Leaving Group ^{18}O Kinetic Isotope Effects on the Nonenzymic Hydrolyses of *p*-Nitrophenyl *N*-Acetyl-α-neuraminide

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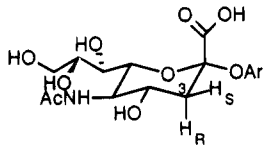
Introduction

We recently undertook a study of the mechanisms of the nonenzymic hydrolyses of aryl glycosides of *N*-acetylneuraminic acid of the α-(naturally-occurring)-configuration (D-enantiomer: structure **1**).[1] We found that four processes were operative in aqueous solution at different pH values: the acid-catalyzed hydrolysis of the neutral molecule, the acid-catalyzed hydrolysis of the anion, the spontaneous reaction of the anion, and a base-catalyzed, probably nonhydrolytic process at pH > 13.



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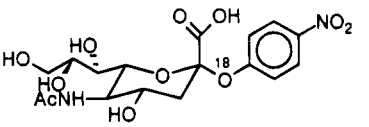
An examination of the isotope effects arising from deuterium substitution at position 3 of the *p*-nitrophenyl compound on its hydrolyses by the first three processes (which all gave *N*-acetylneuraminic acid as product) revealed that the reactive conformation was derived by flattening of the ground-state $^2C_5$ chair, and that some form of nucleophilic participation took place if the C1 carboxylate group was ionized. The chromophoric nitrophenol leaving group is widely used in enzymology, and ^{18}O kinetic isotope effects on its departure can indicate whether this process is wholly or partly rate-determining, and also, if the intrinsic isotope effect can be estimated, they can provide information about transition state structure.[2-5] Isotope effects on nonenzymic processes are important in providing a benchmark for these investigations. We therefore now report measurement of the leaving group ^{18}O kinetic isotope effects on the hydrolysis of *p*-nitrophenyl *N*-acetyl-α-neuraminide (^{18}O-labeled D-enantiomer: structure **2**) at three pH values: pH 1.00, where the process being observed is the H_3O^+-catalyzed hydrolysis of the neutral molecule; pH 2.69, where the process being observed is the H_3O^+-catalyzed hydrolysis of the anion; and pH 6.69, where the process being observed is the spontaneous reaction of the anion. As



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with the previous study of β-deuterium kinetic isotope effects, the ^{18}O effects were measured by the isotopic quasiracemate method,[6] in which the optical rotation of an equimolar solution of the labeled D-enantiomer and unlabeled L-enantiomer is followed over the course of several half-lives of the hydrolysis reaction. This method and our instrumentation impose fairly narrow limits on the absolute value of the rate constant for which isotope effects can be measured, so that it is unfortunately necessary to measure the isotope effects for the different regimes at different temperatures.

Results and Discussion

The results of three independent measurements of the isotope effect under each of the three regimes, together with literature precedents, are given in Table 1.

It is clear that the values of the effect for the acid-catalyzed processes are almost identical to those for hydrolysis of *p*-nitrophenyl β-glucopyranoside. Rosenberg and Kirsch[7] showed that the value at 50 °C was too high to be consistent with true specific acid catalysis in which the protonated glycoside has a real existence, and suggested that proton-transfer was partly rate-limiting. Prompted by an apparent small discrepancy between this value, measured by a mass-spectrometric method, and that for acid hydrolysis of *p*-nitrophenyl α-arabinofuranoside at 80 °C, measured by the isotopic quasiracemate method,[8] we investigated both the solvent isotope effect, and the leaving group ^{18}O isotope effect for the *p*-nitrophenyl β-glucoside as a function of temperature.[9] We were able to show that the mode of acid catalysis was temperature dependent (the solvent isotope effect changing sense at 50 °C) and that our quasi-racemate ^{18}O effects and Rosenberg and Kirsch's mass spectrometric ones defined a common, non-classical temperature-dependence.

The leaving group effects for compound **2** at pH 1.00 and 2.69 are thus in accord with these two hydrolyses being processes in which the protonated substrate is on the borderline of a real existence, as was the case for the glucoside.

The leaving group isotope effect at pH 6.67 is high, indicating that carbon–oxygen bond rupture is complete at the transition state and that there is no contervailing acquisition of zero-point energy, from, for example, protonation. This is entirely in accord with the steep dependence of the rate of hydrolysis of a series of aryl *N*-acetyl-α-D-neuraminides at this pH with leaving group acidity ($\beta_{lg} = -1.3$).[1] This ^{18}O effect is in fact even marginally higher than the equilibrium isotope effect for ionization of *p*-nitrophenyl galactoside into the galactosyl cation and *p*-nitrophenolate (1.0425) calculated by Rosen-

(1) Ashwell, M.; Guo, X.; Sinnott, M. L. *J. Am. Chem. Soc.* **1992**, *114*, 10158.
(2) Rosenberg, S.; Kirsch, J. F. *Biochemistry*, **1981**, *20*, 3189.
(3) (a) Hosie, L; Sinnott, M. L. *Biochem. J.* **1985**, *226*, 437 (b) Kelly, M. A.; Sinnott, M. L.; Herrchen, M. *Biochem. J.* **1987**, *245*, 843.
(4) (a) Caldwell, S. R.; Raushel, F. M.; Weiss. P. M.; Cleland, W. W. *J. Am. Chem. Soc.* **1991**, *113*, 730 (b) Caldwell, S. R.; Rauschel, F. M.; Weiss, P. M.; Cleland, W. W. *Biochemistry*, **1991**, *30*, 7444.
(5) (a) Guo, X.; Sinnott, M. L. *Biochem. J.* **1993**, *294*, 653. (b) Guo, X.; Laver, W. G.; Vimr, E.; Sinnott, M. L. *J. Am. Chem. Soc.* **1994**, *116*, 5572.
(6) (a) Bergson, G.; Matsson, O; Sjoberg, S. *Chem. Scr.* **1977**, *11*, 25. (b) Tencer, M.; Stein, A. R. *Canad. J. Chem.* **1978**, *56*, 2994. (c) Nadvi, N. S.; Robinson, M. J. T. *Abstracts. 4th. International Symposium on Physical Organic Chemistry*; The Chemical Society, London, 1978; C1, p141
(7) Rosenberg, S.; Kirsch, J. F. *Biochemistry* **1981**, *20*, 3196.
(8) Bennet, A. J.; Sinnott, M. L.; Wijesundera, W. S. S. *J. Chem. Soc. Perkin Trans. 2* **1985**, 1233.
(9) Bennet, A. J.; Davis, A. J.; Hosie, L.; Sinnott, M. L. *J. Chem. Soc. Perkin Trans. 2* **1987**, 581.

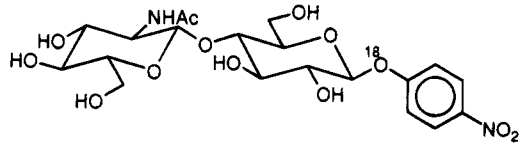
0022-3263/94/1959-7539$04.50/0

Table 1. Leaving Group ^{18}O Kinetic Isotope Effects on the Nonenzymic Hydrolyses of *p*-Nitrophenyl Glycosides

	k_{16}/k_{18} (σ_α(deg), σ_s)[a]	av
this work		
α-*N*-acetylneuraminide, pH 1.00 at 30 °C	1.035 (0.0002, 0.004), 1.035 (0.0002, 0.004), 1.022 (0.0002, 0.002)	1.032 ± 0.008
α-*N*-acetylneuraminide, pH 2.69 at 50 °C	1.026 (0.0006, 0.0026), 1.031 (0.0007, 0.0034), 1.026 (0.0003, 0.0031)	1.028 ± 0.003
α-*N*-acetylneuraminide, pH 6.67 at 60 °C	1.055 (0.0003, 0.0047), 1.053 (0.0003, 0.0050), 1.052 (0.0003, 0.0045)	1.053 ± 0.0016
literature values		
β-D-glucopyranoside, 2 M HCl, at 50 °C[7]		1.0355 ± 0.0015
β-glucopyranoside, 2 M HCl, at 75.1 °C[9]		1.023
at 65.5 °C		1.025[7]
α-arabinofuranoside, 0.1 M $HClO_4$, at 80 °C[8]		1.023 ± 0.003

[a] σ_α is the standard deviation of the experimental points from the best-fit line to eq 1, and σ_s is the standard deviation of the isotope effect calculated from eq 2.

berg and Kirsch.[2] However, these authors themselves have observed an ^{18}O effect for nitrophenolate departure in excess of this (1.0467 ± 0.0015, for hydrolysis of GlcNAcpβ(1→4)Glcpβ-$OpC_6H_4NO_2$ (structure **3**), cata-



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lyzed by hen-egg-white lysozyme[7]). Qualitatively, therefore, it is clear that primary ^{18}O isotope effects of around 5% for the loss of *p*-nitrophenolate from a glycosidic carbon, without proton donation, are precedented, when the transition state is late, as it is in this case, as shown by the β_{lg} value.

More remote precedents for large heavy atom isotope effects for departure of unprotonated leaving groups from carbon are a value of k_{16}/k_{18} of 1.062 for the hydrazinolysis of methyl formate under conditions where expulsion of methoxide is rate-determining,[10] and the value for the ^{18}F isotope effect of k_{18}/k_{19} of 1.026 obtained for the piperidinolysis of 1-fluoro-2,4-dinitrobenzene in tetrahydrofuran by Matsson's group.[11]

Cleland's group has however measured a number of surprisingly low primary effects (<1%) for the departure of *p*-nitrophenolate from phosphorus centers,[4] and Gorenstein *et al.*[12] measured a low 2% isotope effect on the departure of 2,4-dinitrophenolate from the dianion of its phosphate. Very recently, a careful study of primary ^{18}O, secondary ^{18}O, and secondary ^{15}N effects on the hydrolysis of *p*-nitrophenyl phosphate under various pH regimes has been reported by Hengge *et al.*[13] The results for the primary effect in the reactions of the dianion were in line with those of Gorenstein *et al.* and were associated with a substantial secondary ^{15}N effect. Since C–O and P–O bond stretching frequencies are similar, the simplest picture of the physical origin of these effects would predict that the primary leaving group ^{18}O isotope effect would be *greater* for departure from phosphorus than for departure from carbon, since the effect on the reduced mass is greater. This explanation was advanced by Bennet *et al.*[14] to rationalize their 6% leaving group ^{18}O effect on the inactivation of acetylcholinesterase by *p*-nitrophenyl 2-propyl methylphosphonate. Hengge *et al.*[13] rationalized both their low primary ^{18}O and high secondary ^{15}N effects by proposing that the proximity of the negative charge of the metaphosphate moiety increased the weight of the quinonoid resonance structure of the *p*-nitrophenolate ion and thus both increased net bonding at oxygen and decreased it at nitrogen. The much higher primary ^{18}O effects observed for departure of *p*-nitrophenolate from positively-charged carbon qualitatively support this explanation.

Experimental Section

p-Nitrophenyl 2-[^{18}O]-*N*-acetyl-D-neuraminide[5] and *p*-nitrophenyl *N*-acetyl-L-neuraminide[1] have been described, as have the instrumentation and kinetic techniques,[1] although for the present experiments an incurably defective sequence of sodium lamps led us to use the 546 nm mercury line as a light source for all experiments. The data refer to solutions maintained at an ionic strength 0.3 M with sodium perchlorate: at pH 1.00 the solution contained 0.100 M perchloric acid, at pH 2.69 the buffering system was NaOH-malonic acid, and at pH 6.67 NaOH–3-{*N*,*N*-bis(2-hydroxyethyl)amino]-2-hydroxy-1-propanesulfonic acid (DIPSO). The temperature correction to the latter buffer (pH 7.2 at room temperature) was substantial.

Between 3.0 and 4.0 mg/mL of each individual enantiomer were used per run: optical rotation changes per mg/mL concentration of one antipode were 70 mdeg at pH 1.0, 65 mdeg at pH 2.69 and 71 mdeg at pH 6.67. Experimental time courses of optical rotation of an isotopic quasi-racemate in the course of a reaction were fitted to eq 1, using initial values of A and B (optical rotation changes for one enantiomer on complete reaction) measured in a separate experiment, and neglecting fits where A and B altered by more than 10%, or where a "hump" of optical rotation was not discernible.[1]

$$\alpha = A\,e^{-k_L t} + B\,e^{-k_H t} + C \qquad (1)$$

The precision within each individual run was characterized by the standard deviation of optical rotation σ_α and the derived error in the effect σ_s, assuming A and B were invariant.[15] The error in the isotope effect is given by eq 2 where σ_s is the

$$(\sigma_s)^2 \le [\sum_t(\delta\alpha)_t^2 + 2k_H\sum_t|(\delta\alpha)_t(SA_t\,e^{-k_L t} + Bt\,e^{-k_H t})| + (k_H)^2\sum_t(SA_t\,e^{-k_L t} + Bt\,e^{-k_H t})^2]/[(N-1)(\sum_t[Ak_H t]^2\,e^{-2k_L t})] \qquad (2)$$

standard deviation of kinetic isotope effect and $(\delta\alpha)_t = \alpha_i - \alpha_i^{real}$. α_i^{real} is the *i*th point on best fitting curve at time $t = i\Delta t$. S is the kinetic isotope effect (k_L/k_H) and $1\Delta t \le t \le N\Delta t$. The assumption of $\delta k_H \le k_H$ was used in the derivation of eq 2. The second and third terms contribute most of $(\sigma_s)^2$ for a nonbalanced run.

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(10) Sawyer, C. B.; Kirsch, J. F. *J. Am. Chem. Soc.* **1973**, *95*, 7375.
(11) Matsson, O.; Persson, J.; Axelsson, B. S.; Långström, *J. Am. Chem. Soc.* **1993**, *115*, 5288.
(12) Gorenstein, D. G.; Lee, Y.-G.; Kar, D. *J. Am. Chem. Soc.* **1977**, *99*, 2264.
(13) Hengge, A. C.; Edens, W. A.; Elsing, H. *J. Am. Chem. Soc.* **1994**, *116*, 5045.
(14) Bennet, A. J.; Kovach, I. M.; Schowen, R. L. *J. Am. Chem. Soc.* **1988**, *110*, 7892.
(15) Zhang, Y.; Sinnott, M. L.; Bommuswamy, J. *J. Am. Chem. Soc.* **1994**, *116*, 7557.