

Dedicated to Hans Faillard on the occasion of his 60th birthday.

Abbreviations. Neu5Ac, *N*-acetyl-D-neuraminic acid; Neu5Ac2en, 2-deoxy-2,3-dehydro-*N*-acetyl-D-neuraminic acid; Neu5Ac2*P*, *N*-acetyl-β-D-neuraminic acid 2-phosphate; CMP-Neu5Ac, cytidine-5′-monophospho-*N*-acetyl-β-D-neuraminic acid; GLC, gas-liquid chromatography; GLC-MS, gas-liquid chromatography/mass spectrometry; TLC, thin-layer chromatography.

Ultraviolet spectroscopy

Ultraviolet spectra of CMP-Neu5Ac decomposition products, cytidine and cytosine were recorded with an Unicam SP-800 A spectrophotometer at pH 7–9 in 1 mM ammonium hydroxide.

360-MHz ^{1}H-NMR spectroscopy

The ^{1}H-NMR spectra of Neu5Ac2en and Neu5Ac2*P* were recorded in ^2H_2O at 360 MHz with a Bruker HX-360 spectrometer, operating in the Fourier-transform mode at a probe temperature of 25 °C (SON, NMR-facility, State University of Groningen, The Netherlands). Resolution-enhancement of the spectra was achieved by Lorentzian-to-Gaussian transformation according to Ernst [14]. Before analysis, the samples were exchanged three times in ^2H_2O with intermediate lyophilization. Chemical shifts (δ) are given relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate (indirectly to acetone in ^2H_2O: $\delta = 2.225$ ppm).

Treatment of CMP-Neu5Ac in 0.1 M Tris/HCl buffer, pH 8 (preparative scale)

A solution of CMP-Neu5Ac (160 µmol) in 2 ml 0.1 M Tris/HCl buffer, pH 8, was kept for 3 days at 25 °C. Subsequently, the solution was diluted with 100 ml cold water and rinsed slowly through a column of Dowex 1 × 8 (200–400 mesh, HCO_3^- form; 30 × 2.4 cm), equilibrated with 1 mM ammonium hydroxide at 4 °C. After washing the resin with 100 ml of the same solvent the adsorbed compounds were eluted with a linear gradient (0–0.6 M; 1.2 l) of triethylammonium bicarbonate, pH 8, at a flow rate of 30 ml/h. The eluate was monitored at 280 nm and the fractions (10 ml) were analysed colorimetrically for their sialic acid content (Bial, Warren and BH_4/Warren assays). On guidance of the various analyses, fractions A–E (Fig. 1) were obtained and lyophilized. Fractions D and E were re-chromatographed on a column of Sephadex G-25, fine (80 × 2.2 cm), equilibrated and eluted (flow rate 16 ml/h) with 1 mM ammonium hydroxide at 4 °C. For analysis of the effluents the absorbance at 280 nm and the Warren assay were used. The various lyophilized fractions were analysed by TLC silica gel (solvents I and II) and cellulose (solvents III–V), GLC, GLC-MS, colorimetric sialic acid and phosphate estimations, ultraviolet spectroscopy and 360-MHz ^{1}H-NMR spectroscopy.

Small-scale treatment of CMP-Neu5Ac under various neutral and alkaline conditions

Samples of CMP-Neu5Ac (120 nmol) were incubated for 3 h at 52 °C in 200 µl of the following buffers: 0.1 M sodium phosphate buffers of pH 6, 7 or 8; 0.1 M Tris/HCl buffers of pH 7, 8 or 9; 0.1 M sodium bicarbonate buffers of pH 10 or 11, and NaOH/0.1 M KCl solutions of pH 12 or 13. Over pH 10, the pH was maintained at the desired value by the addition of 1 M NaOH. At the end of the incubations the mixtures were diluted with 800 µl cold water and applied on columns of 1 ml Dowex 1 × 8 (200–400 mesh, HCO_3^- form), equilibrated with water at 4 °C. The resin was washed with 6 ml water and the adsorbed compounds were eluted with 6 ml 0.14 M triethylammonium bicarbonate buffer, pH 8 (eluate a), followed by 6 ml 0.4 M triethylammonium bicarbonate buffer, pH 8 (eluate b). Both eluates were lyophilized and analysed for ultraviolet absorbance at 271 nm. The sialic acid content was determined using the Bial assay (eluate a), the Warren assay (eluates a and b) and the $NaBH_4$/Warren assay (eluate b). The nature of sialic acids was checked by TLC. The procedure was calibrated with reference samples of cytidine, CMP, Neu5Ac, Neu5Ac2en and CMP-Neu5Ac. Cytidine, Neu5Ac and Neu5Ac2en were found in eluate a; CMP and CMP-Neu5Ac were found in eluate b.

In a further set of experiments, samples of CMP-Neu5Ac (600 nmol) were incubated with 50 µl of 30% aqueous ammonium hydroxide, 50% aqueous triethylamine and anhydrous triethylamine, respectively. Sealed vials were shaken for 17 h at 52 °C. At the end of incubation, triethylamine or ammonia were evaporated under a stream of nitrogen. The residues were dissolved in cold water (1 ml) and analysed as above.

RESULTS

Identification of the products formed by incubation of CMP-Neu5Ac in 0.1 M buffer, pH 8

The incubation on a preparative scale of CMP-Neu5Ac in 0.1 M Tris/HCl buffer, pH 8, for 3 days at room temperature led to the formation of a number of decomposition products that could be separated on Dowex 1 × 8. Fig. 1a shows the elution profile. The R_F values on TLC in different solvent systems of a series of CMP-Neu5Ac decomposition products are summarized in Table 1. The compound corresponding to

Table 1. *Thin-layer chromatographic data of CMP-Neu5Ac and degradation products*
The solvent systems used were: (I) ethyl acetate/acetic acid/water (5:2:2, by vol.); (II) 1-propanol/acetone/water (5:3:2, by vol.); (III) 1-propanol/1-butanol/0.1 M HCl (2:1:1, by vol.); (IV) acetone/water/1-propanol (3:2:1, by vol.); (V) 95% ethanol/1 M ammonium acetate, pH 7.3 (7:3, by vol.)

Compound	Occurrence in peak of Fig. 1a	R_F on				
		silicagel		cellulose		
		I	II	III	IV	V
CMP-Neu5Ac	D, E					0.20
Neu5Ac	B	0.14		0.60		0.53
Neu5Ac2en	C	0.27		0.71		0.59
CMP	D					0.06
Neu5Ac2*P*	E					0.12
Cytidine	A		0.53		0.53	
Cytosine			0.43		0.44	

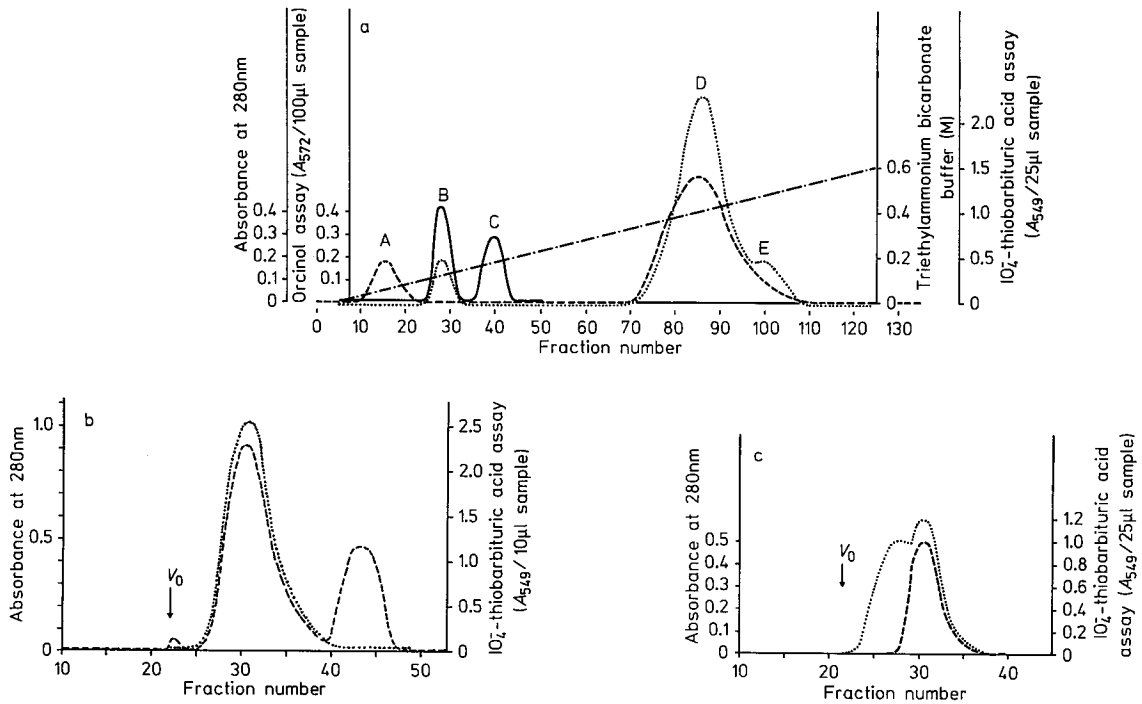

Fig. 1. *Separation of the products obtained on incubation of CMP-Neu5Ac at pH 8 in 0.1 M Tris/HCl buffer for 3 days at 25 °C.* The eluate was monitored for absorbance at 280 nm (——), for sialic acid content using the Bial assay (–) and the Warren assay (·····). (a) Elution diagram on Dowex 1 × 8, HCO_3^- form, using a linear gradient (–·–·–) of triethylammonium bicarbonate buffer, pH 8, at 4 °C. Peaks correspond to: (A) cytidine; (B) Neu5Ac; (C) Neu5Ac2en; (D) CMP and CMP-Neu5Ac; (E) CMP-Neu5Ac and Neu5Ac2*P*. (b) Separation of CMP-Neu5Ac and CMP (peak D of a) on Sephadex G-25 in 1 mM ammonium hydroxide at 4 °C. Fractions 25–38, CMP-Neu5Ac; fractions 39–47, CMP. (c) Separation of CMP-Neu5Ac and Neu5Ac2*P* (peak E of a) on Sephadex G-25 using 1 mM ammonium hydroxide at 4 °C. Fractions 22–27, Neu5Ac2*P*; fractions 29–37, CMP-Neu5Ac

peak A was identified to be cytidine as was evident from the R_F values on TLC (Table 1) and the ultraviolet spectrum (Table 2). The Bial-positive and Warren-positive substance corresponding to peak B was identified as Neu5Ac by TLC (Table 1) and GLC-MS [13]. Peak C, detected with the Bial assay, was identified as Neu5Ac2en by TLC, GLC-MS [13], and by 360-MHz ^{1}H-NMR spectroscopy (Fig. 2; Table 3). The failure of Neu5Ac2en to react in the Warren test has been reported earlier [4, 15, 16]. The material corresponding to peak D was shown to be a mixture of CMP-Neu5Ac and CMP (Fig. 1b, TLC on cellulose, Table 1). The components were separated on Sephadex G-25 yielding an A_{280}-positive, Warren-positive fraction of CMP-Neu5Ac followed by an A_{280}-positive, Warren-negative fraction of CMP. Peak E was further fractionated on Sephadex G-25 (Fig. 1c) and contained CMP-Neu5Ac and a new compound, Neu5Ac2*P* (see below).

Table 2. *Ultraviolet absorption data of cytosine, cytidine and the CMP-Neu5Ac degradation product of peak A in Fig. 1a*
The spectra of the model substances cytosine and cytidine are in good agreement with the spectra described in [22]

Compound	λ_{max1}	λ_{max2}	λ_{min}	Absorbance ratios	
				250/260	280/260
	nm				
Cytosine	267	–	248	0.79	0.58
Cytidine	271	229	250	0.86	0.93
Peak A	271	229	250	0.86	0.93

Identification of N-*acetyl-β-*D*-neuraminic acid 2-phosphate*

The compound present in addition to CMP-Neu5Ac in fraction E (Fig. 1 a and c) reacts positively in the Warren assay. It contains equimolar amounts of Neu5Ac and phosphate (Table 4). Quantitative estimation by the Warren method with and without previous $NaBH_4$ treatment gave identical results for Neu5Ac implying that the compound was an acid-labile glycoside. With and without previous sulfuric acid treatment, identical values for inorganic phosphate were also obtained. After mild acid hydrolysis with formic acid (pH 3, 25 °C, 3 h), Neu5Ac was formed quantitatively. These data point to the presence of a labile phosphate group at position 2 of Neu5Ac.

Conclusive evidence for the structure was obtained from the 360-MHz ^{1}H-NMR spectrum recorded in ^2H_2O (Fig. 3). The values of the vicinal coupling constants of the ring protons (see Table 3) indicate a $^2C_5$(D) chair conformation for the sialic acid derivative. The presence of a phosphate group in axial orientation comes to expression in an extra splitting of the signal of H-3*ax* of the Neu5Ac unit (long-range coupling between phosphorus and H-3*ax*). In addition, the data agree well with those published earlier for cytidine-5′-monophospho-*N*-acetyl-*β*-D-neuraminic acid (Table 3) [7, 17].

Analysis of the products formed from CMP-Neu5Ac in various neutral and alkaline analytical decomposition experiments

The amounts of CMP-Neu5Ac and its decomposition products obtained after incubation in various buffers at

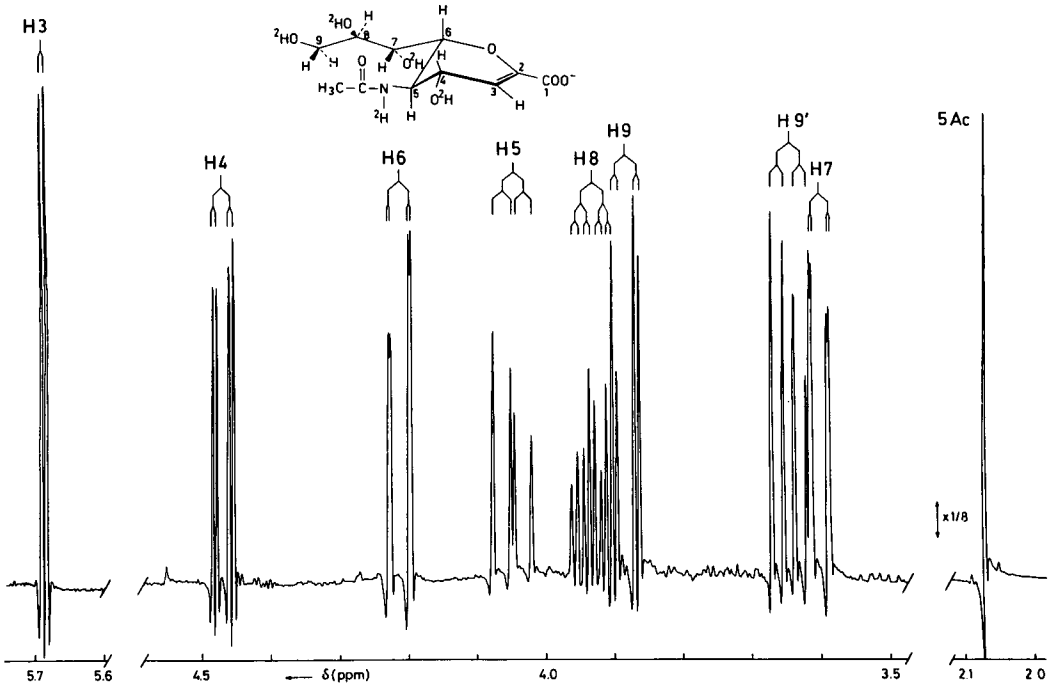

Fig. 2. *Resolution-enhanced 360-MHz ^{1}H-NMR spectrum of Neu5Ac2en in ^2H_2O, recorded at p^{2}H 6 and 25 °C.* For details see Materials and Methods and Table 3

Table 3. *360-MHz ^{1}H-NMR data of CMP-Neu5Ac, Neu5Ac2P and Neu5Ac2en*
Chemical shifts (δ) were measured relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate; coupling constants (J) are given for solutions in ^2H_2O (25 °C) at p^{2}H = 8 (CMP-Neu5Ac), p^{2}H = 7.5 (Neu5Ac2*P*), and p^{2}H = 6.0 (Neu5Ac2en)

Proton	Chemical shift for Neu5Ac unit in			Coupling constant	Value for Neu5Ac unit in		
	CMP-Neu5Ac	Neu5Ac2*P*	Neu5Ac2en		CMP-Neu5Ac	Neu5Ac2*P*	Neu5Ac2en
	ppm				Hz		
H-3*eq*	2.484	2.403	5.690	$^3J_{3eq,4}$	5.0	4.8	2.3
				$^4J_{3eq,P}$	< 1.0	< 1.0	–
H-3*ax*	1.639	1.548	–	$^3J_{3ax,4}$	11.6	11.2	–
				$^4J_{3ax,P}$	6.1	4.2	–
H-4	4.066	4.093	4.470	$^2J_{3eq,3ax}$	−13.4	−12.8	–
H-5	3.92[a]	3.888	4.051	$^3J_{4,5}$	10.4	9.1	8.8
H-6	4.141	4.239	4.213	$^3J_{5,6}$	10.5	10.5	10.9
H-7	3.456	3.386	3.601	$^3J_{6,7}$	1.2	< 1.0	1.0
H-8	3.92[a]	4.028	3.936	$^3J_{7,8}$	9.7	9.6	9.3
H-9	3.90[a]	3.883	3.885	$^3J_{8,9}$	2.8	2.6	2.8
H-9′	3.622	3.581	3.646	$^3J_{8,9'}$	6.2	6.9	6.3
CH_3(5Ac)	2.054	2.045	2.068	$^2J_{9,9'}$	−11.7	−11.7	−11.9

[a] Complex multiplets.

Table 4. *Colorimetric analysis of Neu5Ac2P*
Neu5Ac was determined by the Warren assay [9] and the $NaBH_4$/Warren assay [10]. Inorganic phosphate was determined according to [11] before (a) and after (b) mild acid hydrolysis with formic acid, pH 3 (25 °C, 3 h). Total phosphate was determined as for inorganic phosphate after hydrolysis with 1 M sulphuric acid (100 °C, 1 h). Cytosine was determined by ultraviolet absorption at 271 nm, $\varepsilon = 9 \times 10^3\ M^{-1} \cdot cm^{-1}$; it represents a slight contamination by CMP-Neu5Ac

Residue	Amount
	mol/mol
Neu5Ac	1.00 [9], 0.98 [10]
Inorganic phosphate	0.96 (a), 0.95 (b)
Total phosphate	1.02
Cytosine	0.02

pH 6–13 for 3 h at 52 °C are presented schematically in Fig. 4. At pH 6–7 the well-known acid-catalysed cleavage of CMP-Neu5Ac into CMP and Neu5Ac was observed [2]. Maximum stability of CMP-Neu5Ac was found to be between pH 8 and 11 with concomitant formation of Neu5Ac2en, Neu5Ac and CMP. The yield of Neu5Ac2en increased with increasing pH values. At pH 13, nearly equal amounts (25–30%) of Neu5Ac and Neu5Ac2en were detected. Up to pH 11 the amounts of Neu5Ac and Neu5Ac2en were equal to that of CMP. However, above this value, this was no longer true, probably due to partial decomposition of Neu5Ac known to occur under alkaline conditions [18]. In an incubation study simulating physiological conditions (0.1 M Tris/HCl or sodium phosphate buffers, pH 7.5, 24 h, 37 °C) from 100 µmol CMP-Neu5Ac were obtained: 50 µmol CMP-Neu5Ac left, 49 µmol CMP, 40 µmol

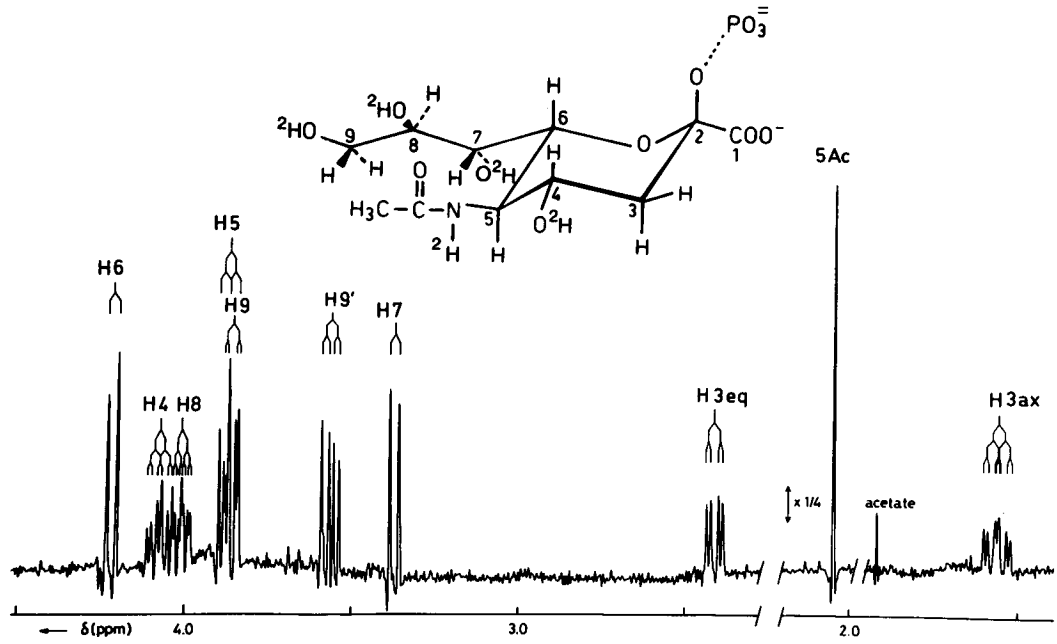

Fig. 3. *Resolution-enhanced 360-MHz ^{1}H-NMR spectrum of Neu5Ac2P in ^2H_2O, recorded at p^{2}H 7.5 and 25 °C.* For details see Materials and Methods and Table 3

Table 5. *Decomposition products of CMP-Neu5Ac treated with concentrated alkali for 17 h at 52 °C*
The values are percentages (molar basis) of the CMP-Neu5Ac input

Compound	Amount found after treatment with		
	30% ammonium hydroxide	50% triethylamine	anhydrous triethylamine
	%		
Neu5Ac2*P* or cytidine	4	2–3	22
Neu5Ac2en	26	17	50
Neu5Ac	40	50	2
CMP-Neu5Ac	6	10	8
CMP	86	83	57

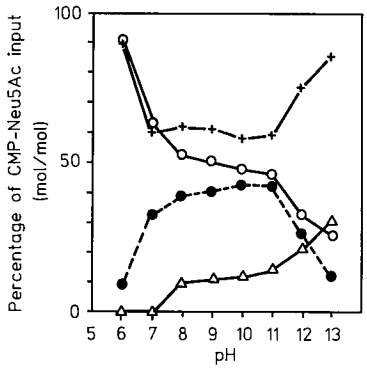

Fig. 4. *Effect of pH on the decomposition of CMP-Neu5Ac in 0.1 M buffers.* For experimental details see text. (●——●) CMP-Neu5Ac; (○——○) Neu5Ac; (△——△) Neu5Ac2en; (+——+) CMP

Neu5Ac and 7 µmol Neu5Ac2en. In none of these small-scale incubations were cytidine or Neu5Ac2*P* observed to be formed in significant amounts. Since the detection limit for Neu5Ac2*P* under the conditions applied is about 2%, the formation of very low amounts of this compound in the small-scale experiments cannot be ruled out.

In contrast to these incubations using dilute buffers, in concentrated aqueous alkali (50% triethylamine or 30% ammonium hydroxide) detectable amounts (2–4%) of Neu5Ac2*P* were formed together with Neu5Ac (40–50%) and Neu5Ac2en (17–26%) (see Table 5). Under anhydrous conditions (triethylamine) even larger amounts (22%) of Neu5Ac2*P* and Neu5Ac2en (50%) were produced. The incubation mixtures contained cytidine in molar amounts equal to Neu5Ac2*P*.

DISCUSSION

The experimental data show that CMP-Neu5Ac can undergo different types of chemical degradation at pH > 7 (see Fig. 5). It may be assumed that a nucleophilic attack of the axial H atom at C-3 of the Neu5Ac unit leads to the formation of Neu5Ac2en and CMP by an elimination process. This reaction, which was not observed to occur with free Neu5Ac [5], is favoured by the *trans* di-axial orientation of H-3*ax* and the O atom at C-2 of Neu5Ac together with the presence of a suitable leaving group. The stabilization of the double bond by the neighbouring carboxylate function reinforces this effect. The nucleophilic species may also attack the P atom. Subsequent rupture of the P-O (C-2) bond yields Neu5Ac and CMP. On the other hand, a cleavage between P-O (ribose C-5) results in the formation of Neu5Ac2*P* and cytidine. An

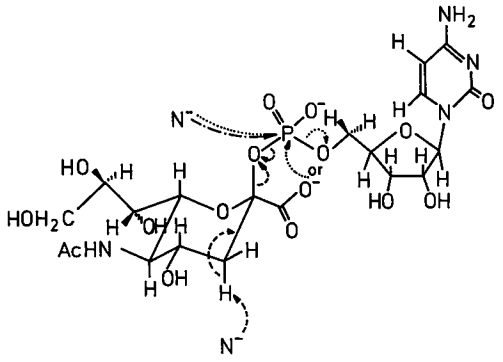

Fig. 5. *Proposed cleavage mechanisms of CMP-Neu5Ac at pH > 7.* ----> gives Neu5Ac2en + CMP; -·-·-> gives Neu5Ac + CMP; ·····> gives Neu5Ac2*P* + cytidine

intramolecular assistance of the carboxylate group in the latter cleavage reaction as depicted in Fig. 5 cannot be excluded.

Significant amounts of Neu5Ac2*P* were detected (a) after prolonged incubation (3 days) of larger quantities of CMP-Neu5Ac at pH 8 and enrichment of the products by preparative chromatography, (b) after incubation in concentrated aqueous alkali or, (c) with highest yield, after incubation in anhydrous triethylamine. From these data it can be concluded that a biological significance for Neu5Ac2*P* is unlikely. However, this new compound represents an interesting addition to the list of neuraminic acid derivatives.

From the investigations it is evident that Neu5Ac2en can be formed under all incubation conditions applied. Furthermore, the studies show that Neu5Ac2en can even be formed from CMP-Neu5Ac by chemical (non-enzymic) decomposition under simulated physiological conditions. The small relative amounts (7%) of Neu5Ac2en found in the decomposition experiments at pH 7.5 are in accordance with the percentage of this compound regularly detected in the free sialic acid fraction from biological tissues and fluids of man [4] and animals [19]. The presence of Neu5Ac2en *in vivo* might reflect a high (local?) production of CMP-Neu5Ac accompanied by a chemical degradation of this labile product at pH values slightly over 7. This can be the case in tissues with a high production of sialoglycoconjugates (e.g. mucus-secreting glands) or in pathological cases [4, 20] where, for instance, a defect in the feedback inhibition mechanism of the UDP-*N*-acetylglucosamine-2-epimerase by CMP-Neu5Ac is suspected [4]. Additional investigations are necessary to decide whether Neu5Ac2en *in vivo* is really a 'chemical artifact' due to such a non-enzymic CMP-Neu5Ac decomposition and to explain the relative amounts of Neu5Ac2en up to 80% observed in the fraction of free sialic acids in some human saliva samples [15]. In these studies, enzymic reactions due to bacterial activity or those of sialoglycoconjugate metabolism should be taken into consideration.

The presence of Neu5Ac2en in biological fluids can have significant metabolic implications, as the compound has a strong inhibitory action on sialidases [21].

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J.-M. Beau, Laboratoire de Biochimie Structurale, Unité d'Enseignement et de Recherches Sciences Fondamentales et Appliquées, Université d'Orléans, F-45046 Orléans Cedex, Loiret, France

R. Schauer, Biochemisches Institut, Medizinische Fakultät der Christian-Albrechts-Universität Kiel, Otto-Meyerhof-Haus, Olshausenstraße 40, D-2300 Kiel 1, Federal Republic of Germany

J. F. G. Vliegenthart and J. P. Kamerling, Afdeling Bio-Organische Chemie, Organisch-Chemisch Laboratorium, Rijksuniversiteit te Utrecht, Croesestraat 79, NL-3522-AD Utrecht, The Netherlands

J. Haverkamp, Afdeling voor Biochemie en Immunochemie, Unilever Research Laboratorium Vlaardingen, Olivier van Noortlaan 120, NL-3133-AT, Vlaardingen, The Netherlands

L. Dorland, Kinderkliniek van de Rijksuniversiteit te Utrecht, "Het Wilhelmina Kinderziekenhuis", Nieuwe Gracht 137, NL-3512-LK Utrecht, The Netherlands