Acid-Catalyzed Solvolysis of CMP-*N*-Acetyl Neuraminate: Evidence for a Sialyl Cation with a Finite Lifetime

Benjamin A. Horenstein* and Michael Bruner

Department of Chemistry, University of Florida, Gainesville, Florida 32611

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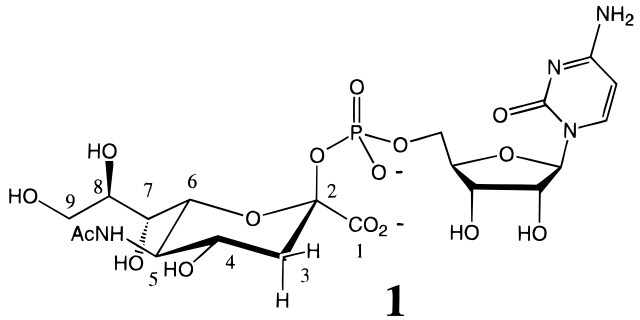
Abstract: An investigation of the mechanism of solvolysis of CMP-*N*-acetyl neuraminate (CMP-NeuAc) is presented that includes synthesis of a family of isotopically labeled CMP-NeuAc's, use of new methodology for measurement of multiple kinetic isotope effects for acid-catalyzed solvolysis of CMP-NeuAc, and a stereochemical analysis of the reaction by solvolysis in aqueous methanol. The CMP-NeuAc isotopomers were synthesized with the following labels: [9-^{3}H], [1-^{14}C-*N*-acetyl], [2-^{14}C], [1-^{14}C], and [9-^{3}H;3,3′-^{2}H] in yields of 78%, 86%, 76%, 85%, and 35%, respectively. The β-dideuterium kinetic isotope effect for solvolysis at pH 5.0, 37 °C, was 1.276 ± 0.008; the primary ^{14}C isotope effect at C2, the anomeric carbon, was 1.030 ± 0.004; and an unusually large *secondary* ^{14}C KIE was observed at C1, the carboxylate carbon, of 1.037 ± 0.004. Analysis of pH versus rate data and rate versus buffer concentration data establish that the solvolytic reaction is specific acid-catalyzed. Solvolysis of CMP-NeuAc at pH 5 or pH 6 in methanol/water mixtures afforded NeuAc, equal quantities of the α- and β-methyl glycosides of NeuAc, and small amounts of the elimination product 2,3-dehydro-*N*-acetyl neuraminic acid. The very large β-^{2}H KIE, small primary ^{14}C KIE, and the large secondary ^{14}C KIE at the carboxylate carbon are consistent with a very late oxocarbenium ion-like transition state in which the carboxylate carbon is in a looser environment than in the ground state. The observation of racemization in the solvolysis reaction supports a reaction pathway that proceeds with the formation of a sialyl cation after the transition state.

Introduction

Unlike glycohydrolases, whose mechanisms have been characterized in detail,[1] glycosyltransferases have not yet undergone the same level of mechanistic scrutiny. A chief difference between the substrates for these two classes of enzymes is that the hydrolases transfer glycones with unactivated leaving groups to water, whereas glycosyltransferases transfer glycones having activated leaving groups to a sugar hydroxyl or amino acid side chain. The emergent paradigm for glycohydrolase mechanisms includes general acid catalysis to effect leaving group departure and transition states with oxocarbenium ion character.[1,2] The same level of knowledge does not yet exist for glycosyltransferases, though some progress has been made.[3] One of the driving forces for mechanistic characterization of glycosyltransferases is the considerable interest in the development of glycosyltransferase inhibitors,[4] given the influence that various oligosaccharide recognition elements can have on biological processes.[5] The activated sugar nucleotide CMP-NeuAc[6] **1** is the key substrate for biosynthesis of sialylated glycoconjugates in which **1** is transferred by sialyltransferases[7] to an acceptor hydroxyl group in a variety of biopolymers including polysialic acids, glycoproteins, and gangliosides.[8] These glycosylation patterns constitute important binding determinants for a variety of cell−cell interactions which include masking of trypanosomal immunogenicity, viral infection and replication, and cell adhesion.[9] As part of the characterization of sialyltransferase mechanisms, it is important to first develop an understanding of the chemical behavior of the sialic acid donor substrate CMP-NeuAc, which has received limited attention.[6,10] CMP-NeuAc has a carboxylate group immediately adjacent to the anomeric carbon. Since a common mechanistic feature for glycosyl transfer involves oxocarbenium ion-like transition states,[1] study of CMP-NeuAc glycosyltransfer provides the opportunity to characterize a natural example of a substrate for glycosyltransfer that possesses functionality that could be involved in catalysis.[11]



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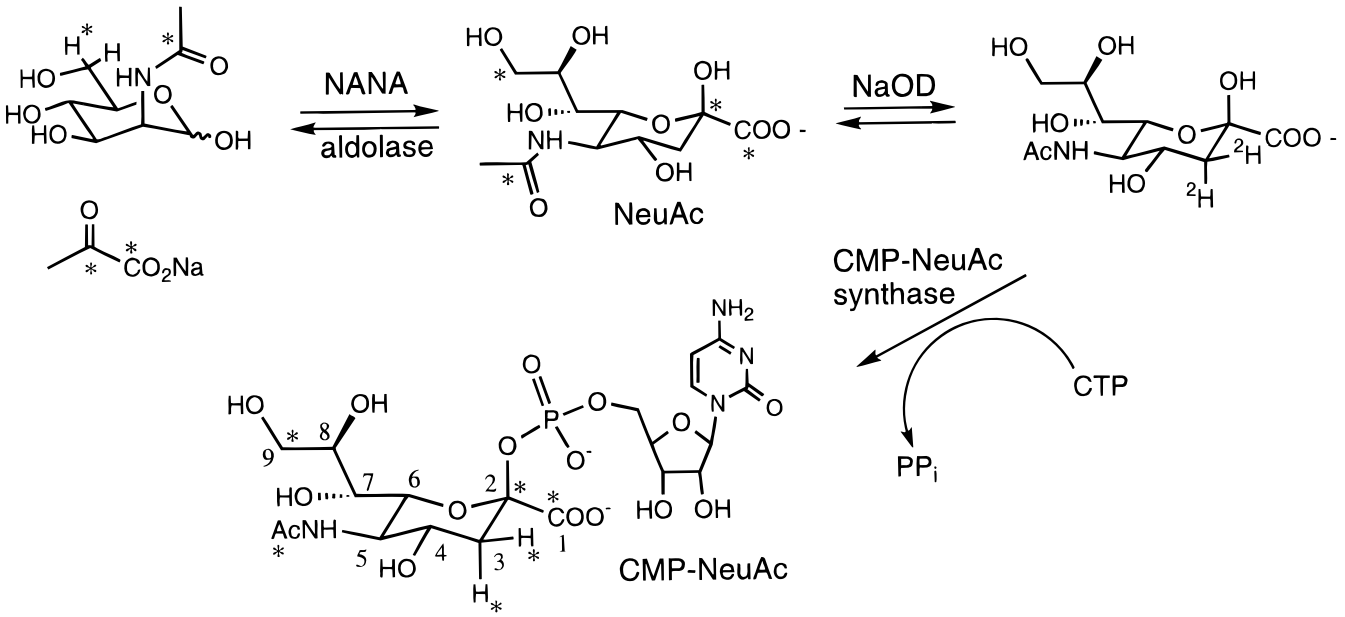

Figure 1. Synthetic route for synthesis of isotopically labeled CMP-NeuAc. Asterisks (*) denote sites of isotopic substitution. *N*-Acetyl neuraminate aldolase (NANA aldolase) is used to synthesize NeuAc from pyruvate and *N*-acetyl mannosamine. The site-specific radiolabeled sodium pyruvate or *N*-acetyl mannosamine precursors employed determine the pattern of substitution in the NeuAc product. Deuterium is introduced into the C-3 position of NeuAc by exchange in alkaline D_2O. CMP-NeuAc is prepared from the isotopically labeled NeuAc's by reaction with cytidine triphosphate, catalyzed by CMP-NeuAc synthase.

As a prelude to characterization of the mechanism of sialyltransferases, we report an investigation of the mechanism of the solvolysis of CMP-NeuAc. A series of ^{2}H-, ^{3}H-, and ^{14}C-substituted CMP-NeuAc isotopomers have been synthesized for use in β-^{2}H and ^{14}C kinetic isotope effect (KIE) experiments, which report on the transition state structure of the reaction. The stereochemistry of the solvolytic reaction was examined by addition of methanol to solvolysis reaction mixtures that lead to formation of both α- and β-methyl glycosides of NeuAc.

Results

Synthesis of CMP-NeuAc Isotopomers. Isotopomers of CMP-NeuAc were synthesized using established enzymatic and chemical methods as shown in Figure 1. The synthesis employs NANA aldolase[12] to provide *N*-acetyl neuraminic acid from sodium pyruvate and *N*-acetyl mannosamine; introduction of radiolabels occurs at this step by use of the appropriate ^{3}H or ^{14}C site-specific labeled reactant, all of which were commercially available. Introduction of ^{2}H into the 3-position of NeuAc was achieved by exchange in alkaline deuterium oxide,[13] which was followed by ^{1}H NMR until complete. CMP-NeuAc's were prepared by reaction of NeuAc with CTP, catalyzed by CMP-NeuAc synthase.[14] The following isotopomers and yields from pyruvate or ManAc were obtained: [9-^{3}H;3,3′-^{2}H] (35%), [2-^{14}C] (76%), [9-^{3}H] (78%), [1-^{14}C-*N*-acetyl] (86%), and [1-^{14}C] (85%). A new compound, the uridine monophosphate analog of CMP-NeuAc (UMP-NeuAc) was prepared in 12% yield by substitution of UTP for CTP. In addition to the studies presented in this paper, the CMP-NeuAc isotopomers and UMP-NeuAc prepared are being employed in mechanistic studies of sialyltransferases, which utilize CMP-NeuAc as the sialyl donor.

Evaluation of the Rate Law for Solvolysis of CMP-NeuAc. Apparent first-order rate constants for solvolysis of CMP-NeuAc at 37 °C were determined over the pH range 3.23−7.16 in 200 mM formate, acetate, and phosphate buffers, which were fit to the following rate law using nonlinear least squares analysis:

$$\log k_{obs} = \log [k_{H1}[H^+]/(1 + K_a/[H^+]) + k_{H2}[H^+]/(1 + [H^+]/K_a) + k_0/(1 + [H^+]/K_a)]$$

Rate constant k_{H1} represents acid-catalyzed reaction of protonated CMP-NeuAc, k_{H2} represents acid-catalyzed reaction of unprotonated CMP-NeuAc (or its kinetic equivalent, water-catalyzed reaction of protonated CMP-NeuAc), k_0 represents the water-catalyzed reaction of unprotonated CMP-NeuAc, and K_a is the acid dissociation constant for a CMP-NeuAc ionizable group, presumably, although not necessarily, the carboxylate. From this analysis, the estimated values for k_{H1}, k_{H2}, k_0, and pK_a were respectively 5.7 ± 1 M^{-1} s^{-1}, 15 ± 1 M^{-1} s^{-1}, $1.0 \pm 0.1 \times 10^{-5}$ s^{-1}, and 4.0 ± 0.2. Measurement of k_{obs} at pH 5.0 in acetate buffers between 0.05 and 1.0 M showed no concentration dependence, indicating the general acid (or base) catalysis is not significant; solvolysis of CMP-NeuAc is specific acid catalyzed. The Supporting Information contains plots of the pH versus rate data and the dependence of k_{obs} on buffer concentration. The calculated % contributions of k_{H1}, k_{H2}, and k_0 to the observed rate at pH's 4, 5, and 6 are as follows: pH 4.0, 27, 72, 1; pH 5.0, 3, 91, 6; pH 6.0, 0, 60, 40.

Kinetic Isotope Effects for Solvolysis of CMP-NeuAc. KIEs for the solvolysis of CMP-NeuAc at pH 5 in 200 mM acetate buffer have been measured with radiolabeled substrates using the dual-label competitive method[15] and are presented in Table 1. A primary ^{14}C KIE of 1.030 ± 0.005 was measured using a mixture of [2-^{14}C] and [9-^{3}H]CMP-NeuAc. A *secondary*

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Table 1. Kinetic Isotope Effects for Solvolysis of CMP-NeuAc[a]

entry	isotopomeric CMP-NeuAc pairs	type of KIE	value[b]
1	[9-^{3}H;3,3′-^{2}H]; [1-^{14}C-acetyl]	β-secondary	1.276 ± 0.008
2	[9-^{3}H;3,3′-^{2}H]; [1-^{14}C-acetyl]	β-secondary	1.278 ± 0.009[c]
3	[9-^{3}H;3,3′-^{2}H]; [1-^{14}C-acetyl]	β-secondary	1.354 ± 0.008[d]
4	[9-^{3}H;3,3′-^{2}H]; [1-^{14}C-acetyl]	β-secondary	1.25 ± 0.02[e]
5	[3,3-^{2}H]; [3,3-^{1}H]	β-secondary	1.25 ± 0.02
6	[2-^{14}C]; [9-^{3}H]	primary ^{14}C	1.030 ± 0.005
7	[1-^{14}C]; [9-^{3}H]	secondary ^{14}C	1.037 ± 0.003
8	[9-^{3}H]; [1-^{14}C-acetyl]	control	1.002 ± 0.010

[a] All KIE experiments utilized the competitive method except entry 5, which used direct rate measurements of ^{2}H versus ^{1}H-substituted CMP-NeuAc. Each KIE value represents the average and standard deviation of three independent experiments. Further details are in the Experimental Section. [b] All experiments except entries 2−4 were run in 200 mM acetate, pH 5.0, 37 °C. [c] 500 mM acetate buffer, pH 5.0, 37 °C. [d] 200 mM acetate, pH 6.0. [e] 200 mM formate, pH 4.0.

^{14}C KIE of 1.037 ± 0.003 was measured using a mixture of [1-^{14}C] and [9-^{3}H]CMP-NeuAc. A large secondary β-dideuterium KIE of 1.276 ± 0.008 was measured using a mixture of [9-^{3}H;3,3′-^{2}H] and [1-^{14}C-*N*-acetyl]CMP-NeuAc. This KIE experiment was repeated at 500 mM acetate buffer, affording an isotope effect of 1.278 ± 0.009. When the pH was raised to 6.0, the secondary β-dideuterium KIE rose to 1.354 ± 0.008; at pH 4.0, the value for this KIE was 1.25 ± 0.02. Since the KIE methodology uses remote labels, it is important to establish that they themselves do not cause an isotope effect. Solvolysis of a mixture of [9-^{3}H] and [1-^{14}C-*N*-acetyl]CMP-NeuAc proceeded with a KIE of 1.002 ± 0.010. This control establishes that if they occur, isotope effects at these reporter labels are small. In order to provide an independent confirmation of the competitive method, separate direct rate measurements were made for the solvolysis of [3,3′-^{2}H] and [3,3′-^{1}H]CMP-NeuAc, with time point aliquots monitored by HPLC. The calculated ratio $^{H}k_{obs}/^{D}k_{obs}$ was 1.25 ± 0.02, in excellent agreement with the value obtained using the competitive method. A possible source of error in the HPLC-based isolation of CMP-NeuAc would be isotopic fractionation during chromatography, which is known.[16] When a mixture of [9-^{3}H] and [1-^{14}C] CMP-NeuAc of known isotopic ratio was chromatographed and the CMP-NeuAc recollected, the ^{3}H/^{14}C ratio was unchanged, having a relative ^{3}H/^{14}C ratio before and after chromatography of 1.001 ± 0.006.

Stereochemistry of CMP-NeuAc Solvolysis. The observed rate constant for solvolysis of CMP-NeuAc at pH 5.0, 200 mM acetate, was (1.43 ± 0.02) × 10^{-2} min^{-1}. Solvolysis of CMP-NeuAc at pH 5 in a 9:1 mol ratio of H_2O/methanol (20% v/v) at 37 °C afforded a 12:1 ratio of NeuAc to NeuAc methyl glycosides by ^{1}H-NMR analysis. A nearly equimolar mixture of α- and β-methyl glycosides was produced, as evidenced by the observation of characteristic peaks in the ^{1}H-NMR for the methoxyl groups and the equatorial hydrogens at C-3 of the neuraminic acid moiety.[17] We also observed that a minor amount (1−3%) of the elimination product 2,3-dehydro-*N*-acetylneuraminic had formed during solvolysis.[10] Solvolysis at pH 5.0 in 5.3:1 H_2O/methanol (30% v/v) afforded a 7.6:1 ratio of NeuAc to NeuAc methyl glycosides ($\alpha/\beta \sim 1$) (Figure 2a). We observed that raising the pH to 6 produced more 2,3-dehydro-NeuAc (9−10%), but the α/β ratio was unchanged from solvolyses conducted at pH 5.0. Solvolysis of CMP-NeuAc at pH 4.0 in aqueous formate buffer showed no formation of 2,3-dehydro-NeuAc. Authentic samples of α- or β-methyl *N*-acetylneuraminic acid glycoside were added to separate aliquots of solvolysis reaction mixtures resulting in increases in the intensity of resonances attributed to these compounds (Figure 2b,c). Control experiments that subjected NeuAc or 2,3-dehydro-NeuAc to the methanolic solvolysis reaction conditions demonstrated that they were stable; hence, the methyl glycosides do not arise from subsequent reaction of either the major or minor reaction products. When a 95:5 β/α ratio of methyl NeuAc glycosides was subjected to the solvolysis reaction conditions no change in the ratio was observed over 13 h at 37 °C.[18] The controls demonstrate that the observed methyl sialosides form from CMP-NeuAc or an intermediate formed during solvolysis and the observed α/β ratios do not reflect equilibration of product.

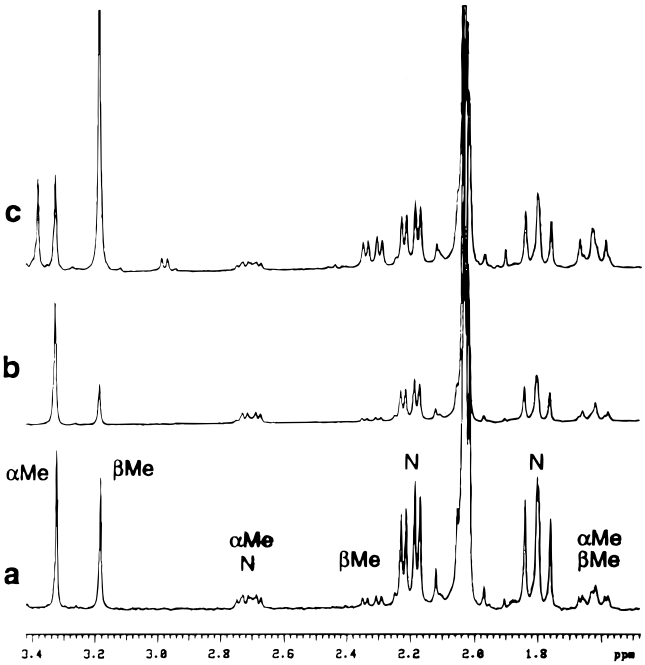

Figure 2. 300 MHz ^{1}H-NMR spectral data for solvolysis of CMP-NeuAc in 30% (v/v) aqueous methanol, pH 5. Spectrum a is of the solvolysis products. Spectrum b shows the result of addition of authentic NeuAc α-methyl glycoside to the reaction mixture. Spectrum c shows the result of addition of authentic NeuAc β-methyl glycoside to the reaction mixture. Key peaks are labeled with the following code: αMe, α-methyl glycoside; βMe, β-methyl glycoside, and N, NeuAc. The most downfield singlet in spectrum c is from an impurity present in the NeuAc β-methyl glycoside standard, which was not purified for this experiment.

Discussion

Kinetic Isotope Effects. Given the presence of a carboxylate group immediately adjacent to the anomeric carbon of CMP-NeuAc, the question arises: what role does the carboxylate play in solvolysis and enzymatic group transfer of NeuAc? One possibility would encompass nucleophilic participation of the carboxylate group in displacement of CMP to form an intermediate, albeit strained, α-lactone.[19,20] At the other limit, the carboxylate group could function as an electrostatic catalyst by stabilization of a transition state or intermediate having oxocarbenium ion character. We sought to apply kinetic isotope

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effects to this problem, as they have proven to be a powerful approach to identify transition state features for glycosyl transfer reactions.[1,2,21]

A primary ^{14}C KIE of 1.030 was observed for solvolysis of CMP-NeuAc. This value is well below the range of 1.08−1.15 expected[22] for an associative S_N2 process, effectively ruling out pathways which proceed with significant bond order to both nucleophile and leaving group. The observed value is in the range anticipated for a dissociative pathway. Acid-catalyzed hydrolysis of glycosides show primary ^{14}C isotope effects of between 1.02 and 1.05, corresponding to transition states with oxocarbenium ion character.[16,23,24] The observed ^{14}C KIE of 1.030 corresponds well with the ^{13}C KIEs of 1.007 and 1.011 reported for acid-catalyzed hydrolysis of α- and β-methyl glucoside, in which it was concluded that there is no nucleophilic assistance in the transition state.[24]

The *secondary* ^{14}C KIE of 1.037 at the carboxylate carbon is remarkable for its size. We considered using the ^{14}C-labeled carboxylate as a reporter label since secondary ^{14}C KIEs at this position were expected to be nearly an order of magnitude smaller.[25] That this KIE was not actually an *inverse* effect of the remote 9-^3H label was established by moving the ^{14}C label to the 5-acetamido group; solvolysis of this compound versus 9-^3H CMP-NeuAc gave a KIE of 1.002 ± 0.010. Since this KIE is normal and relatively large, it indicates that in proceeding from the ground to transition state, the vibrational environment of the carboxyl group becomes substantially looser. The fractionation factor for ionization of a carboxylic acid will be very small, so the large value observed is not reflective of a change in protonation state between the ground and transition states.[26a] The 2° ^{14}C-carboxylate KIE is nearly as large as the 1° ^{14}C KIEs observed for decarboxylation reactions.[26b] While we do not suggest that decarboxylation is occurring in this system, it may be argued that at the transition state the carboxyl group is becoming more CO_2-like.[26c] *Ab initio* modeling[27] of CMP-NeuAc and sialyl cation models indicate that the C−C bond length between the anomeric and carboxylate carbon lengthens by up to 0.05 Å and that the O−C−O bond angle opens by 7−8° upon formation of the sialyl cation. The normal isotope effect measured is consistent with this looser environment for the carboxylate carbon. The basis for such proposed geometric changes is uncertain; perhaps part of the electron demand of the oxocarbenium ion center can be satisfied by induction through the σ-bond between the carboxylate carbon and the oxocarbenium ion carbon. The electron-rich carboxylate oxygens would in turn compensate for the loss of σ-bonding at the carboxylate carbon with additional π-bonding, rendering it more CO_2-like.

Solvolysis of CMP-NeuAc at pH's 4.0, 5.0, and 6.0 proceeded with observed β-dideuterium KIEs of 1.25 ± 0.02, 1.276 ± 0.008, and 1.354 ± 0.008. These values are well within the range expected for β-deuterium effects in carbenium ion-forming reactions.[28] With the observation that some elimination to 2,3-dehydro-NeuAc[10] accompanied solvolysis at pH 5 and pH 6 (but not at pH 4), it was necessary to consider the likely possibility that the β-elimination makes a contribution to the observed isotope effect via expression of a primary hydrogen isotope effect.[29] A general base-catalyzed pathway for β-elimination at pH 5 was judged unlikely by the observation that the KIE was unchanged when the acetate buffer concentration was raised from 200 to 500 mM (entries 1 and 2, Table 1). Beau et al.[10] have shown that production of 2,3-dehydro-NeuAc from CMP-NeuAc is nearly pH independent between pH 8 and pH 10. This, taken with the low [$^-$OH] at pH 5 suggest that at pH 5 the β-elimination reaction is water catalyzed. The observed β-^2H KIEs at pH's 5 and 6 will reflect the weighted average[30] of the individual secondary KIE for solvolysis and the primary KIE for β-elimination:

$$\mathrm{KIE_{obs}} = \mathrm{KIE_1} \times \mathrm{KIE_2}/(\mathrm{KIE_2} \times (F_1) + \mathrm{KIE_1} \times (1 - F_1))$$

where KIE_1 and KIE_2 are the individual KIEs for each reaction, and F_1 is the fraction of reaction proceeding through reaction 1. A derivation for the weighting expression is presented in the Supporting Information section; solution of simultaneous equations for the KIE data at pH 5 and pH 6 with 0.02 and 0.095 mol fractions for elimination, respectively, yielded values of 5.1 and 1.26 for the primary and secondary KIEs. Note that at pH 5, 91% of the reaction proceeds through the k_{H2} pathway and only 3% proceeds through the k_{H1} path (as discussed in the Results section); thus, the estimated secondary KIE of 1.26 is almost entirely a reflection of the KIE for k_{H2}. Solvolysis of CMP-NeuAc at pH 4.0 proceeds without detectable β-elimination and affords a β-^2H KIE of 1.25 ± 0.02, in excellent agreement with the estimated value above. From the rate law for solvolysis, we calculate that, at pH 4, 72% of the reaction proceeds through the k_{H2} term, 29% through the k_{H1} term, and ∼1% through the k_0 term. The observed KIE of 1.25 is then the weighted average of the two concurrent acid-catalyzed solvolysis reactions; knowledge of the relative contributions through the k_{H1} and k_{H2} paths allows limits to be placed on the KIE for each path. For example, if the β-^2H KIE on k_{H2} were 1.10, the KIE for k_{H1} would have to be 1.8, an unrealistic value. We suggest that the lowest reasonable KIE on k_{H2} would be 1.20, which would require that the KIE for k_{H1} be 1.35; a very high value at the upper limit for a β-dideuterium KIE.[19,28] The true value for the k_{H2} β-^2H KIE will be between 1.20 and 1.25; this level of precision is sufficient to conduct a qualitative interpretation of the transition state structure for the k_{H2} solvolysis reaction at pH 5.

In glycoside hydrolysis, a β-^2H kinetic isotope effect arises when the transition state leaving group and nucleophile bond orders sum to less than 1, which results in positive charge development at the anomeric carbon, providing the opportunity for hyperconjugation. The size of this isotope effect derives from the extent of charge development, and a combination of

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(26) (a) Bayles, J. W.; Bron, J.; Paul, S. O. *J. Chem. Soc. Faraday Trans. 1* **1976**, *7*, 1546−1542. (b) Seltzer, S.; Hamilton, G. A.; Westheimer, F. H. *J. Am. Chem. Soc.* **1959**, *81*, 4018−4024. (c) The isoelectronic *N*-methyl pyridine-2-carboxylate undergoes decarboxylation: Quast, H.; Schmitt, E. *Justus Liebigs Ann. Chem.* **1970**, *732*, 43−63.

(27) Calculations were performed at the RHF level with the 3−21g, 6−31g, and 6−31g* basis sets. B. Horenstein, unpublished results.

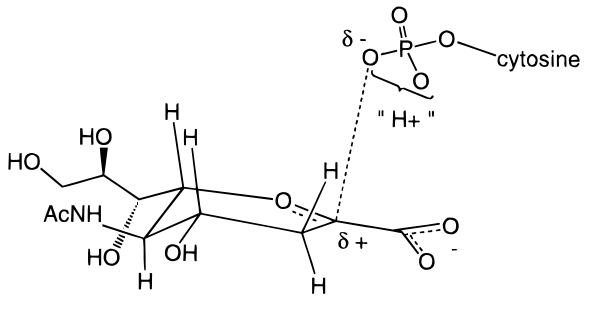
(28) Melander, L.; Saunders, W. H. *Reaction Rates of Isotopic Molecules*; Kreiger: Malabar, FL, 1980; Chapter 6.

(29) Fry, A. *Chem. Soc. Rev.* **1972**, *1*, 163−210.

(30) Melander, L.; Saunders, W. H. *Reaction Rates of Isotopic Molecules*; Kreiger: Malabar, FL, 1980; Chapter 10.

the angular relationship θ between the β C−L bond and the electron deficient p-orbital at the anomeric carbon, and a small conformationally independent inverse inductive effect.[28] A maximal isotope effect will be realized when charge development is complete (zero bond order to the leaving group and nucleophile) and θ is either 0 or 180°. In the present system, we measured isotope effects for C-3 dideuterated CMP-NeuAc, and hence the observed isotope effects should be equal to the product of the individual pro-R and pro-S isotope effects.[31] While the present data do not allow a conformational analysis, what is significant is the large value observed for the dideuterium KIE. It is important to compare the present work to that from Sinnott's group,[19] who reported a series of β-^{2}H isotope effects for solvolysis of α-*p*-nitrophenyl NeuAc. The results allowed an elegant conformational analysis of the solvolysing NeuAc residue and, salient to the present work, provided an indication of the way in which interaction of the carboxyl group with the solvolysing NeuAc oxocarbenium ion can modulate the β-^{2}H KIE. At pH 1.00, the carboxyl group of α-*p*-nitrophenyl NeuAc (pK_a 2.69) is largely protonated, and $(k_H/k_D)_{max}$ was estimated to be between 1.19 and 1.39. At pH 6.67, the carboxyl group is completely ionized, and $(k_H/k_D)_{max}$ was calculated to be 1.098. It was concluded that the transition state must be late with respect to departure of *p*-nitrophenolate but involves nucleophilic participation of the carboxylate, which would reduce hyperconjugation and therefore account for the β-^{2}H KIE. In the present work, the value of 1.20−1.25 for the β-dideuterium KIE on k_{H2} is too large to be consistent with $(k_H/k_D)_{max} = 1.098$, as even disallowing for the inductive component, the maximum dideuterium KIE predicted would be 1.15. A β-^{2}H KIE > 1.20 for solvolysis is too great to be consistent with any significant nucleophilic participation of the carboxylate. By analogy to the work on aryl sialosides,[19] the lack of nucleophilic participation by the carboxylate group could be rationalized if it were significantly protonated, but based on titrations of CMP-NeuAc and the pH versus rate data, the pK_a of the carboxyl group is less than or equal to 4.0; hence the KIEs for solvolysis at pH 5 largely reflect an ionized carboxylate.[32]

In summary, the combination of the large β-dideuterium KIE and the small primary ^{14}C KIE strongly support a transition state structure for CMP-NeuAc solvolysis that is very late, without nucleophilic participation of the carboxylate. To account for a non-nucleophilic carboxylate, we wish to suggest that the transition state for process k_{H2} may involve a conformation of the carboxylate group in which it approaches coplanarity with the oxocarbenium ion plane, as in **2**. Orbital overlap between



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a carboxylate oxygen and the p-orbital of the oxocarbenium ion carbon would be minimized in this conformation, which we suggest would maximize the opportunity for hyperconjugation, qualitatively accounting for the substantially larger β-^{2}H KIE for solvolysis of CMP-NeuAc compared to that observed for aryl sialoside solvolysis, which involves carboxylate group participation.

Solvolysis in Aqueous Methanol. We wished to further characterize the mechanism for CMP-NeuAc solvolysis with a stereochemical probe. If solvolysis of CMP-NeuAc involves nucleophilic participation of the carboxylate, this would require formation of a transient α-lactone, or at least would shield the back face of the solvolysing NeuAc residue.[19] In such a case, the observed stereochemistry of the product should be retention to afford the β-anomer. A direct displacement of CMP by solvent without carboxylate participation would yield the α-anomer, while a transition state that resulted in formation of a sialyl oxocarbenium ion would proceed with results ranging from inversion to racemization depending on the lifetime of the cation. Observation of product stereochemistry by NMR requires that the mutarotation rates be significantly slower than k_{obs} for solvolysis, which was measured to be 1.43×10^{-2} min^{-1}. The rate constants for mutarotation of NeuAc are comparable with $k_\alpha = 7.8 \times 10^{-3}$ min^{-1} and $k_\beta = 6.0 \times 10^{-4}$ min^{-1} reported at pD = 5.4; these values represent a minimum with mutarotation rates increasing above and below this pH.[33] We chose to avoid the complications of mutarotation by incorporating methanol into solvolysis mixtures, which would afford the methyl sialoside ketals that do not mutarotate and are stable to the reaction conditions.

When CMP-NeuAc was solvolysed in methanol/water mixtures at pH 5.0, ∼1:1 ratios of α- and β-methyl glycosides of *N*-acetylneuraminic acid were observed; the selectivity for water versus methanol attack is approximately 1.3. These results are considered in light of the possibilities outlined in Figure 3.

In the first possibility (route A), the carboxylate group participates in displacement of the departing CMP group or forms a tight ion pair that would shield the α face. In this pathway, incorporation of methanol into the solvolysis reaction mixture would be expected to afford only the β-methyl glycoside of NeuAc, which was not observed. Route A is also inconsistent with the large β-dideuterium isotope effect and the small primary ^{14}C isotope effect, as smaller and larger respective values for these KIEs would be predicted due to carboxylate participation.

In the second scenario, there are two competing processes: attack of methanol on the α-face of CMP-NeuAc (route B), and route A; the former giving the α-methyl glycoside in a single displacement and the latter providing the β-methyl glycoside in a double displacement via an intermediate α-lactone or shielded oxocarbenium ion/carboxylate ion pair. This route predicts formation of both anomeric methyl NeuAc glycosides, which is in agreement with the experimental observations. Variation of the α/β-methyl glycoside ratio with methanol concentration would lend support to the idea of competition between routes A and B as the α/β ratio in A should be independent of methanol but in B it should increase in proportion to the methanol concentration. We observed identical α/β-methyl glycoside ratios when the mole fraction of methanol was

(31) (a) Bigeleisen, J. *J. Chem. Phys.* **1955**, *23*, 2264−2267. (b) Bigeleisen, J. *J. Chem. Phys.* **1958**, *28*, 694−699.

(32) Rapid titration of CMP-NeuAc at 4 °C showed an inflection at pH 4.8 and a broader inflection that started at about pH 3.5. The pH 4.8 inflection consumed 1 equiv of acid and is assigned to the cytidine 4-amino group (Dawson, R. M. C.; Elliot, D. C.; Elliot, W. H.; Jones, K. M. *Data for Biochemical Research*; Oxford: New York, 1986) as titration of UMP-NeuAc, which lacks the amino group did not display an inflection around pH 4.8, showing only the lower pH inflection. After completion, titration mixtures were immediately neutralized with Tris base and shown to have less than 5% hydrolysis by HPLC. The upper limit for the carboxyl pK_a is 3.5; the same conclusion was reached by titration of [1-^{13}C]CMP-NeuAc observed by ^{13}C NMR.

(33) Friebolin, H.; Kunzelmann, P.; Supp, M.; Brossmer, R.; Keilich, G.; Ziegler, D. *Tetrahedron Lett.* **1981**, *22*, 1383−1386.

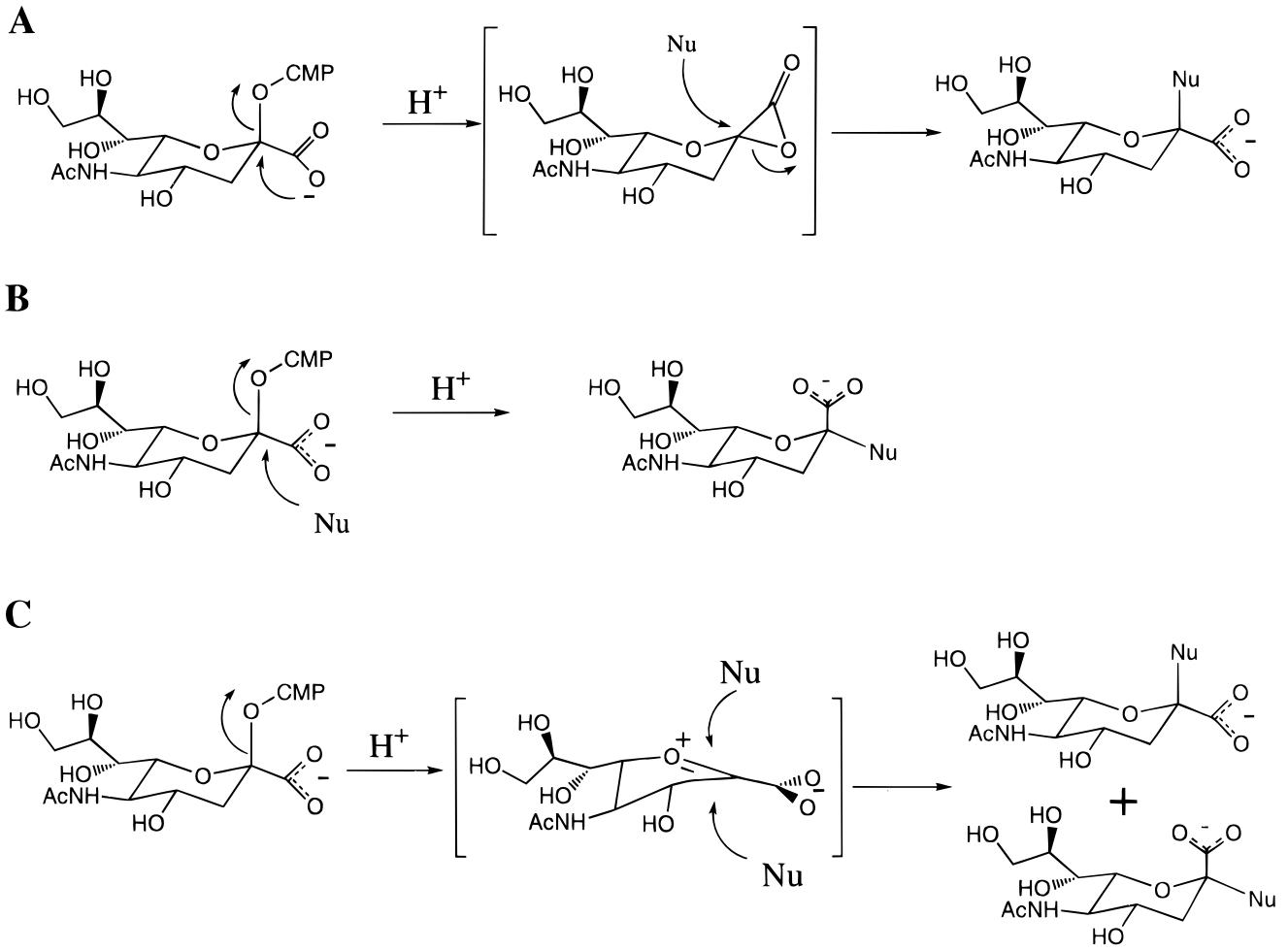

Figure 3. Possible pathways for solvolysis of CMP-NeuAc. Nu = H_2O or methanol; the protonation site is not proven but presumably is a phosphate oxygen. Path A involves nucleophilic participation of the carboxylate to give the β-product on the second displacement. Path B involves backside attack of Nu to afford the α-product. Path C involves formation of a NeuAc oxocarbenium ion, which is attacked from either face by Nu to afford an α/β mixture of products. Path C could also involve NeuAc ion pairs (see Discussion).

increased by over 50%, which shows that there is no methanol concentration dependence over this range. Turning back to the results of the KIE experiments, the large values for the β-^{2}H KIE (1.20−1.25) and the small primary ^{14}C KIE (1.03) are inconsistent with a scenario requiring ∼50% of the reaction manifold to proceed with nucleophilic transition state participation of a negatively charged group.

In the third possibility (route C), solvolysis proceeds through a late oxocarbenium ion-like transition state to form a short-lived neuraminyl cation intermediate, which is trapped by methanol to afford α- or β-methyl glycosides. Route C requires that the neuraminyl cation have a lifetime sufficiently great to allow for equilibration with solvent in order to account for the observed racemization. Another alternative that cannot be ruled out at this time is that the NeuAc cation could ion pair with CMP or intramolecularly ion pair with its carboxylate. If these ion pairs were sufficiently long lived, they could form complexes with solvent methanol or water and then collapse to products. The relative ratio of α/β glycoside would be guided by the steric and electronic factors for the transition states corresponding to attack of methanol on the sialyl cation (or NeuAc cation ion pairs) and would not be sensitive to the concentration of methanol, in agreement with the experimental observations.[34] The selectivity for methanol attack in water/methanol is rather low at 1.3:1; high ratios favoring methanol trapping are associated with stable cations, low ratios are typically associated with highly reactive, short-lived carbenium ion pairs. For a series of cumyl cations, Richard et al. reported corresponding methanol/trifluoroethanol trapping ratios, which revealed a limiting minimum ratio of 2 for cations with a lifetime shorter than 10^{-10} s.[35] Such cations were described as ion pairs that reacted with solvent before diffusion of the leaving group could occur. This could be the case for solvolysis of CMP-NeuAc. Alternative factors that could favor a low methanol/water trapping ratio might include steric effects disfavoring methanol attack at a bulky tertiary center or a localized polarity effect that could favor water for solvation of a charge-separated transition state or tight ion pair.[35]

Route C is the most consistent with the kinetic isotope effect data, as large β-deuterium and small primary ^{14}C KIEs are predicted for a very late transition state with essentially complete charge development and a lack of bonding to either leaving group or nucleophile. When the KIE and solvolysis data are taken together, we conclude that the most likely mechanistic scenario for acid-catalyzed solvolysis of CMP-NeuAc at pH 5 is route C.

One question concerning glycosyl transfer is whether discreet glycosyl oxocarbenium ions exist with a finite lifetime or if glycosyl transfer proceeds through oxocarbenium ion-like

(34) After diffusion of CMP, the sialyl oxocarbenium ion could collapse to give both epimeric α-lactones, which upon solvolysis in aqueous methanol could yield the α and β methyl glycosides.

(35) Richard, J. P.; Amyes, T. L.; Vontor, T. *J. Am. Chem. Soc.* **1991**, *113*, 5871−5873.

transition states that are trapped in the reaction environment. Banait and Jencks[36] have characterized the solvolytic behavior of α-D-glucopyranosyl fluoride and concluded that the glucosyl oxocarbenium ion has a brief existence in pure water, but in the presence of anionic nucleophiles it undergoes a concerted or "enforced" displacement with no appreciable lifetime for the cation. As applied to solvolysis of NeuAc glycosides, these observations would argue for nucleophilic carboxylate group participation, if the lifetimes of the glucosyl and neuraminyl cations were comparable. Two structural differences suggest that the stability of the NeuAc oxocarbenium ion should be distinctly greater than that of the glucosyl oxocarbenium ion. First, the NeuAc oxocarbenium ion is expected to have a longer lifetime as it can formally be considered as a 2-deoxy sugar, which have been estimated to be ∼4 times more stable than oxygenated analogues.[37] Secondly, what sets the NeuAc carbenium ion most apart from "typical" glycosyl oxocarbenium ions is the presence of a carboxylate group immediately adjacent to the site of positive charge formation. Such an arrangement provides the opportunity for a short-range electrostatic stabilization of the oxocarbenium ion. Estimation of the charge stabilization is subject to the choice of solvent dielectric constant. For two point charges at 2.3 Å separation, a dielectric constant of 79 for water yields a value of −1.8 kcal/mol, or a factor of about 20. This is a minimum value for the stabilization, since the two charges are in the same molecule their interaction is not as well screened as a dielectric of 79 would imply. We suggest that the overall stability of the NeuAc cation versus the glucosyl cation is at least 80-fold or 2 orders of magnitude.

Using the minimum estimated 10^2 greater stability of the NeuAc cation over a glucosyl cation and the estimate of the latter's lifetime of $\sim 10^{-12}$ s,[37] we suggest that the sialyl cation has a lifetime of $\geq 10^{-10}$ s, placing it near the border of the lifetime required for diffusional equilibration with bulk solvent.[35] A better estimate for the NeuAc cation lifetime is not yet available but is under investigation. In summary, the observation that CMP-NeuAc affords equal proportions of α- and β-methyl NeuAc glycosides on solvolysis in aqueous methanol is consistent with formation of either a discrete NeuAc cation or NeuAc cation ion pairs.

That CMP-NeuAc solvolysis does not involve a nucleophilic carboxylate, whereas aryl sialoside solvolysis does, might be related to the leaving group ability of CMP versus aryloxy aglycons. *p*-Nitrophenol is a poorer leaving group than CMP and could reasonably be expected to require a degree of nucleophilic assistance for efficient scission of the glycosidic bond. Another difference in these two systems is the potential for unfavorable electrostatic interactions of the phosphate and carboxylate in CMP-NeuAc, which is absent in NeuAc *p*-nitrophenyl glycoside. For the carboxylate to participate nucleophilically, inspection of molecular models indicates that it would have to reside in a conformation that placed the negative charges of the phosphate and carboxylate groups nearby. A conformation of the carboxylate that is near-planar to the incipient sialyl cation plane would minimize this electrostatic repulsion. Aryl sialosides do not have such a directive force and may undergo solvolysis with the carboxylate in a ground state conformation suited for nucleophilic participation.[19] A nucleophilic or non-nucleophilic pathway for sialyl carboxylate group participation is clearly system-dependent, resulting from the interplay of steric and electronic effects.

(36) Banait, N. S.; Jencks, W. P. *J. Am. Chem. Soc.* **1991**, *113*, 7951−7958.

(37) Amyes, T. L.; Jencks, W. P. *J. Am. Chem. Soc.* **1989**, *111*, 7888−7900.

Conclusion

The results of this study suggest that a sialyl cation can exist given the right circumstances and can have implications regarding the mechanism of enzymes involved in sialyl transfer. Sialyltransferases form only α-glycosidic linkages and are therefore inverting enzymes. In this case, it is highly unlikely that the carboxylate group of CMP-NeuAc participates as a nucleophile in sialyl transfer. The results of this study are consistent with an electronic role for the carboxylate group in which it could stabilize a cationic transition state via through-space electrostatic interaction. This conclusion may also pertain to sialidases.[38] Though not reported, it would be of interest to compare the primary carbon isotope effects for retaining enzymes such as influenza neuraminidase with inverting sialidases, which might shed further light on the possibly divergent roles of the sialyl carboxylate group.[39] The kinetic isotope effect methodology described in this paper for solvolysis of CMP-NeuAc is now being applied to the study of sialyltransferases.

Experimental Section

Materials. Buffers and reagents were purched from Sigma and Fisher. NANA aldolase was purchased from Shinko American and Sigma. Authentic standards of CMP-NeuAc, *N*-acetylneuraminic acid α-methyl glycoside, and 2,3-dehydro-*N*-acetyl neuraminic acid were purchased from Sigma. β-Methyl *N*-acetylneuraminic acid glycoside was synthesized by refluxing *N*-acetyl neuraminic acid in dry methanol in the presence of Amberlite IR120-H^+ form, followed by saponification at pH 12.[40] *N*-Acetyl mannosamine isotopomers ([6-^{3}H] and [1-^{14}C-*N*-acetyl]) and sodium pyruvate [1-^{14}C and 2-^{14}C] were purchased from New England Nuclear and Moravek. Cytidine triphosphate (CTP) was purchased from Sigma as the disodium salt with 2.5 equiv of hydration. Liquid scintillation fluid (Liquiscint) was purchased from National Diagnostics. Plasmid pWV200B harboring the expression construct for *Escherichia coli* CMP-NeuAc synthase was a gift from Dr. W. F. Vann at the National Institutes of Health.[41] CMP-NeuAc synthase was purified from *E. coli* JM105 following the published protocol[42] and was judged 90−95% pure based on SDS−PAGE analysis. Amberlite IR120-H^+ form resin used for desalting CMP-NeuAc was first washed with 95% ethanol and then washed extensively with deionized water.

Instruments. ^{1}H-NMR spectra were measured at ambient temperature on a Varian Gemini 300 spectrometer operating at 300 MHz. The acquisition pulse angle was 30°. Spectra were obtained in 99.9% D_2O referenced to the HDO peak (4.80 ppm). NMR analyses of ^{3}H-containing samples were performed in 9-in. flame-sealed tubes. HPLC was performed on a Rainin HPXL gradient unit interfaced to a Macintosh personal computer. A Rainin Dynamax UV-1 detector was employed to monitor separations at 260 nm. Liquid scintillation counting was performed using a Packard 1600 TR instrument; data was written to floppy disk and worked up on the personal computer.

Synthesis of Radiolabeled CMP-NeuAc's and UMP-NeuAc. (A) [9-^{3}H]CMP-NeuAc. Sodium pyruvate (5.3 mg, 50 μmol) and [6-^{3}H]-*N*-acetyl mannosamine (50 μCi, 5 nmol) and 0.1 U of NANA aldolase were incubated for 47 h at 37 °C in 0.1 mL of 10 mM Tris-HCl, pH 7.5, containing 1 mg/mL each of BSA and NaN_3. A portion of the crude [9-^{3}H]NeuAc product (17.5 μCi) was concentrated *in vacuo* and resuspended in 630 μL of 50 mM, pH 8.5, Tris-HCl containing 50 mM $MgCl_2$, CTP (1.89 μmol), and 0.032 U of CMP-NeuAc synthase.

(38) A sialyl cation intermediate has been proposed for influenza neuraminidase: Chong, A. K. J.; Pegg, M. S.; Taylor, N. R.; von Itzstein, M. *Eur. J. Biochem.* **1992**, *207*, 335−343.

(39) (a) Guo, X.; Sinnott, M. L. *Biochem. J.* **1993**, *296*, 291−292. (b) Guo, X.; Laver, W. G.; Vimr, E.; Sinnott, M. L. *J. Am. Chem. Soc.* **1994**, *116*, 5572−5578.

(40) Czarniecki, M. F.; Thornton, E. R. *J. Am. Chem. Soc.* **1977**, *99*, 8273−8279.

(41) Zapata, G.; Vann, W. F.; Aaronson, W.; Lewis, M. S.; Moos, M. *J. Biol. Chem.* **1989**, *264*, 14769−14774.

(42) Liu, J. L.; Shen, G.-J.; Ichikawa, Y.; Rutan, J. F.; Zapata, G.; Vann, W. F.; Wong, C.-H. *J. Am. Chem. Soc.* **1992**, *114*, 3901−3910.

The reaction was let proceed at 37 °C for 2 h. After isolation by anion exchange HPLC, 13.6 μCi (78%) of CMP-NeuAc was obtained.

(B) [9-^{3}H;3,3′-^{2}H]NeuAc. A 50-μCi scale prep of [9-^{3}H]NeuAc was performed as above except a 23-h reaction time was used. The crude [9-^{3}H]NeuAc was diluted with 10 mg of carrier NeuAc and was then concentrated to dryness *in vacuo*. The residue was exchanged in 99.9% D_2O (3 × 0.5 mL) then redissolved in 0.7 mL of D_2O. The solution was brought to a pD of ∼12.5 by addition of 5 μL of 1.8 N NaOD and then transferred to a 5-mm NMR tube to monitor the exchange reaction. After 14 h at 23 °C, ^{1}H-NMR analysis showed that the C-3 axial and equatorial hydrogens were completely exchanged (>98%). The tube was carefully opened, the solution was transferred to a 1.5-mL polypropylene microfuge tube, and then the tube was cooled for 5 min on ice. The pH was adjusted to 7.5 by addition of 100 μL of cold 1 N Tris-HCl and 50 mM $MgCl_2$, pH 7.5. CTP (27 mg, 48.6 μmol) was dissolved in the soution, and the pH was readjusted to 7.5 with 1 N NaOH. After adjusting the volume to 1.0 mL with H_2O, 0.4 U of CMP-NeuAc synthase was added to the reaction mixture. After 7.5 h at 37 °C, the reaction mixture was clarified by centrifugation (2 min, 10000*g*) and purified by anion exchange. From 700 μL of the 1.0-mL reaction mixture was obtained 12.4 μCi (35% from [6-^{3}H]*N*-acetyl mannosamine).

(C) [1-^{14}C]CMP-NeuAc.[43a] The intermediate [1-^{14}C]NeuAc was prepared by NANA aldolase (0.1 U) catalyzed reaction of [1-^{14}C]sodium pyruvate (24 μCi, 3 μmol) with *N*-acetyl mannosamine (7.2 mg, 30 μmol) in 100 μL of 10 mM Tris-HCl (pH 7.5, 1.0 mg/mL BSA, 1.0 mg/mL NaN_3) for 47 h at 37 °C. The crude product reaction mixture was treated with CTP (2.6 mg, 4.5 μmol) and CMP-NeuAc synthase (0.05 U) in a final volume of 1.0 mL (50 mM Tris-HCl, pH 8.5, 50 mM $MgCl_2$) for 3 h at 23 °C. After clarification by centrifugation, anion exchange HPLC afforded 20.4 μCi (85%) of [1-^{14}C]CMP-NeuAc.

(D) [2-^{14}C]CMP-NeuAc. The intermediate [2-^{14}C]NeuAc was prepared by NANA aldolase (0.5 U) catalyzed reaction of [2-^{14}C]sodium pyruvate (100 μCi, 9.2 μmol) with *N*-acetyl mannosamine (44 mg, 184 μmol) in 500 μL of 10 mM Tris-HCl (pH 7.5, 1.0 mg/mL BSA, 1.0 mg/mL NaN_3) for 54 h at 23 °C. The crude product reaction mixture was treated with CTP (7.8 mg, 13.8 μmol) and CMP-NeuAc synthase (0.2 U) in a final volume of 1.0 mL (50 mM Tris-HCl, pH 8.5, 50 mM $MgCl_2$) for 2 h at 23 °C. After clarification by centrifugation, anion exchange HPLC afforded 76.5 μCi (76.5%) of [2-^{14}C]CMP-NeuAc.

(E) [1-^{14}C-*N*-acetyl]CMP-NeuAc. The intermediate [1-^{14}C-*N*-acetyl]NeuAc[43b] was prepared by NANA aldolase (0.35 U) catalyzed reaction of sodium pyruvate (7.8 mg, 73.6 μmol) with [1-^{14}C-*N*-acetyl]-mannosamine (14 μCi, 0.78 μmol) in 100 μL of 10 mM Tris-HCl (pH 7.5, 1.0 mg/mL BSA, 1.0 mg/mL NaN_3) for 4 days at 23 °C. The crude product reaction mixture was treated with CTP (0.9 mg, 1.6 μmol) and CMP-NeuAc synthase (0.1 U) in a final volume of 0.4 mL (100 mM Tris-HCl, pH 8.5, 50 mM $MgCl_2$) for 3 h at 37 °C. After clarification by centrifugation, anion exchange HPLC afforded 12.1 μCi (86%) of [1-^{14}C-*N*-acetyl]CMP-NeuAc.

HPLC Purification of CMP-NeuAc's. CMP-NeuAc was purified from reaction mixtures by HPLC on a MonoQ HR10/10 anion exchange column (Pharmacia) using an initial buffer of 100 mM NH_4HCO_3, 15% methanol, pH 8.0. After CMP-NeuAc eluted from the column, a gradient to 500 mM NH_4HCO_3 was used to elute unreacted CTP from the column. The fractions containing CMP-NeuAc were desalted on Amberlite IR-120H cation exchange resin and filtered through glass wool.[14b] The desalted CMP-NeuAc solution was concentrated at room temperature *in vacuo* on a rotary evaporator equipped with a mechanical vacuum pump. The concentrated CMP-NeuAc ammonium salt was redissolved in 1−2 mL of deionized water and stored at −20 °C. After purification and desalting, aliquots were assayed by HPLC for purity and showed a single symmetrical peak that co-eluted with authentic CMP-NeuAc. Contaminating NeuAc, which would arise from decomposition of CMP-NeuAc, was determined by passing ca. 100 000 cpm of a given CMP-NeuAc through a 2.5-cm column of Dowex-1 (Pi form) in a Pasteur pipet that was eluted with 5 mM phosphate buffer, pH 6.8.[44] CMP-NeuAc is retained, and NeuAc is passed through the column. CMP-NeuAc employed in KIE experiments was greater than 99.5% pure. Slow decomposition of CMP-NeuAc was observed on storage at −20 °C (ca. 0.5%/month).

UMP-NeuAc. NeuAc (2.0 mg, 6.5 μmol), UTP-Na_3 (7.2 mg, 13 μmol), and CMP-NeuAc synthase (0.25 U) were combined in 2.0 mL of 50 mM Tris-HCl (pH 8.5) containing 50 mM $MgCl_2$. The reaction mixture was incubated at 37 °C for 21 h during which time the anion exchange HPLC chromatogram (MonoQ HR10/10, 375 mM NH_4HCO_3, pH 7.8, 2 mL/min, A_{260}) indicated formation of 12% of UMP-NeuAc (retention time 5.2 min). The product was isolated with the above HPLC system, concentrated, and rechromatographed with 150 mM NH_4HCO_3 (retention time 14.2 min). Work is in progress to optimize the yield in this synthesis. The UV−VIS spectrum of UMP-NeuAc showed a λ_{max} of 252 nm, and like CMP-NeuAc, this product was acid labile (0.75 N HCl, 80 °C, 2 min), quantitatively producing UMP (retention time 19.8 min, 150 mM NH_4HCO_3). Attempts to obtain a mass spectrum of UMP-NeuAc have been unsuccessful; however, in addition to chemical behavior consistent with it being a CMP-NeuAc analog, the ^{1}H-NMR spectrum is completely consistent with its proposed identity, being rather similar to CMP-NeuAc with the most discernible differences occurring for the chemical shifts of H5 and H6 of the pyrimidine ring and the C-3 hydrogens of the NeuAc ring. ^{1}H NMR (D_2O): 7.79 (d, J = 8.1, 1 H, H_6-uracil), 5.82 (d, J = 4.5, 1 H, H_1-ribose), 5.78 (d, J = 8.1, 1 H, H_5-uracil), 4.22−4.18 (m), 4.12−4.04 (m), 3.98 (d, J = 10.8), 3.91 (m, 1 H), 3.82−3.7 (m), 3.52−3.42 (m), 3.31 (d, J = 9.9, 1 H), 2.32 (dd, J = 4.5, 13.2, 1 H, H-3$_{eq}$, NeuAc), 1.90 (s, 3 H, NCOCH_3), 1.49 (m, 1 H, H-3$_{ax}$, NeuAc). UV (pH 7.0, H_2O) λ_{max} = 252.

Measurement of Apparent First-Order Rate Constants for Solvolysis. Solvolysis reactions were conducted at 37 °C in 1.5-mL polypropylene microfuge tubes. Reaction mixtures were 30−150 μM in CMPNeuAc, at a given molarity of sodium formate, acetate, or phosphate buffers over the pH range 3.23−7.16. Reactions were initiated by addition of the appropriate volume of buffer to a solution of CMP-NeuAc in deionized water and allowed to proceed for 3−4 half-lives. The course of solvolysis was followed by HPLC (MonoQ HR10/10, 85 mM NH_4HCO_3, 15% methanol, pH 7.8, 2 mL/min, A_{260}) whereby integration of the unreacted CMP-NeuAc (13.6 min) versus the product CMP (18.4 min) allowed calculation of the percent of remaining CMP-NeuAc using eq 1. The extinction coefficients of CMP-NeuAc and CMP were determined to be identical within experimental error. The time points and corresponding progress of reaction data were fit to eq 2 using MacCurveFit to obtain the best fit for the apparent first-order rate constant for solvolysis, k_{obs}; plots of ln % CMPNeuAc versus time showed excellent linearity over 3−4 half-lives:

$$\%\ \text{CMPNeuAc} = A_{\text{CMPNeuAc}}/(A_{\text{CMP}} + A_{\text{CMPNeuAc}}) \times 100 \quad (1)$$

$$\ln\ \%\ \text{CMPNeuAc} = k_{obs}t + \ln\ \%\ \text{CMPNeuAc}_0 \quad (2)$$

Solvolysis of CMP-NeuAc in Methanol/H_2O. Reactions (1.0 mL) were conducted in 1.5-mL polypropylene microcentrifuge tubes and consisted of 6 mM CMP-NeuAc, 200 mM CD_3COOH, pH 5.0 or 6.0, and 20% or 30% (v/v) methanol. The reactions were maintained at 37 °C and monitored by HPLC until CMP-NeuAc consumption was greater than 98% (14 h, pH 5.0; 72 h, pH 6.0). Reaction mixtures were concentrated *in vacuo* and exchanged against D_2O four times to remove traces of methanol. ^{1}H-NMR analysis was used to determine the ratios of α- and β-methyl ketosides[18] of NeuAc, 2,3-dehydro-NeuAc, and NeuAc based on integration of resonances at 5.70 (CH, vinylic 2,3-dehydro-NeuAc), 3.32 (OCH_3, α-methyl ketoside), 3.18 (OCH_3, β-methyl ketoside), and 2.19 (H-3$_{eq}$, β-NeuAc anomer). The total NeuAc concentration was calculated by multiplying the observed integral for the β-NeuAc anomer by 1.087 based on the reported α/β ratio of 92:8.[45] The α- and β-methyl glycosides' identities were confirmed by addition of authentic samples of the glycosides to separate aliquots of solvolysis reaction mixtures.

Kinetic Isotope Effect Experiments. Kinetic isotope effect experiments employed mixtures of radiolabeled isotopomeric substrates and

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were therefore determined by the competitive method.[16] Experiments employed ca. 100 000 cpm each of ^{3}H- and ^{14}C-labeled CMP-NeuAc. ^{14}C heavy-atom isotope effects at the anomeric (C-2) or carboxyl (C-1) carbons used [1-^{14}C] or [2-^{14}C]CMP-NeuAc. Remote-labeled [9-^{3}H]-CMP-NeuAc was used to report on ^{12}C at C1 or C2. The β-dideuterium KIE was measured using [9-3H;3,3′-2H]CMP-NeuAc with remote-labeled [1-^{14}C-*N*-acetyl]CMP-NeuAc being used to report on protium at C-3. A control KIE was performed using a mixture of [9-^{3}H] and [1-^{14}C-*N*-acetyl]CMP-NeuAc where both isotopic labels are remote from the anomeric center. For each experiment, a master mixture of a given ^{3}H/^{14}C-labeled isotopomeric pair of CMP-NeuAcs was prepared from which aliquots were withdrawn for individual reactions and measurement of the reference ^{3}H/^{14}C ratio at time 0. Reaction mixtures (100−500 μL, pH's 5.0 and 6.0) were initiated by addition of the appropriate volume of 1 N acetate buffer to the isotopomeric mixture of CMP-NeuAc. After 40−60% conversion,[46] unreacted CMP-NeuAc was isolated from the reaction mixture on a MonoQ HR10/10 column (100 mM NH_4HCO_3, 15% methanol, pH 8.0, 2 mL/min, A_{260}) and collected directly into liquid scintillation vials (2.0-mL fractions). Care was taken to collect the entire CMP-NeuAc peak.[16] The percent conversion was determined from the ratio of the CMP-NeuAc and CMP peaks using eq 1. The initial ^{3}H/^{14}C ratio was obtained in triplicate by injecting aliquots of the master mixture of labeled CMP-NeuAc onto the MonoQ column and re-collecting the entire CMP-NeuAc peak. The ^{3}H/^{14}C ratios for CMP-NeuAcs were determined by dual-channel liquid scintillation counting (channel A, 0−12 keV; channel B, 12−80 keV) with each tube being counted for 10 min, and all tubes cycled through the counter 6−10 times to afford better counting statistics.[15] The internal ^{133}Ba source was used to estimate the quench parameter for each tube, which varied by ±1%. Triplicate samples of [^{14}C]CMP-NeuAc were used to determine the ratio of ^{14}C counts in channels A and B (A:B_{14}); since ^{3}H is only detected in channel A, the ^{3}H/^{14}C ratio in a given tube was calculated with the following equation:[15] $^{3}\mathrm{H}/^{14}\mathrm{C} = (\mathrm{cpm\ A} - \mathrm{cpm\ B} \times \mathrm{A{:}B_{14}})/(\mathrm{cpm\ B} + \mathrm{cpm\ B} \times \mathrm{A{:}B_{14}})$. The reported value and error of a KIE represents the mean and standard deviation of the mean of three individual KIE experiments taken over 6−10 cycles through the liquid scintillation counter. Equations 3 and 4 were used to calculate the ^{14}C KIEs and β-^{2}H KIEs, respectively. The KIEs were then corrected for % conversion using eq 5.[47]

$$\mathrm{KIE_{observed}} = (^{14}\mathrm{C}/^{3}\mathrm{H})_0/(^{14}\mathrm{C}/^{3}\mathrm{H})_t \quad (3)$$

$$\mathrm{KIE_{observed}} = (^{3}\mathrm{H}/^{14}\mathrm{C})_0/(^{3}\mathrm{H}/^{14}\mathrm{C})_t \quad (4)$$

$$\mathrm{KIE_{corrected}} = \ln(1-f)/\ln((1-f) \times \mathrm{KIE_{observed}}) \quad (5)$$

For determination of the β-dideuterium KIE by the direct-rate method, either [3,3′-^{2}H]CMP-NeuAc or unlabeled CMP-NeuAc was solvolysed at pH 5.0, 37 °C, in 200 mM acetate buffer to afford $^{D}k_{obs}$ and $^{H}k_{obs}$, respectively. Reactions for each isotopomer were run in triplicate. The progress of the reaction was followed by HPLC analysis of time point aliquots as described above for determination of the apparent first-order rate constant for solvolysis, with the same data treatment using eqs 1 and 2. The β-dideuterium isotope effect was calculated from the ratio of the rate constants $^{H}k_{obs}/^{D}k_{obs}$. The error represented for the isotope effect was calculated by propagating the standard error for determining the quotient $^{H}k_{obs}/^{D}k_{obs}$.

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Supporting Information Available: Two figures that present plots of pH versus rate data and buffer concentration versus rate data for solvolysis of CMP-NeuAc; a derivation of a KIE weighting expression for simultaneous reactions proceeding from the same reactant (3 pages). See any current masthead page for ordering information and Internet access instructions.

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