The *N*-Acetyl Neuraminyl Oxecarbenium Ion Is an Intermediate in the Presence of Anionic Nucleophiles

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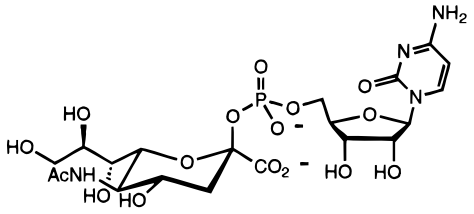
Abstract: Solvolysis of CMP *N*-acetyl neuraminate (CMP-NeuAc) in 1.8 M acetate buffer at pH 5 containing 0.9 M azide results in the formation of both anomers of 2-deoxy-2-azido *N*-acetyl neuraminic acid in addition to *N*-acetyl neuraminic acid as determined by ^{1}H-NMR product analysis. A rate dependence on [azide] was observed with an apparent bimolecular rate constant of $(2.1 \pm 0.3) \times 10^{-3}$ M^{-1} min^{-1} which could only account for half of the azido-NeuAc formed. Comparison of rate, product ratio, and stereochemical data indicate that concurrent pathways for formation of N_3-NeuAc are operative, with 17% of product forming from reaction of azide and the tight ion pair, 12% via the solvent separated ion pair, and 6% from the free NeuAc oxocarbenium ion. From the corrected product ratio data, the lifetime of the oxocarbenium ion was estimated to be $\geq 3 \times 10^{-11}$ s. Solvolysis of CMP-NeuAc at pL = 5.0 afforded an observed solvent deuterium isotope effect (SDIE) $k_{H_2O}/k_{D_2O} = 0.45$, consistent with specific acid catalysis of glycosidic bond cleavage. A SDIE of 0.66 for the apparent bimolecular azide trapping pathway was also observed. An apparent isotope effect of ~1.1 for trapping of the *N*-acetyl neuraminyl oxocarbenium ion by water was determined by product analysis of azide trapping in H_2O and D_2O. An *ab initio* transition state for attack of water on an *N*-acetyl neuraminyl oxocarbenium ion model was located which featured a hydrogen bond between the oxocarbenium ion carboxylate and water; proton transfer was not part of the reaction coordinate. It is proposed that the *N*-acetyl neuraminate carboxylate group stabilizes an intermediate oxocarbenium ion, but the barrier for capture by water is lowered by a transition state hydrogen bond.

Introduction

CMP-NeuAc, **1**, is the sugar-nucleotide[1] donor substrate for sialyltransferases, enzymes responsible for addition of *N*-acetyl neuraminic acid (sialic acid) to oligosaccharide chains of glycoproteins and glycolipids.[2] The structure of **1** is unusual for a sugar nucleotide because the leaving group is a monophosphate, not a diphosphate, and because the glycon NeuAc contains a carboxylate group directly attached to the anomeric center. We are interested in the chemistry of **1** to help understand the mechanistic features of sialyltransferases,[3] and because of its structure, it may represent a useful model for the active site of enzymes which catalyze glycosyltransfer, as described below.



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A long standing question in the area of glycosyltransfer centers on the nature of intermediates which may arise during the hydrolysis and synthesis of the glycosidic bond.[4] The Phillips hypothesis[5] concerning the mechanism of the retaining glycosylase hen egg white lysozyme (HEWL) included the proposition that after cleavage of the glycosidic bond, an enzyme bound oxocarbenium ion was formed which ion-paired with the carboxylate of active site residue Asp 52. It was later found that covalent adducts could be isolated for retaining glycosylases which corresponded to collapse of the ion pair (or by direct capture in the transition state),[6] and the inability to trap a glucosyl oxocarbenium ion in water led to the suggestion that oxocarbenium ions are too unstable to exist with a significant lifetime in the presence of anionic nucleophiles.[7a,b]

On the other hand, a contemporary crystallographic study of lysozyme has suggested that HEWL Asp 52 is too distant to form an acylal covalent intermediate without significant strain.[8] Recently, azide trapping studies of mutants of the retaining glycosylase β-galactosidase have been found to afford galactosyl azide and galactose in ratios paralleling those obtained for trapping of relatively stable carbenium ions in aqueous solution, lending support for the existence of the galactosyl oxocarbenium ion as an enzyme bound intermediate.[9]

(1) Comb, D. G.; Watson, D. R.; Roseman, S. *J. Biol. Chem.* **1966**, *241*, 5637−5642.

(2) Harduin-Lepers, A.; Recchi, M.-A.; Delannoy, P. *Glycobiology* **1995**, *5*, 741−758. Varki, A. *Glycobiology* **1992**, *2*, 25-40. Reglero, A.; Rodriguez-Aparicio, L. B.; Luengo, J. M. *Int. J. Biochem.* **1993**, *11*, 1517−1527. Hayes, B. K.; Varki, A. *J. Biol. Chem.* **1993**, *268*, 16155−16169.

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(4) Sinnott, M. L. *Chem. Rev.* **1990**, *90*, 1171−1202. Withers, S. G. *Pure Appl. Chem.* **1995**, *67*, 1673−1682.

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(8) Strynadka, N. C. J.; James, M. N. G. J. *Mol. Biol.* **1991**, *220*, 401−424.

(9) Richard, J. P.; Huber, R. E.; Heo, C.; Amyes, T. L.; Lin, S. *Biochemistry*, **1996**, *35*, 12387−12401.

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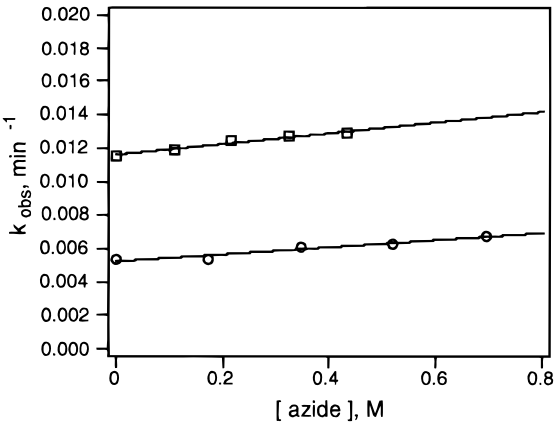

Figure 1. Kinetics for acid-catalyzed solvolysis of CMP-NeuAc, **1**, with varied concentrations of NaN_3 in H_2O and D_2O. The solvolysis reactions were conducted at pL = 5.04, 37 °C, in 1.8 M acetic acid Na acetate buffer as described in the text. [azide] refers to $[N_3^-]$ after correction for pL. The squares (□) correspond to data for D_2O; the circles (o) correspond to data for H_2O. The lines are the best fit to the data by linear regression.

The glycosyltransfer of CMP-NeuAc is salient to this issue in that the NeuAc sugar contains an ionized carboxylate residue adjacent to the anomeric carbon. The carboxylate could function in a number of different capacities which are reflective of the chemical reactivities found in glycosylase active sites. During solvolysis, the carboxylate could stabilize an oxocarbenium ion like transition state and/or oxocarbenium ion intermediate, act as an intramolecular nucleophile, or mediate general acid−base catalysis. The existence of an oxocarbenium ion derived from *N*-acetyl neuraminic acid would provide an example supporting the chemical possibility for existence of enzyme/glycosyl cation ion pairs. Sinnott and colleagues have shown that solvolysis of aryl glycosides of *N*-acetyl neuraminic acid can proceed with nucleophilic participation of the carboxylate group in the transition state, possibly resulting in an intermediate α-lactone.[10] Recently it was shown that acid-catalyzed solvolysis of CMP-NeuAc at pH 5 in water/methanol mixtures provides equal amounts of α- and *β*-methyl glycoside products, consistent with the formation of an oxocarbenium ion intermediate after departure of CMP.[11] In this work we present the results of solvent deuterium isotope effect experiments, azide trapping studies during solvolysis of CMP-NeuAc, and *ab initio* calculations in order to characterize the lifetime of the oxocarbenium ion and the nature of acid/base catalysis over the reaction coordinate.

Results

Azide Rate Dependence for CMP-NeuAc Solvolysis in H_2O and D_2O. The solvolysis CMP-NeuAc (500 μm) at pL 5.04 ± 0.02 at 37 °C in 1.8 M Na acetate buffers containing varied concentrations of NaN_3 was followed by HPLC to afford apparent first-order rate constants. Figure 1 provides a plot of the apparent first-order rate constants versus azide concentration for reactions in H_2O and D_2O. The data show that the rate exhibits a modest dependence on azide concentration. The respective observed rate constants for solvolysis by water, k_{obs} and azide, k_{az} in H_2O and D_2O are $^{H}k_{obs} = (5.2 \pm 0.1) \times 10^{-3}$ min^{-1}, $^{D}k_{obs} = (11.6 \pm 0.1) \times 10^{-3}$ min^{-1}, $^{H}k_{az} = (2.1 \pm 0.3) \times 10^{-3}$ M^{-1} min^{-1}, and $^{D}k_{az} = (3.2 \pm 0.4) \times 10^{-3}$ M^{-1} min^{-1}. The ratios of the intercepts of the plot in Figure 1 show that an inverse solvent isotope effect of 0.45 ± 0.01 is expressed for the hydrolytic reaction, and the ratio of the slopes show that an inverse solvent isotope effect of 0.66 ± 0.12 exists for the azide reaction. We considered the possibility that a salt effect was operative in these experiments since the acetate buffer concentration was fixed, while azide was varied. When the molarity of the acetate buffer was varied along with azide so as to maintain a constant ionic strength of 1.95, identical kinetics were observed within experimental error. If a nonspecific salt effect exists, it is small under the conditions employed in these experiments.

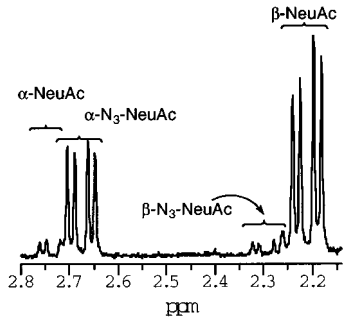

Figure 2. ^{1}H-NMR product analysis for solvolysis of CMP-NeuAc in the presence of 0.9 M total azide in H_2O at pH 5.0. The region of the spectrum shown corresponds to resonances for the C3 equatorial hydrogens of the α/*β* anomers of N_3-NeuAc and NeuAc. The reaction conditions are described in the text.

Product Analysis of Azide Trapping. CMP-NeuAc (10−15 mM) was solvolyzed at pL 5.04 ± 0.02 at 37 °C in 1.8 M Na acetate buffers containing varied concentrations of NaN_3 until HPLC indicated >98% conversion. No azide adducts were detected at low azide concentration (0.005 M). ^{1}H-NMR spectra (Figure 2) of the reaction mixtures containing 0.9 M total azide (actual $[N_3^-]$ = 0.63 M at pH 5.04) showed resonances for α- and *β*-NeuAc equatorial C-3 hydrogens (α- *δ*2.72, dd; *β*- *δ*2.25, dd), and resonances for α- and *β*-N_3-NeuAc equatorial C-3 hydrogens were also observed (α- *δ*2.67, dd; *β*- *δ*2.29, dd).[13] By integration of the equatorial hydrogens at C3, the relative percentages of α- and *β*-NeuAc and α- and *β*-N_3-NeuAc were 4, 61, 32, and 3, respectively. Of the original CMP-NeuAc, 35% was converted into N_3-NeuAc. The results were similar for solvolysis in D_2O at pD = 5.04 (Supporting Information). The total azide employed was 0.9 M, but due to the pK_a shift[14] to 5.15 for hydrazoic acid in D_2O, the actual N_3^- concentration was 0.39 M. The relative percentages of α- and *β*-NeuAc and α- and *β*-N_3-NeuAc were 5, 68, 25, and 2, respectively.

Methanolysis of CMP-NeuAc in the Presence of Azide. CMP-NeuAc was solvolyzed in 18% (v/v) aqueous methanol at pH 5.0 in the presence of 0.9 M azide and in its absence. After complete consumption of CMP-NeuAc, the reaction mixtures were concentrated, worked up as described, and subjected to a product analysis by ^{1}H-NMR. In the absence of azide, ∼1:1 α- and *β*-methyl ketosides of NeuAc were observed, in agreement with previous results.[11] An identical ratio of methyl ketosides were formed in the presence of azide, in addition *to* α- and *β*-N_3-NeuAc and NeuAc in similar proportions as described above for solvolysis in the absence of methanol.

***Ab Initio* Transition State Structure for Capture of an *N*-Acetyl Neuraminyl Oxocarbenium Ion Model by Water.** Previous work has established that a local minimum energy conformation for the *N*-acetyl neuraminyl oxocarbenium ion

(10) Ashwell, M.; Guo, X.; Sinnott, M. L. J. *Am. Chem. Soc.* **1992**, *114*, 10158−10166.

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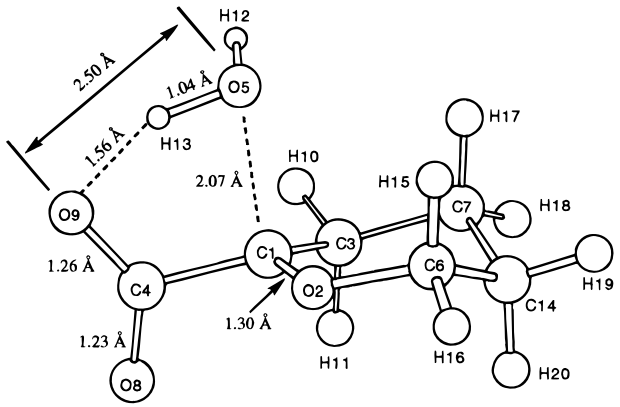


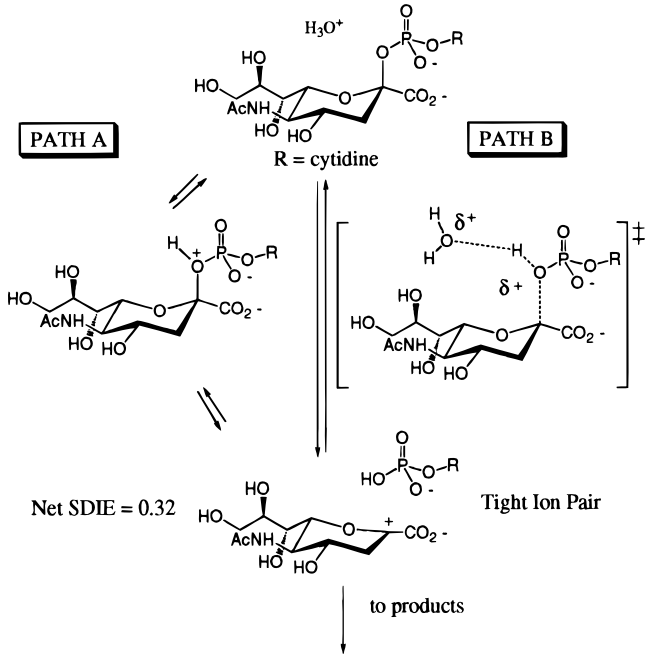
Figure 3. *Ab initio* (B3LYP/6-31G*) transition state structure for attack of water on an *N*-acetyl neuraminyl oxocarbenium ion model. Water oxygen O5 is attacking the anomeric carbon C1. Atom O2 is the pyranosyl ring oxygen, C4, O8, and O9 are the carboxylate group atoms.

model featured a planar arrangement for the C−C−O−C atoms comprising the oxocarbenium ion atoms with the carboxylate group roughly perpendicular to this plane.[13] A water molecule was added to this structure which was then allowed to optimize to a transition state using density functional theory (B3LYP/6-31G*). The structure is presented in Figure 3, along with key geometric parameters. This transition structure was characterized by a single imaginary frequency of −192 cm^{-1}. The atomic displacements for the reaction coordinate featured formation of the C−O glycosidic bond as the major contributor, accompanied by pyrimidalization at the anomeric carbon, and loosening of the oxocarbenium ion C−O bond. The "glycosidic" C−O bond length is 2.07 Å, and the endocyclic C−O bond is 1.30 Å. One water hydrogen atom is hydrogen bonded to the carboxylate group with an O−O internuclear distance of 2.50 Å. The stable vibrational frequencies for the hydrogen bond include an OH stretch of 2628 cm^{-1} and a complex mode at 1808 cm^{-1} which couples the water HOH bend, and hydrogen bonded OH stretch to the asymmetric C═O stretch of the carboxylate.[15] Transfer of the water hydrogen to the carboxylate was not a component of the transition state.

Discussion

Solvent Isotope Effects for Solvolysis of CMP-NeuAc. The solvolysis of CMP-NeuAc at pH 5 proceeds with an observed inverse solvent deuterium kinetic isotope effect (SDIE) of 0.45, in reasonable agreement with the experimental inverse SDIE of 0.57 measured for solvolysis of α-D-glucose-l-phosphate.[16] The result indicates specific acid catalysis and is consistent with our earlier conclusion.[11] The results are distinct from those recently reported for solvolysis of benzylic acetate containing an ortho carboxylate group,[17] and for aqueous decomposition of CMP-KDO, a sugar-nucleotide similar to CMP-NeuAc by virtue of a carboxylate at the anomeric carbon and a CMP leaving group.[18]

Scheme 1



A reasonable and productive protonation site is the glycosidic phosphate oxygen which would lead to the tight ion pair as the first product. A partial reaction sequence is presented in Scheme 1. The classic specific acid catalyzed reaction would involve rapid equilibrium protonation as shown in path A of the scheme. It has been suggested[19] that the pK_a of the glycosidic phosphate oxygen might be too low to afford a high enough concentration of the CMP-NeuAc conjugate acid to be kinetically competent, possibly requiring catalysis in the transition state as shown in path B. At this time we cannot rule out either possibility. The calculated equilibrium solvent isotope effect is ∼0.32 for hydronium ion catalyzed generation of the tight ion pair. This is somewhat more inverse than the experimentally observed value of 0.45. Expression of the isotope effect may reflect kinetic complexity as outlined in Scheme 2.[22] If internal return k_{-1} of the tight ion pair is similar to the net forward chemistry of the ion pair k_2, the size of the isotope effect will be sensitive to the relative values of k_{-1} and k_2, varying between the equilibrium isotope effect for formation of the ion pair and the kinetic isotope effect for cleavage of the glycosidic bond when $k_{-1} > k_2$ and $k_{-1} < k_2$, respectively. Support that this scheme may be operative follows from the observation that the solvent

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(13) Horenstein, B. A. *J. Am. Chem. Soc.* **1997**, *119*, 1101−1107.
(14) Ritchie, C. D. *Physical Organic Chemistry*, 2nd ed.; Marcel Dekker: New York, 1990; p 281.
(15) The Supporting Information includes coordinates for visualization of these vibrational modes.
(16) Bunton, C. A.; Humeres, E. *J. Org. Chem.* **1969**, *34*, 572−576.
(17) Fife, T. H.; Bembi, R.; Natarajan, R. *J. Am. Chem. Soc.* **1996**, *118*, 12956−12963.
(18) Lin, C.-H.; Murray, B. W.; Ollman, I. R.; Wong, C.-H. *Biochemistry* **1997**, *36*, 780−785.
(19) A referee suggested that the glycosidic phosphate oxygen could have a pK_a of −9 to −10. If this pK_a is correct, the concentration of CMP-NeuAc in reactive protonated form at pH 5 would be so low as to require a rate constant of 10^{10}−10^{11} s^{-1} for glycosidic bond cleavage to maintain overall kinetic competency. For a discussion of the timing of proton transfers and solvent isotope effects, see: Schowen, R. L. *Prog. Phys. Org. Chem.* **1972**, *9*, 275−332.
(20) Alvarez, F. J.; Schowen, R. L. In *Isotopes in Organic Chemistry*; Buncel, E., Lee, C. C., Eds.; Elsevier: Amsterdam, 1987; Vol. 7, Chapter 1. Kresge, A. J.; More O'Ferrall, R. A.; Powell, M. F. In *Isotopes in Organic Chemistry*; Buncel, E., Lee, C. C., Eds.; Elsevier: Amsterdam, 1987; Vol. 7, Chapter 4.
(21) We point out that the rate law for solvolysis has two kinetically equivalent expressions, the first involving spontaneous reaction of CMP-NeuAc protonated (presumably) at the carboxylate group, and the second for acid-catalyzed reaction with the carboxylate ionized.[11] Our analysis of the solvent isotope effects employed the second form of the rate law because it is more chemically reasonable.
(22) The steady state assumption was applied to the tight ion pair, and the expression shown in the scheme was obtained by dividing the rate expression for reaction in H_2O by that for reaction in D_2O. The rate constant k_2 represents the net rate for reactions of the tight ion pair in the forward direction.

Scheme 2

$$\text{CMP-NeuAc} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} |\,\text{CMP}^-\ \ \text{NeuAc}^+\,| \xrightarrow{k_2} \text{products}$$

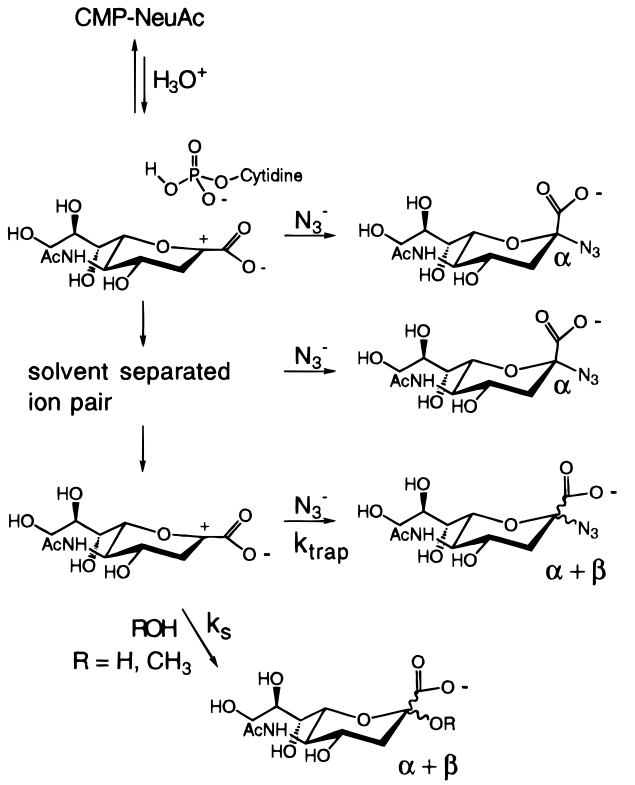
$$\text{KIE}_{\text{observed}} = \frac{^{H_2O}k_1\,(^{D_2O}k_{-1} + k_2)}{^{D_2O}k_1\,(^{H_2O}k_{-1} + k_2)}$$

isotope effect is more normal for azide trapping than for solvolysis (0.66 vs 0.45); the net rate constant k_2 would be higher with a contribution from azide capturing some of the ion pair, with a resultant change in the observed isotope effect away from the equilibrium isotope effect. Further study will be required to resolve the structural features of specific acid catalysis in this system and the possible effect of kinetic complexity on observed kinetic isotope effects.

Azide Trapping of the *N*-Acetyl Neuraminyl Oxocarbenium Ion. A fascinating feature of CMP-NeuAc is the presence of a carboxylate group adjacent to the anomeric center and how it may influence glycosyltransfer. Kinetic isotope effects for CMP-NeuAc solvolysis are most consistent with a very late oxocarbenium ion-like transition state in which the carboxylate group is coplanar with the oxocarbenium ion plane. Based on the observation that methanol could be incorporated into NeuAc during solvolysis of CMP-NeuAc to afford nearly equimolar amounts of α- and β-glycoside, and by conformational analysis, we propose that the transition state breaks down to an oxocarbenium ion intermediate with the carboxylate group orthogonal to the oxocarbenium ion plane.[11,13]

We employed azide ion trapping experiments to obtain further evidence for the nature of intermediates on the solvolytic pathway.[23a−c] The data of Figure 1 show that solvolysis of CMP-NeuAc has only a modest rate dependence on [azide], reasonably via reaction of azide with the tight ion pair to afford α-N_3-NeuAc. However, ^{1}H-NMR product analysis data show that only half of N_3-NeuAc formed during solvolysis can be accounted for by kinetics. The balance of the N_3-NeuAc is assigned to arise from reaction of azide with the solvent separated ion pair and the free oxocarbenium ion. The conclusion that some reaction occurs with the free oxocarbenium ion follows from the ^{1}H-NMR analysis which identified β-azido NeuAc, which most reasonably would derive from capture of the free oxocarbenium ion. It would be difficult to justify formation of β-azido NeuAc from the solvent separated ion pair since the leaving group CMP is anionic and on the β-face. Utilizing the NMR product ratios and the N_3-NeuAc accounted for by kinetics, we assign the relative proportions of NeuAc and N_3-NeuAc formed from the tight ion pair, solvent separated ion pair, and free oxocarbenium ion, respectively, as 65:17:12:6. Scheme 3 presents a mechanistic scheme which is consistent with the results. Solvent ROH only attacks the free oxocarbenium ion; the ion pairs are not captured by solvent. This point is supported by the observation that methanolysis of CMP-NeuAc in the presence and absence of azide gave identical ratios of α- and β-methyl NeuAc glycoside products. If solvent capture of ion-pairs were significant, addition of the much better nucleophile azide would compete with solvent for formation of the α-methyl glycoside, with an attendant change in the α/β ratio of methyl glycosides, which was not observed. Interestingly, azide appears selective over solvent for trapping ion-pairs, but solvent is more selective than azide for the free oxocarbenium ion. In part this may be a manifestation of the carboxylate group conformation: it may be near-planar in the ion pair due to unfavorable electrostatics with the phosphate of neighboring CMP, while the free oxocarbenium ion should favor a "twisted" conformation.[l3] Unfavorable electrostatic interactions between the carboxylate and azide may exist for azide attack on the free oxocarbenium ion; this is discussed further later. The results show that the *N*-acetylneuraminyl oxocarbenium ion exists in various intermediate forms, but most importantly, indicate that a free oxocarbenium ion can exist in aqueous solution *even in the presence of a strong anionic nucleophile.* The free oxocarbenium ion is not especially long lived, and in the following discussion we consider its lifetime.

Scheme 3



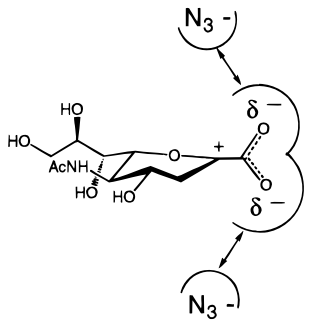
Lifetime and Reactivity of the *N*-Acetyl Neuraminyl Oxecarbenium Ion. The ratio of NeuAc to α/β N_3-NeuAc produced from the free oxocarbenium ion intermediate will be equal to the ratio of rate constants for their formation, k_s/k_{trap}-[N_3^-]. With knowledge of k_{trap}, k_s can then be calculated; the reciprocal of k_s is equal to the oxocarbenium ion lifetime. The product ratio NeuAc/α/β N_3-NeuAc was 0.093, and the value for k_{trap} is required to solve for k_s. Values close to 5×10^9 M^{-1} s^{-1} have been measured experimentally[24] for reaction of a carbenium ion and azide in aqueous solution. This value is probably too high for trapping the *N*-acetyl neuraminyl oxocarbenium ion because (1) the *N*-acetyl neuraminyl oxocarbenium has a net molecular charge of zero, not +1, and (2) the preferred conformation of the carboxylate group of the *N*-acetyl neuraminyl oxocarbenium ion should be orthogonal to the oxocarbenium ion plane[13] as in **II**. On both counts, k_{trap} for

(23) (a) Young, P. R.; Jencks, W. P. *J. Am. Chem. Soc.* **1977**, *99*, 8238−8248. (b) Ta-Shma, R.; Rappoport, Z. *J. Am. Chem. Soc.* **1983**, *105*, 6082−6095. (c) Richard, J. P. *Tetrahedron* **1995**, *51*, 1535−1573.

(24) McClelland, R. A.; Banait, N.; Steenken, S. *J. Am. Chem. Soc.* **1986**, *108*, 7023−7027. McClelland, R. A.; Kanagasabapathy, V. M.; Steenken, S. *J. Am. Chem. Soc.* **1988**, *110*, 6913−6914.

(25) The sensitivity of diffusion controlled rates to electrostatics is well-known, see: Rice, S. A. In *Comprehensive Chemical Kinetics*; Bamford, C. H., Tipper, C. F. H., Compton, R. G., Eds.; Elsevier: Amsterdam, 1985; Vol. 25, Chapter 3.

the *N*-acetyl neuraminyl oxocarbenium ion should be smaller than for trapping simple carbenium ions because the electrostatics are less favorable.[25] To the best of our knowledge, more appropriate experimental rate constants have not been reported, so we employ the known value of 5×10^9 M^{-1} s^{-1} as an *upper* limit for k_{trap}.



II

With this consideration in mind, the calculated first-order rate constant for water trapping of the cation, k_s, is 3.4×10^{10} s^{-1}; the lifetime (a lower limit) is 3×10^{-11} s, slightly briefer than the earlier estimate.[11] This lifetime is just sufficient[26a,b] to account for the results of trapping by aqueous methanol in which an approximately 1:1 ratio of methyl glycosides was observed and proposed to arise by reaction of methanol with a diffusionally equilibrated oxocarbenium ion. The *N*-acetyl neuraminyl oxocarbenium ion lifetime of $\geq 3 \times 10^{-11}$ s is at least 30 times greater than the estimated lifetime[7a] for the glucosyl oxocarbenium ion in pure water($\sim 10^{-12}$ s). It has been proposed that a 2-deoxy sugar oxocarbenium ion will be more long lived than the corresponding 2-hydroxy species by a factor of 4.[7a] So in the comparison between the glucosyl oxocarbenium ion and that of NeuAc, when the 2-deoxy factor is taken into account, the relative stability conferred by the carboxylate group is 30/4, which at 37 °C would be worth about −1.2 kcal/mol as a minimum value.

Water and Oxocarbenium Ion Carboxylate Group Interaction. Although the *N*-acetyl neuraminyl oxocarbenium ion is more long lived than the glucosyl oxocarbenium ion, it is surprising that it does not have a much longer lifetime than it does. Even accounting for solvation, the immediately proximate carboxylate group and oxocarbenium ion carbon should enjoy a substantial stabilizing interaction that other unsubstituted glycosyl cations do not. In comparison to a 2-deoxy pyranosyl oxocarbenium ion, a 2-deoxy α-carboxylate substituted pyranosyl oxocarbenium ion was estimated to be −17 kcal/mol more stable by *ab initio* calculations which included an aqueous solvation model.[13] The implication is that the thermodynamic stability is unrealized because of other factors. If the carboxylate were to function as an intramolecular general base it would shorten the lifetime by facilitation of oxocarbenium ion capture by water (but not azide). This might be detectable by a solvent isotope effect, but an experimental difficulty faced in establishing this is that capture of the oxocarbenium ion by solvent is a fast step, rendering it kinetically silent by conventional techniques. We considered that competitive azide trapping might provide a means of accessing this rapid step, where azide trapping in H_2O versus D_2O could provide $^{H_2O}k_s/^{D_2O}k_s$. From the data obtained in D_2O, $^{D_2O}k_s$ was estimated[27] to be 3×10^{10} s^{-1}, quite close to the value for $^{H_2O}k_s$ giving an apparent solvent isotope effect of 1.1. This result suggests[28] that there is little proton transfer during attack of water on the oxocarbenium ion.

We modeled the attack of water on the *N*-acetyl neuraminyl oxocarbenium ion with *ab initio* calculations and located the transition state structure shown in Figure 3. The attacking water is in a hydrogen bond to the carboxylate group with the hydrogen residing squarely on the water oxygen, and proton motion within the hydrogen bond is not a part of the reaction coordinate but is a stable vibration. Thus the transition state hydrogen bond is very similar to a ground state hydrogen bond, which would be expected to result in an isotope effect of approximately unity,[29] in agreement with the experimental results. The hydrogen bonded array of water and carboxylate can still be considered as a catalytic grouping on an electrostatic basis.[2lc,30] As glycosidic bond formation occurs, charge is transferred to the attacking water. A hydrogen bond to the negatively charged carboxylate would serve to stabilize the shift in charge that occurs during progressive formation of the new glycosidic C−O bond, lowering the barrier for reaction.

It has been pointed out that general base catalysis is not anticipated to be significant for unstable carbenium ions.[30b] Because of the proximate carboxylate in the *N*-acetyl neuraminyl oxocarbenium ion, and its potential for facilitating addition of water, we suggest that the separate consideration of oxocarbenium ion stability and general base catalysis may not be possible in the present system because these factors are likely to be intertwined. A substantial *intrinsic* lifetime of the *N*-acetyl neuraminyl oxocarbenium ion is predicted because it contains a proximate stabilizing carboxylate group, but this is largely offset because this same group is likely to facilitate water addition to the oxocarbenium ion, shortening its lifetime.

The proposed interrelationship of *N*-acetyl neuraminyl oxocarbenium ion stability and intramolecular catalysis by the carboxylate group may find parallel in enzyme-catalyzed glycosyl transfer. Azide trapping studies[9] on mutants of β-galactosidase which had the catalytic general acid/base Glu 461 mutated to either glycine or glutamine showed a marked ability to trap the galactosyl-enzyme intermediate to afford azido- galactoside and galactose products; the wild type enzyme does not incorporate azide. In part, removal of the Glu carboxylate group favors reaction with anionic nucleophiles, but also the apparent first-order rate constant for capture of the galactosyl-enzyme complex by water was reduced: >74-fold for E461G for reaction with water and 40 000-fold for E461G for reaction with trifluoroethanol.

(26) (a) The rate constant for trapping the free oxocarbenium ion by water cannot be faster than the rate for diffusional separation of the tight ion pair; an estimate[26b] of the diffusional separation rate constant is 1.6×10^{10} s^{-1} for azide and a substituted benzyl carbenium ion. This is likely to be a lower limit for CMP and the N-acetyl neuraminyl oxocarbenium ion due to less favorable electrostatics. (b) Richard, J. P.; Jencks, W. P. *J. Am. Chem. Soc.* **1984**, *106*, 1373−1383.

(27) A value of 4.2×10^9 M^{-1} s^{-1} for trapping by azide in D_2O was used to account for the 1.2-fold higher viscosity of D_2O versus H_2O at 37 °C. Kirshenbaum, I. *Physical Properties of Heavy Water*; McGraw Hill: New York, 1951.

(28) A near unity solvent isotope effect could be the result of fortuitous cancellation of nonunity contributions and must therefore be considered cautiously.

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Conclusions

The key features of the solvolytic pathway of CMP-NeuAc at pH 5 are (1) glycosidic bond cleavage is specific acid catalyzed; (2) formation of N_3-NeuAc from CMP-NeuAc occurs from ion pairs and the free oxocarbenium ion; (3) the free oxocarbenium ion has a lifetime of $\geq 3 \times 10^{-11}$ s; and (4) capture of the oxocarbenium ion by water does not appear to utilize intramolecular general base catalysis, but the rate may be facilitated by favorable electrostatic interaction between the attacking water and carboxylate group. The *N*-acetyl neuraminyl oxocarbenium ion exists as an intermediate in the presence of azide and its own carboxylate group, providing an example of the chemical feasibility of the intermediacy of this oxocarbenium ion in sialyltransferase and neuraminidase active sites. However, the lifetime of the intermediate should be short in the presence of catalytic functionality that facilitates transfer to the glycosyl acceptor.

Experimental Section

Materials. Buffers and reagents were purchased from Sigma and Fisher. Plasmid pWV200B harboring the expression construct[31] for E. *coli* CMP-NeuAc synthase was a gift from Dr. W. F. Vann at the National Institutes of Health. CMP-NeuAc synthase[32] and CMP-NeuAc[11] were prepared as previously described.

Instrumental. ^{1}H-NMR spectra were measured at ambient temperature on a Varian Gemini300 spectrometer operating at 300 MHz. The acquisition pulse angle was 30°. Spectra were obtained in 99.9% D D_2O referenced to the HDO peak (4.80 ppm). A preacquisition saturation of the HDO line was used for solvent suppression. 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.

Measurement of Apparent First-Order Rate Constants for Solvolysis. Solvolysis reactions were conducted at 37° C in 0.5 mL polypropylene microfuge tubes. Reaction mixtures were 500 μM in CMPNeuAc, 1.8 M CD_3COONa buffer, pL = 5.04 ± 0.02. 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, A260) whereby integration of the unreacted CMP-NeuAc (13.6 min) versus the product CMP (18.4 min) allowed calculation of the percent 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.

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

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

Solvolyses of CMP-NeuAc for Product Analysis. Reactions (1.0 mL) were conducted in 1.5 mL polypropylene microcentrifuge tubes and consisted of 10 mM CMP-NeuAc, 1.8 M CD_3COONa pL 5.0 in the presence of the appropriate amount of NaN_3 and/or methanol. For reactions in D_2O, 0.4 was added to the observed pH meter readings obtained with a combination glass electrode (Orion).[33] The reactions were maintained at 37 °C and monitored by HPLC until CMP-NeuAc consumption was greater than 98% (14 h, pH 5.0). Reaction mixtures were passed through a Pasteur pipette containing Amberlite IR120-H^+ resin to remove cations, then concentrated *in vacuo*, and exchanged against D_2O three times for NMR analysis. The desalting step was necessary as it was observed that if this step were neglected, the solution was quite alkaline after concentration, resulting in exchange of the β-hydrogens in NeuAc in D_2O, complicating the analysis. ^{1}H-NMR analysis (Figure 2) was used to determine the ratios of α- and β-N_3-NeuAc and of NeuAc based on integration of the respective C3 equatorial hydrogens in the following way. The resonances for α- and β-N_3-NeuAc were assigned by the reported chemical shifts for these compounds.[12] The upfield half of the α-NeuAc resonance (doublet of doublets, dd) was buried under the α-N_3-NeuAc dd, while the upfield half of the β-N_3-NeuAc dd was too close to the β-NeuAc dd to satisfactorily integrate these latter two resonances. The area for α-NeuAc was taken to be twice the area of the resolved downfield half of its dd; the area corresponding to the resolved half of the α-NeuAc was then subtracted from the area of the overlapping α-N_3-NeuAc and α-NeuAc multiplets to provide the corrected integration for α-N_3NeuAc. The corrected integral areas for β-N_3-NeuAc and β-NeuAc were determined in the same way. The equilibrium ratio of α- and β-NeuAc was calculated to serve as an internal check of the method: we calculate 6:94 and 7:93 α/β ratios after solvolysis in H_2O and D_2O, respectively; the literature reports 5:95 and 8:92 equilibrium ratios for α/β NeuAc in aqueous solution,[34] indicating that the method is reliable.

Calculations. *Ab initio* calculations were performed using Gaussian 94,[35] revision C.3 on a Silicon Graphics Indigo XZ workstation and an IBM RS 6000 SP. Calculations employed the 6-31G* basis set and density functional theory (DFT) with the Becke 3 parameter exchange functional and the Lee-Yang-Parr correlation functional.[36,37] The transition state structure for attack of water on the α-carboxylate substituted pyranosyl oxocarbenium ion[13] was optimized to tight criterion (Figure 3). A frequency calculation on the transition state structure afforded a single imaginary frequency which corresponded to atomic motion on the desired reaction coordinate. Cartesian coordinates for the transition state structure and selected sets of Cartesian displacements for vibrational modes of interest are provided in the Supporting Information.

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Supporting Information Available: ^{1}H-NMR spectral data for product analysis of azide trapping in D_2O. ^{1}H-NMR spectral data for methanol incorporation in the absence and presence of azide. Cartesian coordinates for selected normal vibrational modes of the calculated transition state structure (4 pages). See any current masthead page for ordering and Internet access instructions.

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