Pathways for the Hydrolysis of Glycosides of *N*-Acetylneuraminic Acid

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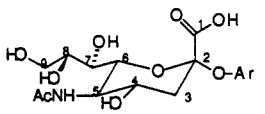
Abstract: The hydrolyses of aryl α-glucosides of *N*-acetylneuraminic acid proceed by four processes: (i) the H_3O^+-catalyzed hydrolysis of the neutral molecule; (ii) the H_3O^+-catalyzed hydrolysis of the anion (the kinetically equivalent neutral hydrolysis of the neutral molecule being ruled out by a small solvent isotope effect and β_{lg} values close to zero); (iii) the spontaneous hydrolysis of the anion; (iv) a base-catalyzed process possibly involving nucleophilic attack of the ionized C9-OH at position 6. Secondary deuterium kinetic isotope effects (d_2, *pro-R*, and d_1, *pro-S*, at C3; d_1 at C4) have been measured for the *p*-nitrophenyl glycoside undergoing processes i–iii by the isotopic quasi-racemate method, with the *p*-nitrophenyl L-*N*-acetylneuraminide being made by the Vasella route from L-glucose (*Helv. Chim. Acta* **1986**, *69*, 1172, 1191, and 1205). The effects support a flattened $^2C_5$ ring conformation for these processes and require some nucleophilic assistance by the C1 carboxylate group for processes ii and iii, despite the strained α-lactone ring that results. The implications for catalysis by sialidases and retaining glycosidases such as lysozyme are discussed.

Introduction

The basic mechanistic chemistry involved in the nonenzymic hydrolysis of simple pyranosidic[1] and furanosidic[2] *O*-glycosides was established by the middle 1970s, especially with respect to the site[1,2] and timing[3] of any protonation step.[4] More recent mechanistic concerns have centered on the lifetime of glucopyranosyl and related cations[5,6] and the failure of the antiperiplanar lone pair hypothesis to predict relative rates[7] and transition-state structures[8] in the reactions of pyranosyl and tetrahydropyranyl derivatives. The mechanistic picture which is emerging is one in which the glucopyranosyl cation has a real existence just in water, but not in less polar solvents, and even in water is too unstable to exist in the presence of anionic nucleophiles.[5,6,8] In accord with this picture, simple tetrahydropyranyl derivatives, in which the cation is not destabilized by the inductive effects of several hydroxyl groups, appear to react by simple S_N1 ($D_N + A_N$) or A_1 pathways[9] in water. Although there is as yet no consensus as to the existence or absence of antiperiplanar lone pair effects in reactions of pyranosyl derivatives, it is now conceded that "it is not necessary to take literally the earliest formulation of the theory" (of stereoelectronic control).[10]

Despite the extensive study of aldosyl derivatives, very little mechanistic attention has been paid to the hydrolyses of glycosides of sialic acid (e.g., I).[11] In these compounds the reaction center possesses a carboxylate group in place of the anomeric hydrogen. Further, the adjacent position is not oxygenated. In the case of simple aldopyranosides, removal of the electron-withdrawing 2-OH group greatly stabilizes the glycosyl cation, so that acid-catalyzed hydrolyses are faster by some 10^3.[4,12] At the same time, the recent work of Banait and Jencks[6] shows that the glucopyranosyl cation, at least, is too unstable to exist in the presence of an anionic nucleophile. Despite the disfavored α-lactone structure that would result from full bond formation, therefore, some sort of participation by the carboxylate group in the hydrolysis of sialic acid glycosides seems not a priori unreasonable.

It thus appears that the existing knowledge of glycoside hydrolysis cannot be confidently extrapolated to hydroyses of sialic acid glycosides. Sialic acid residues are important in many aspects of cell–cell and cell–protein recognition.[13] Furthermore, the same basic structural motif exists in the glycosides of many bacterial polysaccharides and lipopolysaccharides,[14] the most well-known being the 3-deoxy-D-*manno*-2-octulosonic acid (KDO) linkages (II) of lipid A; a deaminated version of sialic acid (KDN), in which the acetamido group is replaced by a hydroxyl group, is also known.[15]



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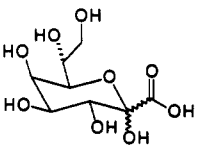

II

We therefore now report an investigation of the pathways for the nonenzymic hydrolyses of aryl glycosides (I) of *N*-acetylneuraminic acid. The α-anomeric configuration shown is the configuration of the sialyl linkage which occurs naturally.

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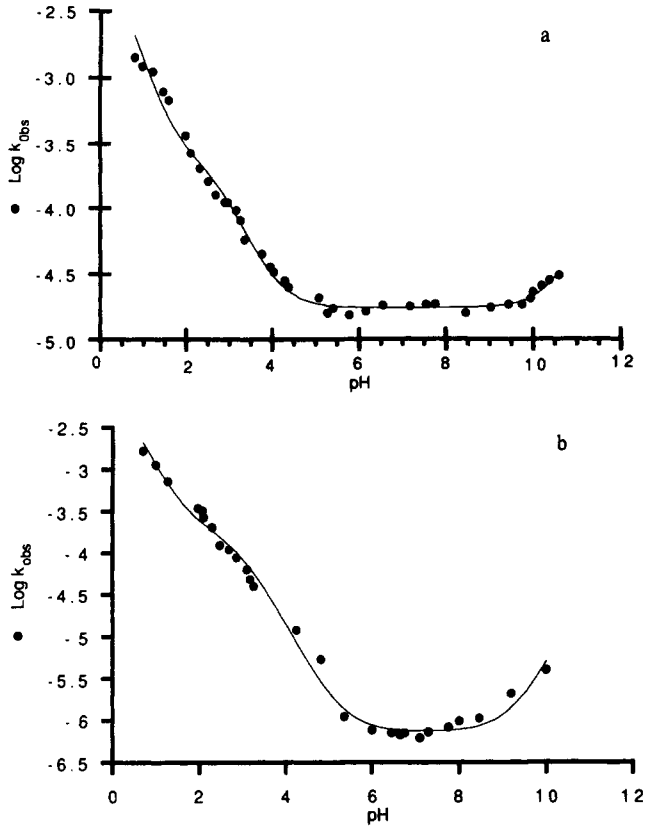

Figure 1. Dependence of first-order rate constant for the hydrolysis of aryl α-glycosides of *N*-acetylneuraminic acid on pH at 50.0 °C, ionic strength maintained at 0.3 M with sodium perchlorate. (a) Data for the *p*-nitrophenyl glycoside. (b) Data for the *p*-cyanophenyl glycoside.

Results and Discussion

Figure 1a displays the observed first-order rate constant for the liberation of *p*-nitrophenol from its *N*-acetylneuraminide (I), as measured as a function of pH at 50 °C. Data refer to 0.300 M buffer solutions derived from neutral, monobasic acids, with an ionic strength maintained at 0.300 M with sodium perchlorate. The continuous line is the least-squares best fit to eq 1.

$$\log k_{obs} = \log \{k_{H^+}[H^+]/(1 + K_a/[H^+]) + k_0/(1 + K_a/[H^+]) + k_-/(1 + [H^+]/K_a) + k_{OH^-}K_w/[H^+](1 + [H^+]/K_a)\} \quad (1)$$

The value of pK_a derived from this least-squares best fit (2.85) corresponds, within experimental error, to the pK_a value (2.86) obtained from the half-neutralization point of the glycoside itself. We can therefore discern that *p*-nitrophenol is liberated by four processes: (1) the acid-catalyzed reaction of the neutral molecule, with $k_{H^+} = 1.18 \times 10^{-3}$ M^{-1} s^{-1}; (2) the spontaneous reaction of the neutral molecule, or its kinetic equivalent, the proton-catalyzed reaction of the anion derived by ionization of the C-1 carboxylic acid group, with $k_0 = 2.27 \times 10^{-4}$ s^{-1}; (3) the spontaneous reaction of the anion, with $k_- = 1.74 \times 10^{-5}$ s^{-1}; and (4) a base-catalyzed process, with $k_{OH^-}K_w = 3.87 \times 10^{-16}$ M s^{-1}.

We could not detect buffer catalysis at pH 2.69 or 6.75; k_{obsd} in 0.150, 0.300, and 0.600 M buffer was, respectively, 0.95, 1.07, and 1.05×10^{-4} s^{-1} at pH 2.69 and 1.91, 1.81, and 1.66×10^{-5} s^{-1} at pH 6.75. The acidic site in the neutral acid buffer at pH 6.75 was a tertiary amine (DIPSO), so that the experiment at this pH merely serves to establish that measured first-order rate constants do not contain a general-acid-catalyzed term. The acid at pH 2.69 was malonic, so that the null result here (where the dibasic acid has a statistical advantage that will make general-acid catalysis easier to observe) is reasonably compelling than any Brønsted α value will be close to unity.

The products at pH 1.00, 2.69, and 6.75 were *N*-acetylneuraminic acid and *p*-nitrophenol only; those at pH 13 were *p*-nitrophenolate, *N*-acetylneuraminic acid, and a second carbohydrate moiety, which was also unstable.

The data for the *p*-cyanophenyl glycoside (Figure 1b) can be fit with $pK_a = 2.90$, $k_{H^+} = 9.8 \times 10^{-4}$ M^{-1} s^{-1}, $k_0 = 1.81 \times 10^{-4}$ s^{-1}, $k_- = 7.36 \times 10^{-7}$ s^{-1}, $k_{OH^-}K_w = 4.29 \times 10^{-16}$ M s^{-1}. These data provide reassurance that the four pH regimes do not alter markedly with leaving group pK_a, except for a narrowing of the region for the k_- process.

The nature of the transition states in processes 1–4 was examined with the aid of solvent deuterium isotope effects and β_{lg} values and in processes 1–3 with the aid of secondary deuterium kinetic isotope effects. Deuterium was specifically introduced at high enrichment into the *p*-nitrophenyl glycoside I at C-4, into both positions at C-3, and also into the *pro-R* and *pro-S* positions at C-3 individually (in the $^2C_5$ conformation in which glycoside I is drawn, the *pro-R* hydrogen is axial). These effects were measured by the isotopic quasi-racemate method,[16] which requires that chiral products be stable under the reaction conditions; for this reason, we were not able to measure small secondary effects on the pH 13 process, since reducing sugars are not stable to alkali. We confirmed, however, that there was no detectable change in the optical rotation of a solution of *N*-acetylneuraminic acid for a period of 4–5 half-lives of the hydrolysis reaction at pH 1.0, 2.69, and 6.75.

Solvent isotope effects at pH 1.0 or 13.0 refer to 0.100 M concentrations of $HClO_4$ or NaOH in H_2O and D_2O (those at pL 2.69 and 6.75 from corrected pH meter readings). Rates measured at 1.00, 0.67, 0.33, and 0.00 atom fraction deuterium gave solvent isotope effects (expressed as k_{H_2O}/k_{D_2O}) of 1.36, 0.96, 0.86, and 1.71 at pH 1.00, 2.69, 6.75, and 13.0, respectively. These effects are too small to be able to detect curvature of the proton inventories with the precision of the data available. Measurements at pL 6.59 at 70.0 °C gave both a solvent isotope effect of 0.90_8 and also activation parameters $\Delta H^{\ddagger} = 142$ kJ mol^{-1} and $\Delta S^{\ddagger} = 48$ J mol^{-1} K^{-1} for the pH-independent process.

Plots of k_{obsd} versus leaving group pK_a for a series of five aryl glycosides of *N*-acetylneuraminic acid at pH 1.00, 2.69, 6.67, and 13.0 are shown in Figure 2a–d. The derived β_{lg} values are -0.0_1, 0.01_6, -1.3_2, and -0.17, respectively.

The combination of solvent isotope effects and β_{lg} values enables the broad molecular nature of processes 1–4 to be determined. The acid-catalyzed hydrolysis of aryl pyranosides,[1] and of those aryl furanosides that react by initial exocyclic cleavage,[2] has long been known to give rise to β_{lg} values that are near zero or slighly positive, since the buildup of positive charge occasioned by protonation of the leaving group oxygen atom is almost offset by the buildup of negative charge arising from C–O cleavage. Generally, the form of acid catalysis has been assumed to be specific for glycosides, but Rosenberg and Kirsch[17] pointed out that their leaving group ^{18}O kinetic isotope effect on the acid-catalyzed hydrolysis of *p*-nitrophenyl β-glucopyranoside was too big for the oxygen atom to be fully protonated, even if the C–O bond was fully cleaved, and they proposed a general-acid-catalyzed mechanism. This suggestion was shown to be correct when both this effect and the solvent deuterium kinetic isotope effect were found to have an anomalous temperature dependence,[18] with the solvent effect changing sense around 50 °C.

The measured solvent isotope effect of 1.36 for the proton-catalyzed hydrolysis of the neutral molecule is thus in accord with the precedent of *p*-nitrophenyl β-glucopyranoside. The difference in solvent isotope effect (1.36 versus 1.00 at 50 °C) is small, but in a sense is in accord with proton transfer being marginally earlier than with the glucoside. The β_{lg} value for the acid-catalyzed hydrolysis of β-glucopyranosides is positive ($+0.27 \pm 0.04$),[1,4] differing from the (within experimental error zero) β_{lg} value for

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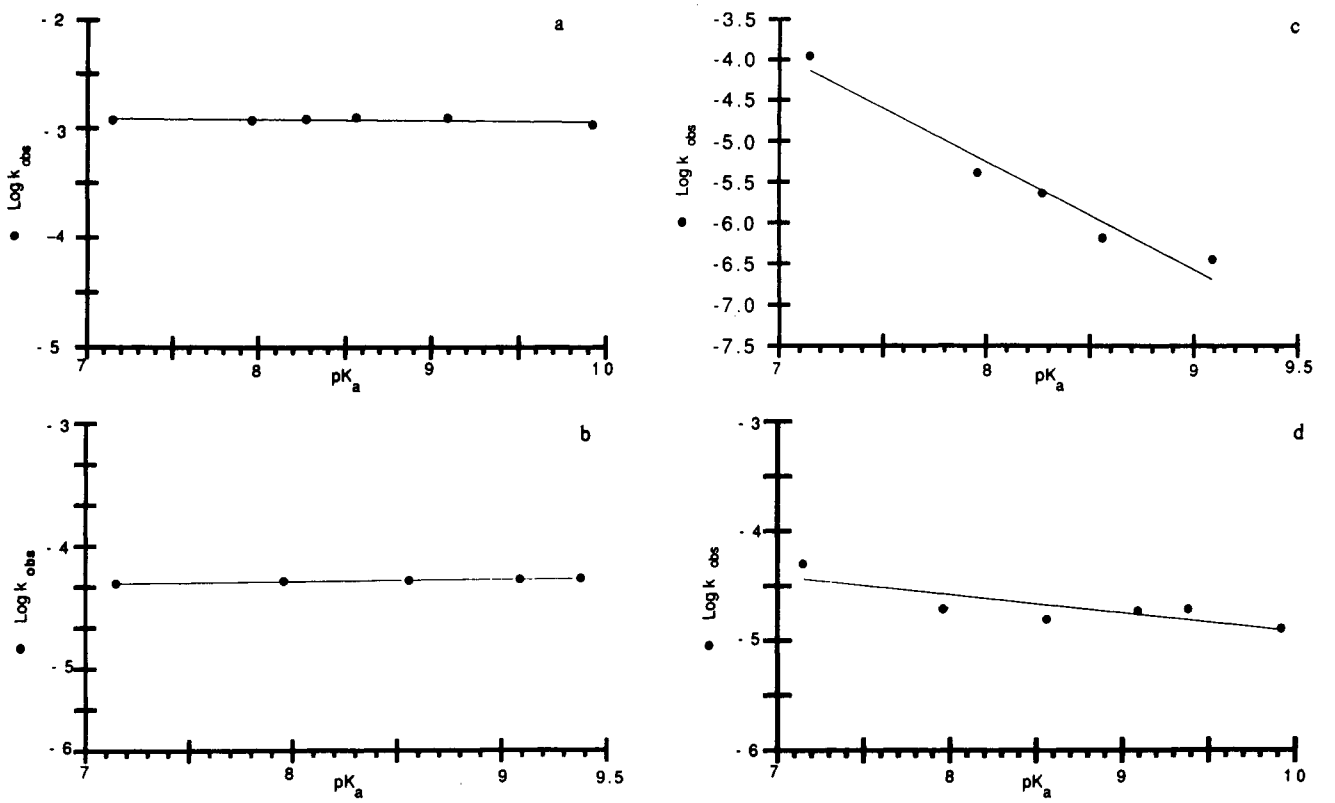
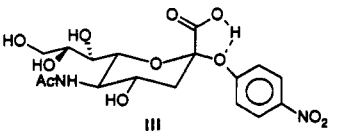


Figure 2. Dependence of the first-order rate constant for hydrolysis of aryl α-glycosides of *N*-acetylneuraminic acid upon the p*K* of the parent phenol. The phenols are as follows, in order of increasing pK_a: *p*-nitrophenol (7.15), *p*-cyanophenol (7.96), *m*-nitrophenol (8.27), 3,4-dichlorophenol (8.56), *m*-chlorophenol (9.09), *p*-chlorophenol (9.38), and phenol (9.92). (a) pH 1.0 at 50.0 °C. (b) pH 2.6 at 50.0 °C. (c) pH 6.67 at 60.0 °C. (d) pH 13 at 50.0 °C.

the proton-catalyzed reactions of neutral aryl *N*-acetyl-α-D-neuraminides.

The pH-independent reaction of the monoanion of aryl neuraminides (process 3) clearly has close parallels with the pH-independent reaction of aryl tetrahydropyranyl ethers: the β_{lg} values are similar (-1.3_2 compared with -1.18 ± 0.4,[9] respectively). However, there is a real but small difference in the solvent isotope effect on the reactions with *p*-nitrophenolate as the leaving group in the two cases. For the simple tetrahydropyranyl derivative the effect is direct ($k_{H_2O}/k_{D_2O} = 1.1$).[19] A small direct effect is to be expected if there is any hydrogen-bonding solvation to the anionic leaving group which is not present in the neutral molecule, and it is unremarkable for this reason. For the anion of the sialic acid glycoside, however, the effect is the inverse, 0.86. There is no reason to suppose that hydrogen bonding to the departing leaving group should be any less important in this reaction than in the tetrahydropyranyl case. If this is so, then some other process giving rise to an inverse solvent isotope effect of around 0.7_8 must be involved. It is known that the protons in hydrogen-bonded systems XO–H···O⁻–Y have fractionation factors of less than unity (in suitable cases as low as 0.3).[20] Therefore, if the C-1 carboxylate group were to participate in the displacement reaction as a nucleophile, an inverse contribution to the net solvent isotope effect would be expected, arising from the release of a water molecule from hydrogen bonding to the carboxylate into bulk water. Stable α-lactones are known,[21] but all that we propose at this stage is that there is some participation by the C-1 carboxylate in the displacement reaction, sufficient to weaken a carboxylate–water hydrogen bond. The positive entropy of activation for the spontaneous reaction of the monoanion is further circumstantial evidence for release of hydrogen-bonded water into bulk solvent—the value (48 J mol^{-1} K^{-1}) being significantly higher than that for tetrahydropyranyl *p*-nitrophenyl ether (9.2 ± 2.5 J mol^{-1} K^{-1}).[19]

The apparent spontaneous reaction of the neutral molecule (process 2) could arise from participation of the un-ionized carboxylic acid as an intramolecular general acid directly, as in structure III, through a water chain, or from the proton-catalyzed reaction of the anion. A distinction between these two kinetically equivalent transition states can be made on the basis of the absence of an appreciable solvent isotope effect at pL 2.69 (k_{H_2O}/k_{D_2O} = 0.96). This rules out the mechanism of structure III (and a fortiori proton transfer through a water chain involving more transferred protons), since the directly comparable formic acid catalyzed hydrolysis of tetrahydropyranyl *p*-nitrophenyl ether exhibits an isotope effect of 3.4.[19] Even though the proximity of the proton-catalyzed regime 1 and "spontaneous" regime 2 constrained us to work at constant pL rather than constant buffer ratio in measuring this solvent isotope effect, it is unlikely that the solvent isotope effect on substrate pK_a disguises a solvent isotope effect of this magnitude on the bond-breaking process: an increase of the substrate pK_a by the usual 0.4 units[58] (from 2.86 to 3.26) on transfer to D_2O would by itself give rise to an inverse effect of 0.76 at pL 2.69. The true solvent isotope effect on process 2 would then be 1.31, in line with the solvent isotope effect on the acid-catalyzed reaction of the neutral molecule.



III

The β_{lg} value for the acid-catalyzed hydrolysis of the anion differs little from that for the acid-catalyzed hydrolysis of the neutral molecule, in accord with the processes being essentially similar. Two-parameter analysis of the rates of hydrolysis of mixed acetals, which involve the initial departure of substituted salicylic acids, suggests that intramolecular general-acid catalysis by

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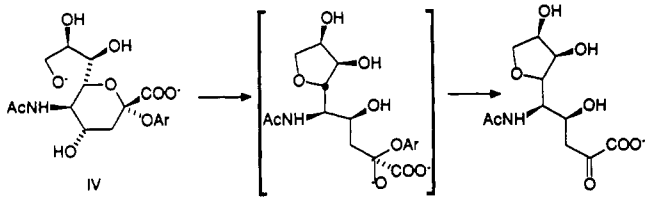
COOH of aryl acetal hydrolysis is associated with a β_{lg} value of −0.4.[22] The observed value of β_{lg} of 0.01_6 for this process is thus further evidence against reaction via III.

The observation of a base-catalyzed pathway for liberation of phenolate from all five aryl glycosides (I) was a considerable surprise. Base-catalyzed hydrolyses of aryl glycosides usually proceed via intramolecular nucleophilic attack of a suitably placed ionized hydroxyl on the anomeric center,[4,23,24] yet there appears to be no suitably disposed group in I to so participate. In the specific case of the nitrophenyl glycoside, nucleophilic attack on the aromatic ring by hydroxide ion is well-precedented,[25] and there is evidence that this pathway accounts for some of the *p*-nitrophenol and all of the *N*-acetylneuraminic acid liberated from glycoside I in base. Figure 2d shows that the β_{lg} value for the base-catalyzed hydrolyses is not really well defined, with the point for *p*-nitrophenol lying above the line defined by the other four points. The solvent isotope effect establishes that *p*-nitrophenol is indeed liberated by two processes with different temperature coefficients since, although it is 1.71 at 50.0 °C, it is 1.25 at 70.0 °C, and such a temperature dependence cannot be accounted for by a single chemical event. We therefore suggest that some of the base-promoted liberation of *p*-nitrophenolate arises from an S_N2Ar reaction at C-1 of the *p*-nitrophenyl ring. In accord with this, base-catalyzed hydrolysis of the phenyl glycoside, which cannot react by an S_N2Ar pathway, yields no *N*-acetylneuraminic acid.

Any mechanism for generation of phenolate other than by attack of hydroxide at the aromatic ring must account for the shallow dependence of rate for this process on leaving group ability (Figure 2d). As the reaction is carried out at pH 13, protonation of the leaving phenol is very unlikely, so that under these conditions the low β_{lg} value can be taken as evidence that the C–OAr bond is unlikely to be broken in the rate-determining stage. The absence of a primary kinetic isotope effect on the base-catalyzed reaction of the *p*-nitrophenyl compound consequent upon deuterium substitution at position 3($k_{H_2}/k_{D_2} = 1.1_5$[59]) also precludes any major contribution from an eliminative mechanism yielding 2,3-dehydro-*N*-acetylneuraminic acid.[60] A mechanism which fits the facts now available is that the ionized C9 hydroxyl group attacks C6 as a nucleophile in an intramolecular S_N2 reaction and opens the pyranose ring, expelling the anion of a hemiacetal of pyruvate and the phenol. This rapidly loses phenolate to yield a β-hydroxy ketone, which is rapidly degraded under the experimental conditions (pH 13 and 50 °C).

The pK_a of the anomeric hydroxyl group of glucose is 12.3,[26] and the loss of sugar hemiacetal anions as leaving groups is precedented in the well-known base-catalyzed removal of O-linked carbohydrate links of glycoproteins in mild base. We therefore very tentatively suggest the process (IV) as a pathway for the cleavage of glycosides of *N*-acetylneuraminic acid in base.



IV

The structure of the transition states for processes 1–3, our main concern, was further probed by measurements of β- and γ-deuterium kinetic isotope effects on the hydrolysis of the *p*-nitrophenyl compound at pH 1.00, 2.69, and 6.67. Results of individual quasi-racemate runs are given in Table I. With existing polarimetric equipment, the optimum reaction time for isotopic quasi-racemate measurements is dictated within fairly narrow limits by instrumental integration time and stability, so that the effects at pH 1.00 had to be measured at 30 °C and the effects at pH 6.67 at 60 °C. If the effects are classical, i.e., $T \ln k_H/k_D$ = constant, then the consequence of this change in temperature is less than the error in the measurement of the effects. Effects calculated on individual runs are reproducible to about 10% of the effect; also, the effects of the *pro-R* and *pro-S* deuteriums are, as they should be for secondary effects, additive. Thus, at pH 1.0 the β-effect arising from two deuteriums (mean 1.10_2) is, within experimental error, the product (1.09_5) of the mean effects of the *pro-R* deuterium (average 1.06_8) and the *pro-S* deuterium (average 1.02_5). Where no effect is recorded, we estimate that an effect of around 0.5% could have been detected (see the Experimental Section for more details). Specimen primary data are displayed in Figure 3a–e.

Table I. Secondary Isotope Effects k_H/k_D for *p*-Nitrophenyl *N*-Acetylneuraminide

3-substituent	pH 1.00 at 30 °C KIE	av	pH 2.69 at 50 °C KIE	av	pH 6.67 at 60 °C KIE	av
pro-R d_1	1.07_0	1.06_8	1.08_5	1.08_6	1.07_5	1.07_3
	1.06_7		1.08_4		1.07_6	
	1.06_6		1.08_8		1.07_0	
pro-S d_1	1.02_5	1.02_5	1.00_0	1.00_0	1.00_0	1.00_0
	1.02_7		1.00_0		1.00_0	
	1.02_3		1.00_0		1.00_0	
d_2	1.10_6	1.10_2	1.08_5	1.08_2	1.07_6	1.07_5
	1.09_3		1.08_5		1.07_1	
	1.10_6		1.07_8		1.07_8	
4-d_1	0.97_3	0.97_6	0.99_9	1.00_0	1.00_0	1.00_0
	0.98_0		1.00_0		1.00_0	
	0.97_6		1.00_0		1.00_0	

β-Deuterium kinetic isotope effects have their origin in two phenomena, hyperconjugation and the inductive effect of deuterium, and can be considered in terms of eq 2.[27] The first term

$$\ln (k_H/k_D) = \cos^2 \theta \ln (k_H/k_D)_{max} + \ln (k_H/k_D)_i \quad (2)$$

represents hyperconjugation of the C–L σ-orbital with an electron-deficient p orbital on an adjacent carbon atom; θ is the dihedral angle between the C–L bond and this orbital. $\ln (k_H/k_D)_{max}$ is the maximal hyperconjugative effect obtained when the C–L bond and the p orbital are exactly eclipsed and increases as the positive charge on the adjacent carbon atom increases, with the associated weakening of the C–L bond.

The second term in eq 2 represents a small, geometry-independent inductive deuterium effect. It is found experimentally that deuterium has apparently a small electron-donating inductive effect, but there is no consensus as to its theoretical origin (one view is that the anharmonicity of the C–L bond vibration, even in the zeroth vibrational state, results in the center of electron density of the C–L σ-bond lying slightly more toward the carbon atom in the case of the C–D bond than the C–H bond).

The present measurements were in part an attempt to address the ambiguities inherent in a single β-deuterium kinetic isotope effect measurement, in that one has one experimental parameter (the effect) and three unknowns (θ, $(k_H/k_D)_{max}$, and $(k_H/k_D)_i$). In this system, however, the only estimate that has to be made empirically is that of $\ln (k_H/k_D)_i$. The effects for each of the pair of diastereotopic hydrogen atoms at C-3 give us two simultaneous equations and two unknowns for the hyperconjugative portion of the effect:

$$\ln (k_H/k_{D_R}) = \cos^2 \theta \ln (k_H/k_D)_{max} \quad (3)$$

$$\ln (k_H/k_{D_S}) = \cos^2 (\theta + 120°) \ln (k_H/k_D)_{max} \quad (4)$$

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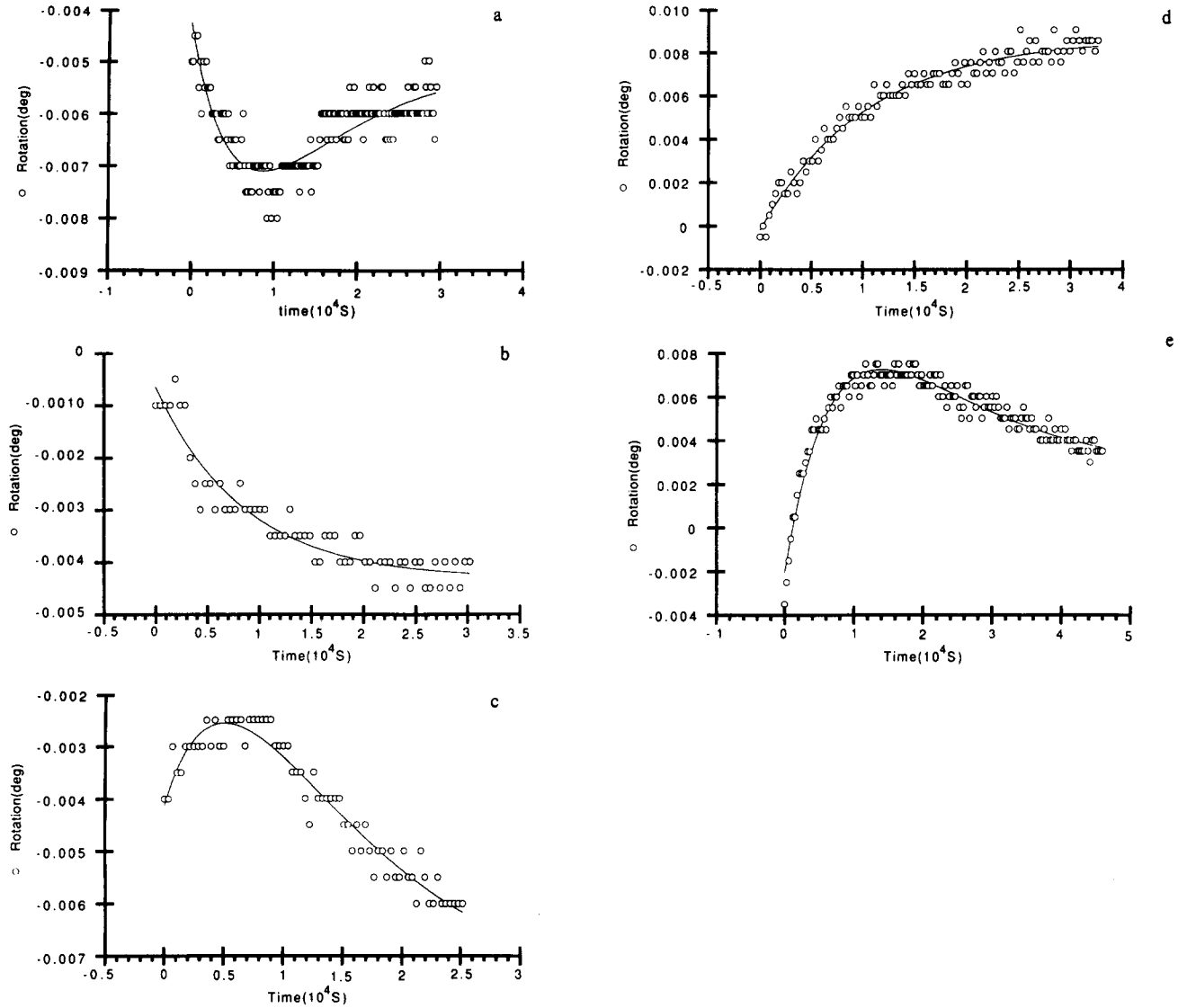
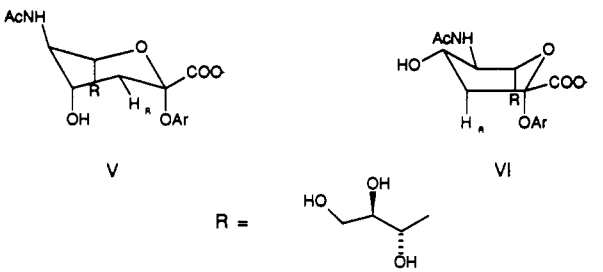


Figure 3. Specimen isotopic quasi-racemate runs in the hydrolysis of *p*-nitrophenyl α-*N*-acetylneuraminide: (a) 4-d_1 at pH 1.0; (b) 4-d_1 at pH 2.6; (c) 3-*pro-S* at pH 1.0; (d) 3-*pro-S* at pH 6.75; (e) 3-d_2 at pH 6.75.

Since the inductive contribution to the effect is geometry independent, qualitative conclusions about the geometry of the transition states of all three processes can immediately be reached from the relative magnitudes of the effects for the *pro-R* and *pro-S* hydrogens. In the $^2C_5$ preferred conformation of the ground-state α-*N*-acetylneuraminide, the leaving group is not antiperiplanar to either of the sp^3 lone pairs on oxygen; nonetheless, the β-deuterium kinetic isotope effects are significantly bigger for the *pro-R* deuterium than for the *pro-S* deuterium. This is in accord with transition states in which the $^2C_5$ conformation is distorted toward the $^4H_5$ conformation expected for the sialyl cation: a transition state, in short, similar to that deduced for the acid-catalyzed hydrolysis of methyl β-glucopyranoside.[8] (In the $^2C_5$ conformation itself, the dihedral angles made by C3–H_R and C3–H_S with the C2–OAr bond are approximately equal, but in the $^4H_5$ half-chair conformation of the cation, θ is small for the C3–H_R bond and close to 90° for the C3–H_S.)

Significantly, all transition states derived from the alternate $^5C_2$ chair conformation and some of the skew-boat pseudorotational itinerary in which the leaving group is antiperiplanar to an oxygen lone pair can be rejected on arguments based simply on the relative magnitudes of the effects from the two diastereotopic hydrogens. In the $^5C_2$ chair (V) there is an oxygen lone pair antiperiplanar to the leaving group, but the *pro-S* hydrogen at C3 is antiperiplanar to it whereas the *pro-R* hydrogen makes a dihedral angle of about 60°. Distortion toward the $^5H_4$ half-chair expected for the cation would tend to equalize the *pro-R* and *pro-S* effects,



but not reverse their magnitudes.

On the skew-boat pseudorotational itinerary, there is a lone pair on the oxygen atom approximately antiperiplanar to the leaving group in the region $^{4,0}B \leftrightarrow {}^2S_4 \leftrightarrow B_{2,5} \leftrightarrow {}^3S_5$. The necessary planarity of C6, O6, C2, and C3 in the cation, however, means that these conformations must distort to either $^{2,6}B$ or $B_{2,6}$ (or a half-chair) in the transition state. Only the $^{4,0}B$ conformation (VI) permits a transition state in conformity to the antiperiplanar lone pair hypothesis and the present β-deuterium kinetic isotope effects. The $B_{2,5}$ and $^3S_5$ conformations have the *pro-S* hydrogen at C3 antiperiplanar to the leaving group, and the $^2S_4$ conformation is such that the C–OAr bond would make a dihedral angle of approximately 30° with the C3–H_R bond and about 150° with the

(28) Kresge, A. J.; Weeks, D. P. *J. Am. Chem. Soc.* **1984**, *106*, 7140.

C3–H_S bond, from which approximately equal results would be expected. On the basis of these considerations alone, therefore, the only possible transition-state conformation that accords with ALPH in this system is some derivative of a ground-state $^{4,0}B$ conformation (VI). This conformation has the leaving group and the trihydroxypropane side chain both pseudoaxial, so that the conformation must be very high in energy, if not prohibitively so.

To locate θ more precisely requires an estimate of ln $(k_H/k_D)_i$. A guide is given by the data of Kresge and Weeks,[28] who found a kinetic isotope effect (k_H/k_D) of 0.965 in pure water, and 0.935 in 50% aqueous dioxane, on the acid-catalyzed hydration of CH_2=CLOEt. The initial step is the generation of the oxocarbonium ion without a change in hybridization at the cationic center: the effect is therefore largely inductive. If it is assumed that the inductive effect of deuterium, like other inductive effects, is attenuated by a factor of approximately 0.4 per carbon–carbon bond, then these data provide a precedent for ln $(k_H/k_D)_i$ for an acetal hydrolysis of –0.0.27 to –0.014. Williams[29] estimated the inductive contribution to the equilibrium isotope effect for $(CL_3)_2C$–X = $(CL_3)_2C^+ + X^-$ in the gas phase from ab initio MO calculations (RHF/4-31G level). The inductive component of the overall effect was calculated to be variable: ln $(k_H/k_D)_i$ per deuterium being –0.07 for X = F and –0.041 for X = H_2O^+. From these calculations, and the doubling of the effect on the change of the solvent from pure water to 50% aqueous dioxane in Kresge and Weeks' experiments,[28] it does appear that the inductive effect depends in a major way on comparatively minor details of the reaction under consideration and that it can be surprisingly large. Sunko's group[30] reported the kinetic effect of an equatorial deuterium β to an equatorial leaving group in the acetolysis of a conformationally restricted cyclohexyl brosylate to be k_H/k_D = 0.944. Just as the inductive contribution to β-deuterium kinetic isotope effects appears variable, so the size of γ-effects (which should be about 0.4 of the inductive component of β-effects) appears very variable. Sunko et al.[31] reported direct effects of around 1–2% per endo deuterium on the acetolysis of 7-norbonyl triflate, which they attributed to relief of steric strain, whereas Gregoriou and Varveri[32] failed to detect any kinetic isotope effect at all in the aqueous trifluoroethanolysis and aqueous hexafluoroisopropanolysis of 4,4-dideuterio-2-octyl brosylate.

The purpose of the introduction of deuterium at C4 was to provide an estimate of ln $(k_H/k_D)_i$ directly in this system. No detectable effect was found at pH 2.69 or 6.67, and therefore at these two pH values the inductive component of the β-deuterium kinetic isotope effects is probably small. On the basis that it is, it is possible to deduce that, at both pH values, the dihedral angle θ is 30° and that the value of $(k_H/k_D)_{max}$ is within experimental error (1.09_8 at pH 6.67 and 1.11_5 at pH 2.69). The value of θ is most plausibly accommodated if the conformation of the sugar ring is a flattened $^2C_5$ chair conformation, in which the C1 carboxylate group is participating in the displacement reaction.

The 2.3% inverse γ-deuterium effect at C4 at pH 1.0 is large but not unprecedentedly so: on the assumption that inductive effects are attenuated by a factor of 0.4 per carbon–carbon bond, it corresponds to an estimate for ln $(k_H/k_D)_i$ of –0.058, exactly in line with the experimental β-deuterium effect[30] on the acetolysis of the conformationally restricted cyclohexyl brosylate. Calculation of the values of θ and $(k_H/k_D)_{max}$ using this value of ln $(k_H/k_D)_i$ gives values of 57° and 1.5, respectively. Since the hyperconjugative component of the β-deuterium effect of a CL_3 group is $^3/_2$ ln $(k_H/k_D)_{max}$, the calculations of Williams[29] on the equilibrium β-deuterium effects for the generation of the isopropyl cation from various precursors are germane to the question of whether the $(k_H/k_D)_{max}$ value of 1.5 is unrealistically high. The maximum value of $(k_H/k_D)_{max}$ compatible with Williams' calculations is 1.39, suggesting that the estimate of ln $(k_H/k_D)_i$ in our system is indeed too high and that therefore there is some conformational component to the deuterium effect at C4. In cyclohexane itself, the equatorial conformation of deuterium is favored by 8.3 ± 1.5 cal mol^{-1},[33] corresponding to an equilibrium isotope effect of 1.2%, but in some cyclohexanones an axial deuterium conformation is favored by the same factor.[34]

If no correction is made for $(k_H/k_D)_i$, then at pH 1.0 θ is estimated to be 52° and $(k_H/k_D)_{max}$ to be 1.19. The value of θ is thus insensitive to estimates of the inductive component of the β-deuterium kinetic isotope effect; moreover, even if there is assumed to be no inductive effect, the value of ln $(k_H/k_D)_{max}$ is nearly double that for the reactions of the anion. This immediately means that the electron deficiency of the reaction center is much higher at pH 1.0 than at pH 2.69 or 6.67, where the substrate carboxylate is ionized. Moreover, $(k_H/k_D)_{max}$ measure the electron deficiency of a particular p-type orbital, not of the atom as a whole, and therefore the different inductive effects of COOH and COO^- are not relevant, since they are exerted through the σ-framework, orthogonal to the orbital in question. The very different value of $(k_H/k_D)_{max}$ for reactions of the anion, then, establishes that this p-type orbital is much less electron deficient when the carboxylate group is ionized.

There are two possible reasons for this: that the carboxylate group is participating nucleophilically or that the transition state for reactions of the anion is earlier than that for the reactions of the neutral molecule. The large, negative β_{lg} value (–1.3) for the spontaneous reaction of the anion, however, indicates a transition state that is absolutely very late. If the transition state for the acid-catalyzed reaction of the anion were earlier than that for the acid-catalyzed reaction of the neutral molecule, then the former process should be characterized by a more positive β_{lg} value, if the degree of proton transfer to the leaving group is the same. The failure to observe buffer catalysis at pH 2.69 even with malonic acid suggests that any Brøsted α value is close to unity, so that it is reasonable to assume that the degree of proton transfer in the two acid-catalyzed reactions is indeed comparable. Therefore, their identical β_{lg} values are evidence against an earlier transition state for the reaction of the anion.

Whereas the solvent isotope effect data described in the first part of this discussion merely suggested a desolvation of the ionized carboxylate group in the transition state for the reactions of the anion, our β-deuterium kinetic isotope effects require this ionized carboxylate of the substrate to participate as a nucleophile in the hydrolysis of the *p*-nitrophenyl glycoside, despite the highly strained α-lactone that results. This form of neighboring group participation has a long-standing, indeed classical, precedent in the hydrolysis of 2-bromopropionate ion,[35] but the precedent refers to nucleophilic displacement at an unactivated secondary position. The present work shows that such participation is effective in a displacement which, in its absence, would only require the generation of a 2-deoxyglycosyl cation, a comparatively stable species which is likely to have a real existence in water.

That nucleophilic participation by a carboxylate can occur, even when an α-lactone would result and even when the reaction center would of itself give a relatively stable oxocarbonium ion, has immediate implications for the catalytic mechanism, not only of sialidases but of all those glycosidases which yield the product sugar in the same anomeric configuration as the substrate. The paradigmal retaining glycosidase is lysozyme, and the Vernon model for the glycosyl enzyme intermediate, an ion pair of a glycosyl cation and the side-chain carboxylate of Asp 52, has survived[36] in the face of accumulating evidence that, in general, such intermediates are covalent. This evidence now includes site-directed mutagenesis of the homologous aspartate in bacteriophage T4 lysozyme to a cysteine to yield a mutant enzyme

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with a neutral nucleophile and near-wild-type catalytic activity,[37] α-deuterium kinetic isotope effects on the hydrolysis of glycosyl enzymes in the wrong sense for reaction of an sp^2-hybridized cation with a nucleophile,[38] the isolation and characterization of a covalent 2-deoxyglucosyl enzyme,[39] and various covalent 2-fluoro-2-deoxyglycosyl enzymes (including one shown to be catalytically competent).[40]

The supposition on which the Vernon model was based was that in a system in which a covalent bond could be formed between a glycosyl cation and a carboxylate group only at the expense of considerable strain then the stabilization by the carboxylate must be electrostatic. Model building into the D (catalytic) subsite of lysozyme, on the basis of the X-ray crystal structures of lysozyme and its chitooligosaccharide complexes,[41] suggested that this enzyme was just such a system. More recently, Strynadka and James[42] have refined the structure of the complex of lysozyme and the cell-wall trisaccharide (NAM.NAG.NAM) to 1.5-Å resolution. In this complex subsite D, the catalytic site is occupied by a NAM residue. In a careful analysis of the structure, it was possible to discern that $O^{\delta 2}$ of Asp 52 could approach the anomeric carbon to within 2.3 Å simply by a change of dihedral angles ψ^1 ane ψ^2 of the aspartate residue, but that closer approach would disrupt the hydrogen-bonded network in which $O^{\delta 1}$ was embedded. Moreover, attach would have to be by the lone pair on $O^{\delta 2}$ anti to $O^{\delta 1}$, in conflict with stereoelectronic ideas about the different basicities of *E* and *Z* lone pairs of carboxylates.[43] On these grounds, Strynadka and James on balance favored the original Vernon proposal of an electrostatically stabilized glycosyl cation rather than a covalent intermediate.

The strain energy of an α-lactone exceeds by a large but unquantified amount the 114 kJ mol^{-1} of strain energy of oxirane[44] and is well in excess of the energetic cost of disrupting any plausible hydrogen-bonded system in an enzyme; nonetheless, nucleophilic participation by the C1 carboxylate group in reactions of *N*-acetylneuraminides happens. Additionally, such participation must involve attack by a supposedly less basic anti lone pair of the carboxylate, in accord with recent work which has indicated that the difference in the kinetic basicity of *E* and *Z* carboxylate lone pairs is minimal.[45] Our results, then, undermine the assumptions which led Strynadka and James, and previous workers, to favor an ion-paired glycosyl enzyme intermediate in lysozyme catalysis.

The sialidase of influenza virus has been crystallized, and a full X-ray structure has been published.[46] A site-directed mutagenesis study of this enzyme led to a proposed mechanism in which there was no enzymic nucleophile approaching the reaction center from the β-face of the sugar ring; rather, Glu 277, on the α-face of the ring, was considered to stabilize the neuraminyl cation electrostatically.[47] Our present results can bring these ideas in line with chemical precedent by allowing the carboxylate group of the substrate to act as the nucleophile.[48] Participation by substrate carboxylates in reactions catalyzed by those glycosidases whose substrates possess them (such as pectate and alginate hydrolases, in addition to sialidases) should perhaps be considered as a general mechanistic possibility.

Very recently the acid-catalyzed hydration of vinyl ethers containing an α-carboxylic acid group—such as methoxyacrylic acid—has been studied.[49] The process, like the hydrolysis of glycosides of *N*-acetylneuraminic acid, formally generates an oxocarbenium ion with an α-carboxylate. Ionization of the carboxylate group accelerates the rate of hydration of these vinyl ethers by about 3 orders of magnitude, whereas the second-order rate constant for acid-catalyzed hydrolysis of *p*-nitrophenyl *N*-acetylneuraminic acid anion (1.61×10^{-1} M^{-1} s^{-1}) is only 2 orders of magnitude greater than that for hydrolysis of the neutral molecule. The stereochemistry of the two reactions is however very different: the C–COOH bond in the vinyl ether system starts off in the plane of the oxocarbenium species and in the *N*-acetylneuraminide case is perpendicular to it.

Experimental Section

Buffer Systems. The neutral acid–sodium hydroxide buffer systems were used between the following pH values as indicated: 2.06–3.48, malonic acid; 4.0–4.31, succinic acid; 4.46–5.36, acetic acid; 5.5–6.30, 2-(*N*-morpholino)ethanesulfonic acid (MES); 7.0–8.2, 3-[*N*,*N*-bis(2-hydroxyethyl)amino]-2-hydroxy-1-propanesulfonic acid (DIPSO); 9.0–10.3, 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (CAPSO); 10.6–11.13, 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS). Buffer pH values were measured at room temperature, and then for each buffer system the temperature correction for the buffer acid pK_a, obtained by measuring the pH difference between room temperature and 50 °C, was applied. The temperature correction for carboxylic acid based buffers was negligible, but that for amine-based buffer systems was substantial; however, these buffers were used exclusively to define the pH-independent portion of the curve. From pH 0.8 to 1.9 perchloric acid–sodium perchlorate was used, and at pH 13, sodium hydroxide–sodium perchlorate was used; the pH was calculated from stoichiometric quantity of strong acid or base. The ionic strength was maintained at 0.3 M with sodium perchlorate.

Product Analysis. This was carried out by HPLC (Spectraphysics system) for solutions at pH 1.0 and 2.69, using an RP 18 column, elution by water at a flow rate of 0.2 mL min^{-1}, and refractive index detection. *N*-Acetylneuraminic acid was identified by comparison with a genuine sample. *N*-Acetylneuraminic acid could also be detected by its absorbance at 220 nm, so that UV detection, together with TLC, was used for studies on the phenyl glycoside and initial studies on the *p*-nitrophenyl glycoside.

Measurements of First-Order Rate Constants. These were measured in 1-cm path length cuvettes in a Perkin-Elmer Lambda 6 spectrophotometer, fitted with a Peltier effect thermostated cell block. Wavelengths used were 340 nm for the *p*-nitrophenyl compound below pH 7 and 400 nm above it, 262 nm for the *p*-cyanophenyl compound below pH 7 and 280 nm above it, 340 and 220 nm for the phenyl and *m*-nitrophenyl compounds below pH 7, and 280 nm for all other runs. Data points were fitted to eq 5, using the nonlinear least-squares fitting program Kaleidagraph (version 2.0) (Abelbeck software, bought from Synergy (PCS Inc.)) run on a Macintosh SE30 personal computer. It was confirmed in the case of the *p*-nitrophenyl glycoside at pH 1.00, 2.69, and 6.67 that the rate constant obtained polarimetrically corresponded to the rate constant obtained spectrophotometrically.

$$A = A_0 + B(1 - e^{-kt}) \quad (5)$$

Kinetic Measurements by Polarimetry. A JASCO DiP-370 photoelectric polarimeter was fitted with jacketed cells through which water, thermostated by water circulated from a Fisher "Isotemp" water circulator, was passed. Depending on the absorbance of the solution, sodium light (λ = 589 nm) or the mercury line at 546 nm was used. Notwithstanding its electronic sophistication, we find this instrument to be far

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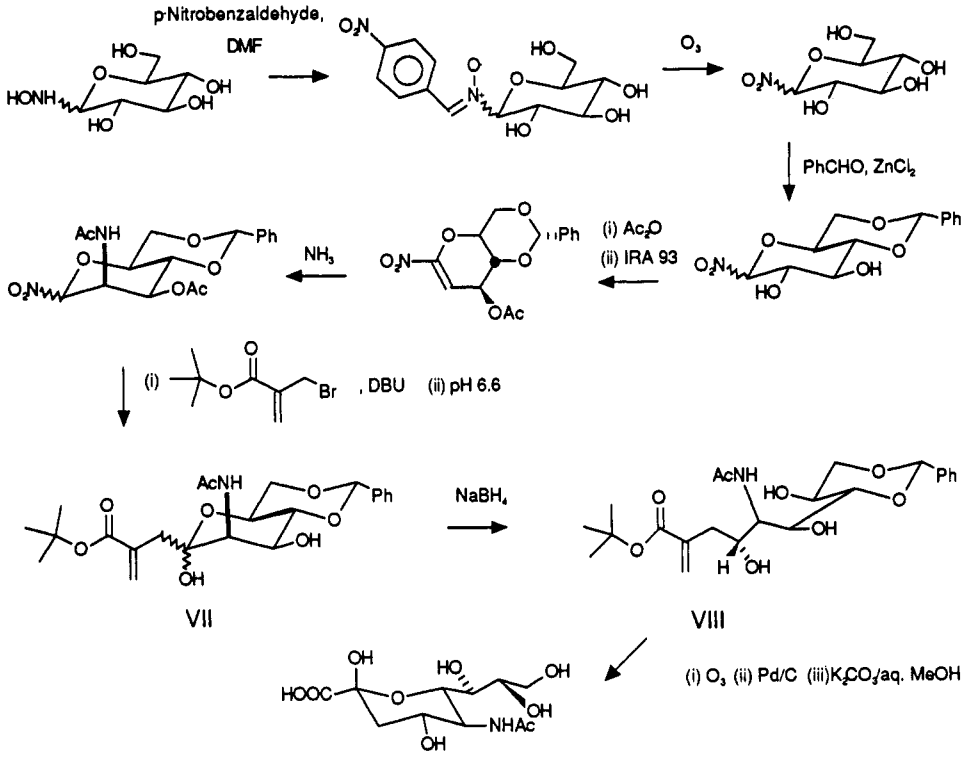
(48) The stereochemistry of the reaction catalyzed by the influenza enzyme appears to be unknown. It is known to be retention for the *Clostridium perfringens* and *Arthrobacter ureafaciens* enzymes (Friebolin, H.; Brossmer, R.; Keilich, G.; Ziegler, D.; Supp, M. *Hoppe-Seyler's Z. Physiol. Chem.* **1980**, *361*, 697), but hydrophobic cluster analysis of the gene sequences puts the *C. perfringens* and influenza neuraminidases in different classes (Henrissat, B. *Biochem. J.* **1991**, *280*, 309), so that no assumptions about stereochemistry can be made.
(49) Kresge, A. J.; Leibovitch, M.; Sikorski, J. A. *J. Am. Chem. Soc.* **1992**, *114*, 2618.

Scheme I



less satisfactory for measurements of kinetic isotope effects by the isotopic quasi-racemate method than the Perkin-Elmer 241 MC instrument used previously.[8] The manufacturer's stated noise level of 0.0005° can with care be achieved, but it is sensitive to perturbation by temperature and the exact alignment of the lamp and with small sample volumes necessitates the use of an expensive "high-intensity" mercury lamp with a short design lifetime.

Calculation of Isotopic Effects. Experimental time courses of optical rotation of an isotopic quasi-racemate in the course of a reaction were fitted to eq 6, using Kaleidagraph, with good initial estimates of A and k_H from measurements in a separate experiment. The fits to such an

$$\alpha = Ae^{-k_H t} + Be^{-k_D t} + C \qquad (6)$$

expression if the parameters are completely undetermined are very ill-conditioned, but the isotopic quasi-racemate technique allows close initial estimates to be made. It was checked visually that the experimental points were evenly distributed about the calculated "best fit" line and also that $A \approx -B$. Previous curve-fitting procedures we had used[8] treated A, B, and k_1 as largely or completely indetermined. This was necessitated by the programs then available, but of course made unnecessary demands on the data.

If exactly equal concentrations of labeled D- and unlabeled L-glycoside are present at the beginning of the run and there are no isotope effects on molar optical rotations, then the size of the maximum in optical rotation (Π) observed is given approximately by

$$\Pi = (\delta k)\Delta\alpha/2.7k$$

where $\Delta\alpha$ is the total change in optical rotation for one antipode, and the rate constants for labeled and unlabeled substrates are $k + \delta k$ and k, respectively. It was checked that the computed effect corresponded to the effect estimated visually from this approximation.

***N*-Acetyl-L-neuraminic Acid.** This was made from L-glucose, essentially following the route described by Vasella et al.[50-52] for the D enantiomer, as set out in Scheme I. However, we found it advantageous to make a few minor modifications. As was recognized by Beer et al.,[50,53] better overall yields from glucose are obtained by benzylidenating the nitro compound, rather than making the nitro compound from, 4,6-*O*-benzylideneglucose;[51] this is an important consideration in the L series. Moreover, in our hands the reaction of glucose oxime with *p*-nitrobenzaldehyde sometimes worked as described[50] and sometimes failed, for unknown reasons. We found the following procedure more consistent.

***N*-(*p*-Nitrobenzylidene)-β/α-glucopyranosylamine *N*-Oxide.** Finely ground *p*-nitrobenzaldehyde (8.52 g, 1.1 equiv) was dissolved in undistilled DMF (50 mL) and added to a rapidly stirred suspension of glucose oxime (10.0 g) in DMF (100 mL). A yellow solution was formed after 5 min, and the stirring was reduced. The product began to crystallize after 5 min. After an additional 10 min, diethyl ether (200 mL) was added, and stirring was continued for another 5 min. The product (15.4 g, 92%), pure enough for the subsequent step (ozonolysis to 1-deoxy-1-nitro-β-glucopyranose[50]), was filtered off and dried under vacuum.

4,6-*O*-Benzylidene-1-deoxy-1-nitro-β/α-D-glucopyranose. Benzaldehyde (55 mL) was stirred with powdered anhydrous $ZnCl_2$ (23.4 g) until it had "set". 1-Deoxy-1-nitro-β-glucopyranose (22.5 g) as a slurry in benzaldehyde (21.0 mL) was added, with cooling. After 5 h at room temperature, 20% aqueous sodium bisulfite (12 mL) was added. The mixture was extracted with chloroform (2 × 50 mL), and the chloroform was washed with brine, dried (Na_2SO_4), and evaporated. The residue was chromatographed on silica gel with light petroleum ether/ethyl acetate (5:2 → 1:1) as eluent to give, after recrystallization from acetone/hexane, the product (15.5 g, 49%) as a mixture of predominantly the β anomer, which was carried through the sequence[51] until the stereochemistry was destroyed in the elimination to give the nitroglucal, whose properties were identical to those reported.[51]

***N*-Acetyl-L-neuraminic acid** as prepared by this route had $[\alpha]^{21}_D$ $+30.9°$, $31.9°$, $33.0°$ (*c* 1.2, 1.4, 1.6, H_2O) and was spectroscopically identical to the natural D material ($[\alpha]^{24}_D$ −32° (*c* 1, H_2O).

Introduction of Isotopes. [4-^{2}H]-*N*-Acetyl-D-neuraminic Acid. The synthesis of *N*-acetylneuraminic acid was optimized in the D series, which also enabled us to introduce deuterium at position 4. To a solution of *tert*-butyl 5-acetamido-7,9-*O*-benzylidene-2,3,5-trideoxy-2-methylidene-D-*manno*-4-nonulosonate (VII) (3.36 g) in 4:1 (v/v) dioxane/water (57 mL) at 0 °C was added a solution of $NaBD_4$ (0.915 g) and acetic acid (0.858 mL) in the same solvent (168 mL) over 28 h with stirring. After workup as described,[51] the crude *tert*-butyl 5-acetamido-7,9-*O*-benzylidene-2,3,5-trideoxy-2-methylidene-D-*glycero*-D-*galacto*-nononate (VIII), contaminated with <7% of its C4-epimer, was ozonolyzed. *tert*-butyl 5-acetamido-7,9-*O*-benzylidene-3,5-dideoxy-[4-^{2}H]-D-*glycero*-D-*galacto*-2-nonulosonate was chromatographed twice on silica gel with a gradient of methanol in chloroform (20:1 → 10:1). ^{1}H NMR (400 MHz, ($(CD_3)_2SO$) of this compound and its unlabeled analogue revealed that the multiplet at δ 3.93–3.83 (H4, H5, and H6) was simplified and

(50) Beer, D.; Bieri, J. H.; Macher, I.; Prewo, R.; Vasella, A. *Helv. Chim. Acta* **1986**, *69*, 1172.
(51) Baumberger, F.; Beer, D.; Chisten, M.; Prewo, R.; Vasella, A. *Helv. Chim. Acta* **1986**, *69*, 1191.
(52) Baumberger, F.; Vasella, A. *Helv. Chim. Acta* **1986**, *69*, 1205.
(53) The better overall yields obtained by this route are mentioned in the discussion section of ref 50, which cites Christen, M. Diplomarbeit, Universität Zürich, Zürich, Switzerland, 1985.

reduced in intensity and that the doublet of doublets at δ 1.97 and 1.58 (*pro-S* and *pro-R* H3) became simple doublets in the deuterated compound.

Incorporation of Deuterium at Position 3.[54] A solution of *N*-acetyl-D-neuraminic acid (0.50 g) in D_2O (10 mL) was adjusted to pD 11.6 (pH meter reading 11.2) by addition of 40% NaOD in D_2O. After 3.5 h at room temperature, the ^{1}NMR signal at δ 1.70 had disappeared, but the signal at δ 2.15 remained unchanged in intensity. Dowex 50x8-100 cation exchanger (H^+ form) was added, the suspension was stirred, the resin was filtered off and washed, and the filtrate and washings were lyophilized to yield a material which was used directly for production of methyl ([3-*pro-R*-^{2}H]-5-acetamido-3,5-dideoxy-β-D-*glycero*-D-*galacto*-nonulosyl chlorid)onate.

After 48 h at pD 11.6 both signals of the C3-bound protons of *N*-acetylneuraminic acid had disappeared, but no signs of decomposition were apparent, and the dideuterio compound was obtained on workup as above. The specifically *pro-S* deuterated compound was made by back-exchanging the dideuterated compound in water at pH 11.2 for 5 h. One proton (by NMR integration) could be incorporated without the *pro-S* proton signal becoming apparent.

Synthesis of Glycosides. Samples of *N*-acetylneuraminic acid were converted to the fully acetylated methyl ester by literature procedures[55] and thence to methyl 5-acetamido-3,5-dideoxy-β-*glycero*-*galacto*-nonulosyl chlorid)onate by reaction with acetyl chloride for 24 h at room temperature.[56] The glycosyl chlorides, used without purification, were converted to the various fully protected aryl neuraminides under the phase-transfer conditions of Rothermel and Faillard.[57] After chromatography on silica gel (ethyl acetate as eluant) fully protected glycosides were obtained in 50-60% yields from the chloride. Zemplén deacetylation[56] gave the aryl *N*-acetyl-α-neuraminide methyl esters (methyl(aryl 5-acetamido-3,5-dideoxy-α-*glycero*-*galacto*-2-nonulosid)onates), which were crystallized and characterized. Portions of the methyl esters were hydrolyzed[57] as required; the fully deprotected glycosides are unstable.

It was advantageous to keep all nitrophenyl glycosides in the dark.

Comparison of the 3-H proton resonances in unlabled *p*-nitrophenyl *N*-acetyl-α-neuraminide by ^{1}H NMR (400 Mz, CD_3OD; *pro-R* δ 1.89 (dd, apparent t, *J* = 12.0, 12.0 Hz), *pro-S* δ 2.97 (dd, *J* = 12.0, 3.0 Hz) with those of the deuterated analogues failed to reveal any back-exchange of deuterium under the alkaline conditions of the glycosidation reaction (0.1 M NaOH), in which production of glycoside by elimination to the acetylated 2,3-dehydroneuraminic acid methyl ester, followed by addition of *p*-nitrophenol, was a possibility.

Characterization Data of Samples of *p*-Nitrophenyl *N*-Acetyl-α-neuraminide. The compounds were repeatedly recrystallized from methanol/ether, between room temperature and -50 °C. Melting points are with decomposition (and hence particularly sensitive to trace impurities); rotations refer to 0.1% solutions in methanol: L, mp 134-136 °C, $[\alpha]^{25}_D$ -73.5°; D-[4-^{2}H], mp 134-136 °C, $[\alpha]^{25}_D$ +73.5°; D-[3-$^2H_2$], mp 134-136 °C, $[\alpha]^{25}_D$ +73.5°; D-[3-*pro-R*-^{2}H], mp 133.5-135.5 °C, $[\alpha]^{25}_D$ +74.5°; D-[3-*pro-S*-^{2}H], mp 134-137 °C, $[\alpha]^{25}_D$ +74.0°. Eschenfelder and Brossmer[46] report mp 113-115 °C, $[\alpha]^{25}_D$ +69° for the unlabeled compound in the D series.

Characterization Data of Methyl (Aryl 5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-nonulopyranosid)onates. The methyl esters were recrystallized from ether/petroleum ether and the free acids from methanol/ether (neither with heating). Rotations refer to 0.1% solutions in methanol. *m-Nitrophenyl*: mp 101.1-102.4 °C; $[\alpha]^{25}_D$ +9.1°; MS $M^{\cdot+}$, 444. Anal. Calcd for $C_{26}H_{32}N_2O_{15}$: C, 50.98; H, 5.27. Found: C, 50.63; H, 5.19. Free acid mp 116-118 °C. *p-Cyanophenyl*: mp 105.4-106.4 °C, $[\alpha]^{25}_D$ +34.5°; MS $M^{\cdot+}$, 411. Anal. Calcd for $C_{27}H_{32}N_2O_{13}$: C, 53.87; H, 5.36. Found: C, 53.09; H, 5.46. Free acid mp 126-128 °C. 3,4-*Dichlorophenyl*: mp 102.4-103.9 °C, $[\alpha]^{25}_D$ +47°; MS $M^{\cdot+}$, 468. Anal. Calcd for $C_{26}H_{31}Cl_2N_2O_{13}$: C, 49.06; H, 4.91. Found: C, 48.99; H, 4.93. Free acid mp 134.5-136.5 °C. *m-Chlorophenyl*: mp 101.8-102.9 °C; $[\alpha]^{25}_D$ +7.5°; MS $M^{\cdot+}$, 433. Anal. Calcd for $C_{26}H_{32}ClNO_{13}$: C, 51.87; H, 5.36. Found: C, 51.78; H, 5.40. Free acid mp 114-115 °C. *p-Chlorophenyl*: mp 104.3-105.4 °C; $[\alpha]^{25}_D$ +54°; MS $M^{\cdot+}$, 433. Anal. Calcd for $C_{26}H_{32}ClNO_{13}$: C, 51.87; H, 5.36. Found: C, 51.65; H, 5.20. Free acid mp 124-126 °C. *Phenyl*: mp 104-107 °C (lit.[57] mp 102-106 °C); $[\alpha]^{25}_D$ +2.0°; MS $M^{\cdot+}$, 399. Anal. Calcd for $C_{26}H_{33}NO_{13}$: C, 55.02; H, 5.86. Found: C, 54.41; H, 5.88. Free acid mp 140-142 °C (lit.[55] mp 141-143 °C).

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Biosynthesis of Acivicin. 3. Incorporation of Ornithine and N^δ-Hydroxyornithine[1]

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Abstract: The biosynthesis of the antibiotics acivicin (**1**) and 4-hydroxyacivicin (**2**) has been studied in *Streptomyces sviceus*. Initial experiments identified ornithine (**11**) as the primary precursor, rather than glutamic acid or glutamine. Incorporation of [5-^{13}C,^{15}N]ornithine established the retention of the ω-amino nitrogen. Incorporation of [2,3,3-$^2H_3$]-, [3,3,4,4-$^2H_4$]-, [3*R*-^{2}H]-, and [3*S*-^{2}H]ornithines revealed the complete loss of H-2, replacement of the 3*S* hydrogen with the isoxazoline oxygen, and partial loss of the 3*R* hydrogen. Incorporation of $^{18}O_2$ revealed the derivation of the isoxazoline oxygen and the 4-hydroxyl group of **2** from molecular oxygen, although partial exchange of the former had occurred. N^δ-Hydroxyornithine (**18**), rather than either *erythro*- or *threo*-β-hydroxyornithine, was shown to be the first committed intermediate in the pathway. [^{14}C]-**1** was metabolized to [^{14}C]-**2**, but the reverse did not occur. A linear pathway from **11** through **18** to **1** and then to **2** is proposed to account for these findings.

Acivicin (AT-125) (**1**)[2,3] and 4-hydroxyacivicin (**2**),[4] antibiotics produced by *Streptomyces sviceus*, are members of a small group of metabolites containing an isoxazolidine ring at various levels of oxidation. Acivicin has potent anticancer activity[5] and has found

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