Solvolysis of the Quinoline 5,6- and 7,8-Oxides: Effect of the Ring Nitrogen

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Abstract: pH-rate profiles for the hydrolysis of quinoline arene oxides were measured in 1:9 dioxane-water at 25 °C (0.1 M $NaClO_4$) and compared with the parent carbocyclic compound naphthalene 1,2-oxide. Unlike naphthalene 1,2-oxide whose hydrolysis rate shows a first-order dependence on hydronium ion concentration (k_H) below pH 5, rates for the quinoline 5,6- and 7,8-oxides show a k_H reaction (0.14 and 1.54 M^{-1} s^{-1} for quinoline 5,6- and 7,8-oxides, respectively), but plateau below pH 2-3. pH-independent rate constants in the low-pH plateau are 2.7×10^{-5} and 1.7×10^{-3} s^{-1} for the 5,6- and 7,8-oxides, respectively. On the basis of studies of the *N*-methyl cation of the 5,6-oxide ($k_{obsd} < 4 \times 10^{-7}$ s^{-1} at pH 1.8), it is concluded that the low-pH plateau is due to lack of reactivity for the N-protonated quinoline oxides. Also unlike naphthalene oxide, the quinoline oxides show a reaction with hydroxide ion (k_{OH} rate) that results in the formation of trans dihydrodiols. In acid, quinoline 5,6-oxide, but not quinoline 7,8-oxide, gives a significant yield (20%) of the trans dihydrodiol product and provides the first example of a benzo-ring arene oxide that forms dihydrodiol product under acidic conditions. Other products of the acid-catalyzed reaction of the 5,6-oxide are 5- (73%) and 6- (7%) hydroxyquinolines. The 7,8-oxide upon solvolysis in acid gave exclusively 8-hydroxyquinoline. The 100-1000-fold decrease in reactivity of the quinoline oxides, relative to naphthalene oxide in the pH range of 1-10 , can be accounted for in terms of the presence of their ring nitrogens and the differences in the pK_a values of these nitrogens.

Unlike naphthalene, its aza analogue quinoline is considered to have significant mutagenic and carcinogenic activity.[1] Both are substrates for the cytochrome P450 system in mammalian liver which converts them to arene oxides.[2] In the case of the larger polycyclic aromatic hydrocarbons, aza substitution can either enhance or diminish tumorigenic activity depending on the position of substitution.[3] Tumorigenic activity of these larger carcinogenic hydrocarbons is generally considered to result from covalent interaction of their metabolically formed bay-region diol epoxides with biopolymers.[4] The present study compares the pH-dependent hydrolysis rate of naphthalene 1,2-oxide (**1**) with those obtained for the quinoline 5,6- (**2**) and 7,8-oxides (**3**) and provides the first examples of the effect of the pK_a of a ring nitrogen on the pH-rate profiles of azaarene oxides.

Results and Discussion

The pH-rate profiles for the arene oxides (Figure 1) were generated from hydrolysis data obtained in 1:9 dioxane-water at 25 °C at ionic strength 0.1 M ($NaClO_4$). As shown previously in other solvents,[5] the rate profile for naphthalene 1,2-oxide is biphasic with both a hydronium ion catalyzed (k_H) and a pH-independent (k_0) route for solvolysis as described by eq 1. In

$$k_{obsd} = k_H a_{H^+} + k_0 \quad (1)$$

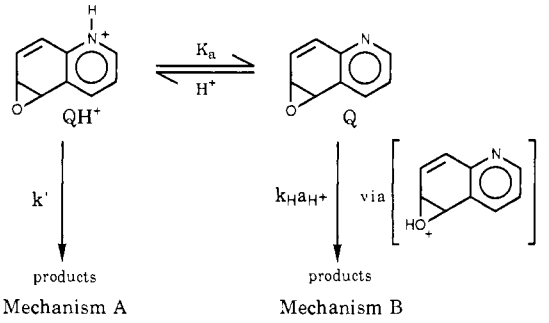
contrast the quinoline oxides (**2**, **3**) exhibit a break in the rate vs pH profiles at pH 3-4. For these compounds, the presence of nitrogen in the adjacent ring allows for the possibility of two mechanisms for the acid-catalyzed reaction as illustrated for the 5,6-oxide (**2**) (Scheme I). Mechanism A consists of a spontaneous or solvent-assisted opening of QH^+ (k') to account for the low-pH plateau whereas mechanism B is a hydronium ion catalyzed (k_H) reaction of neutral Q (via protonation at oxygen) in which QH^+ is unreactive. The rate law for mechanism A at pH 0-5 is given by eq 2 where f_{QH^+} is the fraction of quinoline oxide in the N-protonated form. For mechanism B the rate law is given by eq

$$k_{obsd} = k' f_{QH^+} = \frac{k' a_{H^+}}{a_{H^+} + K_a} \quad (2)$$

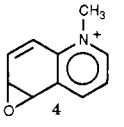
3 where f_Q is the fraction of quinoline oxide that is not protonated at nitrogen. In order to distinguish between these mathematically

$$k_{obsd} = k_H a_{H^+} f_Q = \frac{K_a k_H a_{H^+}}{a_{H^+} + K_a} \quad (3)$$

Scheme I



identical (kinetically equivalent) mechanisms, the *N*-methyl cation of the 5,6-oxide (**4**) was synthesized as a model for the N-protonated 5,6-oxide (QH^+). If mechanism A is correct, then



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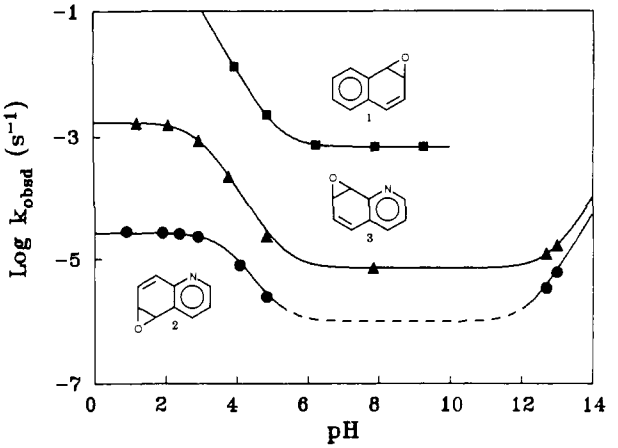


Figure 1. Dependence of the pseudo-first-order rate constants for hydrolysis of **1**, **2**, and **3** on pH in 1:9 dioxane–water at an ionic strength of 0.1 M ($NaClO_4$) at 25 °C. The lines are theoretical curves calculated on the basis of eq 1 (for **1**) and 4 (for **2** and **3**), and the rate constants in Table I. The line for **2** is based on the upper limit for k_0 (broken line at neutral pH) given in the table.

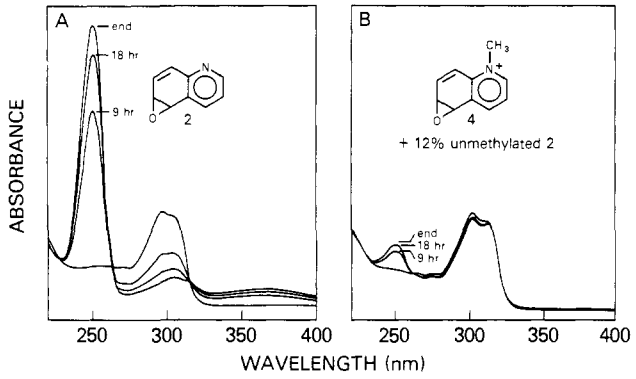


Figure 2. Spectrophotometric comparison of the acid hydrolysis (pH 1.85) of quinoline 5,6-oxide (A) and of its *N*-methyl cation (B) in the kinetic medium. Due to the presence of unmethylated oxide (plot B) an initial reaction was observed with $k_{obsd} = 2.98 \times 10^{-5}$ s^{-1} corresponding to the hydrolysis of the small amount of **2** present.

k_{obsd} for **4** at pH values below neutrality should be essentially that seen for **2** at the low-pH plateau where it is fully protonated (all QH^+). If mechanism B is correct, only the neutral form that has no proton on nitrogen reacts, via general or specific acid catalysis by hydronium ion at the epoxide oxygen, and hence **4** would be kinetically inert. Spectrophotometric comparison of the hydrolyses of **2** and **4** at pH 1.85 (cf. Figure 2) as well as at pH 4.10 revealed **4** to be essentially stable in acid. The k_{obsd} for **4** at pH 1.82 was $< 4 \times 10^{-7}$ s^{-1} (limiting value as measured by HPLC) whereas k_{obsd} for **2** in the low-pH plateau is 2.7×10^{-5} s^{-1} (Figure 1). Thus we conclude that mechanism B is correct for the acid hydrolysis of **2**.

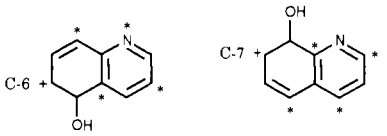
Quinoline 7,8-oxide (**3**) has a pH–rate profile similar to that of the 5,6-oxide (**2**) but is ca. 10–100-fold more reactive both in the acid plateau and in the k_H region. Similarly, a pH-independent reaction (k_0) was observed for **3** ($t_{1/2}$ at pH 7.85 = 27 h), whereas pH-independent hydrolysis was not conclusively demonstrated for **2** but was estimated to be at least ten times slower for **2** than **3**. The leveling off of the pH–rate profile at low pH for both **2** and **3** is a consequence of the pK_a of the ring nitrogen, which when protonated renders the oxides unreactive. Since rates in the acid plateau are determined by the product $k_H K_a$, the higher values of both these constants (Table I) result in **3** being more reactive in this region. The pK_a of the ring nitrogen in the quinoline oxides can be calculated from the kinetics of hydrolysis by the relationship $1/k_{obsd} = 1/k_H a_{H^+} + 1/k_H K_a$ with the negative *x*-intercept of a plot of $1/k_{obsd}$ vs $1/a_{H^+}$ being equal to $1/K_a$. On the basis of product studies described later, arene oxides **1–3** favor opening to the allylic rather than the benzylic carbocation. Most of the

Table I. Rate Constants for Hydrolysis of Arene Oxides in 1:9 Dioxane–Water[a]

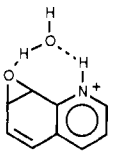
compound	k_H, M^{-1} s^{-1}	k_0, s^{-1}	k_{OH}, M^{-1} s^{-1}	pK_a
naphthalene oxide (**1**)	110	6.8×10^{-4}		
quinoline 7,8-oxide (**3**)	1.54	7.2×10^{-6}	9.8×10^{-5}	2.96[b]
quinoline 5,6-oxide (**2**)	0.14	$< 1 \times 10^{-6}$ [c]	$\sim 6 \times 10^{-5}$	3.72[d]
N-methyl cation (**4**)			3.0	

[a] At 25 °C, ionic strength 0.1 M ($NaClO_4$). [b] Determined kinetically. [c] Estimated from the intercept of a plot of k_{obsd} vs $[c_{OH}]$. [d] Determined by spectrophotometric titration of the 5,6-oxide at 306 nm. A value of 3.79 was determined kinetically.

decrease in k_H for **2** relative to **3** is probably a consequence of decreased stabilization of the major carbocation (C-6) from **2**,



since one of the resonance contributors for this carbocation places positive charge on nitrogen. Such is not the case for the carbocation (C-7) from **3**. Enhanced reactivity of **3** relative to **2** as a result of intramolecular proton transfer from nitrogen to oxygen via one or more water molecules as illustrated below is possible, but it does not appear necessary to explain the observed result.



A probable basis for the increased basicity of **2** relative to **3** (Table I) stems from the ability of N-protonated **2** to delocalize charge into the double bond of the oxirane bearing ring, a resonance contributor not possible for the N-protonated form of **3**.

The reactivity index $\Delta E_\pi/\beta$ from Hückel molecular orbital calculations has been used to estimate the relative ease of cation formation from epoxides in carbocyclic systems.[6] With appropriate adjustments, estimates of this index can also be obtained for analogous nitrogen-containing systems.[7] Values of $\Delta E_\pi/\beta$ calculated[8] for allylic cation formation from **1**, **2**, and **3** are 0.960, 0.919, and 0.960, respectively. Thus, this simple treatment fails to predict any differences in reactivity between naphthalene oxide and **3** although it does qualitatively indicate that **3** should be more reactive than **2**, as is experimentally observed. We suggest that the failure of Hückel molecular orbital calculations to predict the 100-fold decrease in reactivity of **3** relative to **1** results from the fact that positive charge is not delocalized onto nitrogen in the allylic cation derived from **3**, and thus the effect of nitrogen substitution in this compound may be primarily *inductive* in nature.

Although a base-catalyzed rate for naphthalene oxide was not observed up to pH 14 in water,[5] probably due to a relatively high k_0 rate,[9] compounds **2** and **3** undergo facile reaction with hydroxide ion in 1:9 dioxane–water (Table I). Thus, the rate law over the entire pH range is given by eq 4. *N*-Methyl cation **4** is remarkably

$$k_{obsd} = \left(\frac{K_a}{a_{H^+} + K_a}\right)(k_H a_{H^+} + k_0 + k_{OH} a_{OH^-}) \qquad (4)$$

susceptible to reaction with hydroxide ion (pH 9.6–12.4), with a rate ($k_{OH} = 3.0$ M^{-1} s^{-1}, based on a_{OH^-} = antilog (14 − pH))

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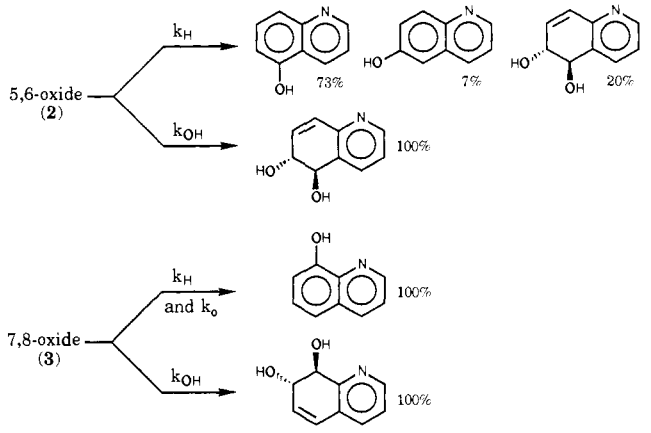
Scheme II



over 50 000 times that of the unmethylated 5,6-oxide (**2**).

Under conditions of both acid-catalyzed (k_H) and spontaneous (k_0) solvolysis, phenols are the exclusive products from **1**. The predominance (98:2) of 1-hydroxy- over 2-hydroxynaphthalene[2a,5] is a reflection of the enhanced stability of the allylic carbocation at C-2 over the benzylic carbocation at C-1. This enhanced stability is presumably derived from the fact that two resonance contributors are possible for the allylic carbocation at C-2 which do not involve the fully aromatic ring, whereas only one such contributor is possible for the benzylic carbocation at C-1. Product distributions from the quinoline oxides are shown in Scheme II. Very much like **1**, quinoline 7,8-oxide (**3**) produces 8-hydroxyquinoline via the comparable allylic carbocation C-7 in both pH ranges. Quinoline 5,6-oxide (**2**) behaves quite differently in acid, where 20% of the product consists of the *trans*-5,6-dihydrodiol. No cis dihydrodiol was detectable by NMR. This is the first example where a benzo-ring arene oxide forms a dihydrodiol under acid conditions, although such products are common from K-region arene oxides in acid.[10] In addition, a significant amount of 6-hydroxy- relative to 5-hydroxyquinoline (7:73) is formed, presumably due to decreased stability of the allylic C-6 cation compared to the benzylic C-5 cation. The allylic carbocation has one reasonance contributor which places positive charge on nitrogen. Simple Hückel molecular orbital calculations[8] also indicated that in all three cases, the allylic cation is favored over the benzylic cation. The differences in E_π between the cations at the two positions varied from 0.140 β (for **2**) to 0.232 β (for **3**) with an intermediate value of 0.181 β for **1**. The larger difference in π energy between the allylic and benzylic cation from **3**, relative to **2**, is consistent with the qualitative predictions as well as the experimental observations described above. The base-catalyzed hydrolysis reaction (k_{OH}) produced exclusively trans dihydrodiol from either quinoline oxide. At 0.1 M sodium hydroxide, a calculated 50% of the observed reaction rate for quinoline 7,8-oxide (**3**) is due to the k_0 process (Figure 1), which produces 8-hydroxyquinoline. The experimentally observed product distribution of 45% *trans*-7,8-dihydrodiol and 55% 8-hydroxyquinoline at 0.1 M base is consistent with exclusive dihydrodiol formation via the k_{OH} process.

In summary, the quinoline oxides **2** and **3** are dramatically less reactive (ca 100–1000-fold) than their naphthalene 1,2-oxide counterpart in the pH range of 1–10. Unique to the quinoline oxides is the presence of a low-pH plateau in their pH–rate profiles where the N-protonated species is unreactive, and an unusual susceptibility to attack by hydroxide ion in a k_{OH} process. Much of their novel chemistry can be understood on the basis of the presence and pK_a of the ring nitrogen.

Experimental Section

Rates of reaction were measured in 1:9 dioxane–water at 25 °C and ionic strength 0.1 M ($NaClO_4$). Dioxane was distilled from sodium and stored frozen. Constant pH was maintained with (1–3) × 10^{-3} M formic, acetic, Tris, MES (4-morpholineethanesulfonic acid), CHES (2-(cyclohexylamino)ethanesulfonic acid), EPPS (4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid), and CAPS (3-(cyclohexylamino)-1-propanesulfonic acid) buffers adjusted to the desired pH with sodium hydroxide. No dependence of the rate on buffer at these buffer concentrations was observed. For kinetic studies of **2** and **3** above pH 12, pH values were estimated by use of the relationship pH = (14 − log c_{OH^-}) where c_{OH^-} is the stoichiometric hydroxide ion concentration. Measured pH values of 2.5–20 mM sodium hydroxide solutions in the kinetic medium agreed within 0.1 pH unit with values calculated from this relationship. Rates of reaction were followed spectrophotometrically on a Cary 219 spectrophotometer and/or by HPLC on a Hewlett Packard 1090 liquid chromatograph. ^{1}H NMR spectra were obtained on a Varian 300-MHz instrument. Quinoline oxides and trans dihydrodiols[2b] as well as naphthalene oxide[11] were synthesized by published procedures.

Quinoline 5,6-Oxide (2). Kinetics. Acid hydrolysis was carried out over a pH range of 0.89 to 4.83, and rates were measured spectrophotometrically at 250 nm. Rates of hydrolysis in base were determined at two concentrations of sodium hydroxide (0.1 and 0.05 M) by following the decrease in the ratio of oxide to *p*-nitrobenzyl alcohol (internal standard) by HPLC on a 4.6 × 250 mm Du Pont Zorbax phenyl column, 0.025 M Tris-OAc (pH 7.5):MeOH, gradient 80:20 to 30:70 in 25 min, 1 mL/min, 298 nm (isosbestic point for the oxide and diol product). Samples (0.5 mL) were quenched with 0.1 mL of 1.0 M BES (*N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid) prior to injection of 0.1 mL. Retention times (min): diol (9.6), standard (19.0), oxide (20.4). A pK_a of 3.72 was determined for the oxide by spectrophotometric titration at 306 nm over a pH range of 1.85 to 5.81, under the kinetic conditions of solvent and ionic strength.

Products. The above chromatographic conditions were used to determine the ratio of the *trans*-5,6-dihydrodiol of quinoline to 5- and 6-hydroxyquinolines produced in an acid hydrolysis mixture at pH 2.37 after 45 h ($6t_{1/2}$). The 5,6-dihydrodiol was shown by UV spectroscopy to be stable under these conditions. Calibration of the chromatograms at 298 nm was achieved by injecting a known molar mixture of the commercially available 5- and 6-hydroxyquinolines, which indicated that the area of integration of the 6-hydroxyquinoline peak must be multiplied by 1.35 in order to reflect the molar ratio of 5- to 6-hydroxyquinoline. A similar experiment, using a mixture of the 5,6-dihydrodiol and 5-hydroxyquinoline that was analyzed by ^{1}H NMR integration, indicated that the area of integration of the 5-hydroxyquinoline peak must be multiplied by 1.56 in order to reflect the molar ratio of 5-hydroxyquinoline to 5,6-dihydrodiol. Thus, in order to determine the product ratios at 298 nm, the area of integration of 5-hydroxyquinoline must be multiplied by 1.56 and that of 6-hydroxyquinoline by 2.11 to determine the molar ratio of 5- and 6-hydroxyquinoline to 5,6-dihydrodiol. The distribution of products from the acid hydrolysis was 7.3% 6-hydroxyquinoline, 19.8% 5,6-dihydrodiol, and 72.9% 5-hydroxyquinoline. As indicated above the kinetics in base were monitored by HPLC at an isosbestic point for the oxide and the 5,6-dihydrodiol and produced a quantitative recovery of the 5,6-dihydrodiol. Base hydrolysis (2 M NaOH) was also used to obtain the 5,6-dihydrodiol on a preparative scale.

Quinoline 7,8-Oxide (3). Kinetics. Acid hydrolysis was carried out over a pH range of 1.20 to 4.83, and rates were measured spectrophotometrically at 250 nm. Rates of hydrolysis in base were determined spectrophotometrically at 253 nm at two concentrations of sodium hydroxide (0.1 and 0.05 M). These rates were confirmed by monitoring the decrease in the ratio of oxide to p-nitrobenzyl alcohol (internal standard) by HPLC (3.9 × 300 mm Waters μBondapak C-18 column, 0.025 M Tris-OAc (pH 7.5):MeOH, gradient 80:20 to 30:70 in 25 min, 1.0 mL/min, 264 nm). Samples were quenched as for the 5,6-oxide. Retention times (min): 7,8-dihydrodiol (5.0) standard (8.9), 7,8-oxide (10.5). The spontaneous hydrolysis rate was measured spectrophotometrically (239 nm) in both 10^{-3} and 2 × 10^{-3} M EPPS buffer at pH 7.85.

Products. Due to the extremely poor chromatographic characteristics of the *trans*-7,8-dihydrodiol of quinoline and of 8-hydroxyquinoline, product determination was carried out after first converting the hydrolysis products to the corresponding acetates with pyridine/acetic anhydride. Acid hydrolysis was carried out with stirring overnight (>$100t_{1/2}$) under the standard kinetic conditions in 0.1 M perchloric acid. The 7,8-dihydrodiol was shown by UV spectroscopy to be stable under these conditions. The solution was neutralized, saturated with salt, and extracted with CH_2Cl_2. The organic phase was dried (Na_2SO_4), solvent was removed under reduced pressure, and the product was acetylated. Product distribution was determined by HPLC (4.6 × 250 mm Astec Cyclobond

(10) Boyd, D. R.; Jerina, D. M. In *Small Ring Heterocycles*, Hassner, A., Ed.; John Wiley and Sons, Inc.: New York, 1985; Vol. 42, Part 3, pp 197–282.

(11) Yagi, H.; Jerina, D. M. *J. Am. Chem. Soc.* **1975**, *97*, 3185–3192.

1 (β-cyclodextran) column, 0.025 M Tris-OAc (pH 7.5):MeOH (70:30), 1.0 mL/min, 285 nm) of the acetates. Authentic 7- and 8-acetoxyquinolines were prepared by standard acetylation (pyridine/acetic anhydride) of commercially available phenols (retention times (min): 8-acetoxy (6.0), 7-acetoxy (8.5)). Structures of the standards were confirmed by NMR. The only compound detected from the acid hydrolysis reaction was 8-acetoxyquinoline. A mixture of the 8-acetoxyquinoline and *trans*-7,8-diacetoxy-7,8-dihydroquinoline obtained from base hydrolysis was chromatographed on the cyclodextran column eluted with 85:15 (0.025 M Tris-OAc (pH 7.5):MeOH). This resulted in a separation of the enantiomers of the 7,8-diacetoxy derivative from 8-acetoxyquinoline (retention times (min): enantiomer 1 (8.5), enantiomer 2 (9.5), and 8-acetoxy (10.5)).

Base hydrolysis was carried out under the standard kinetic conditions in 0.1 M NaOH (3.2 mg of oxide in 50 mL) for 66 h ($5.7t_{1/2}$). The solution was then neutralized, saturated with salt, and extracted with EtOAc. The organic phase was dried (Na_2SO_4), solvent was removed under reduced pressure, and the products were acetylated. The 300-MHz ^1H NMR spectrum (CD_3COCD_3) of the mixture revealed a product ratio of 55:45 (8-acetoxyquinoline:*trans*-7,8-diacetoxy-7,8-dihydroquinoline).

***N*-Methylquinoline 5,6-Oxide Cation (4). Synthesis.** Reaction of the 5,6-oxide with excess dimethyl sulfate in 1 M phosphate buffer at pH 7.4 for 1.5-2.0 h gave a product with a UV spectrum very similar to that of the protonated but unmethylated oxide. Mass spectral data were obtained on a Californium-252 PDMS after dissolving in MeOH and electrospraying onto an aluminized Mylar film: m/z 160 (M^+), 144 ($-CH_4$ or O), and 142 ($-H_2O$).

Kinetics. Acid hydrolysis of **4** was attempted at pH 1.85 and 4.10 under standard conditions and was monitored spectrophotometrically (220-400 nm) as illustrated in Figure 2. The spectrophotometric experiment at pH 1.85 showed a very small increase in absorbance at 250 nm, which was attributed to a 12% contamination with unmethylated oxide. For solutions with an initial absorbance of 1 at the λ_{max} ca. 300 nm, the change in absorbance at 250 nm was 1.16 for unmethylated **2** and 0.14 for **4** contaminated with **2**; equal extinction coefficients at λ_{max} for **4** and protonated **2** were assumed. As further evidence that the N-methylated oxide (**4**) was stable at acidic pH (1.82), the reaction was monitored by HPLC (4.6 × 250 mm Du Pont Zorbax phenyl column, eluted with 0.05 M NH_4OAc (pH 5.00):CH_3CN, gradient 80:30 to 20:70 in 25 min, at 1 mL/min, 301 nm). No significant change (≤6%) in the ratio of N-methylated oxide (rt 5.8 min) to *p*-nitrobenzyl alcohol (internal standard, rt 12.4 min) was observed over 66.5 h, indicating that the N-methylated oxide is essentially stable in acid ($k_{obsd} < 4 \times 10^{-7}$ s^{-1}). Base hydrolysis was carried out over a pH range of 9.64 to 12.35 and monitored spectrophotometrically at 270 nm.

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Hydrogen/Deuterium Fractionation Factors of the Aqueous Ligand of Cobalt in $Co(H_2O)_6^{2+}$ and Co(II)-Substituted Carbonic Anhydrase

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Abstract: We have measured the hydrogen/deuterium fractionation factor for the rapidly exchanging, water ligands of cobalt in $Co(H_2O)_6^{2+}$ and in two Co(II)-substituted isozymes of carbonic anhydrase. The fractionation factor was determined from NMR relaxation rates at 300 MHz of the protons of water in mixed solutions of H_2O and D_2O containing these complexes. In each case, the paramagnetic contribution to $1/T_2$ was greater than to $1/T_1$, consistent with a chemical shift mechanism affecting $1/T_2$. The fractionation factors obtained from T_1 for $Co(H_2O)_6^{2+}$ and for the isozymes of Co(II)-substituted carbonic anhydrase were close to the fractionation factor for bulk water which is unity. The fractionation factors obtained from T_2 were 0.73 ± 0.02 for $Co(H_2O)_6^{2+}$ and 0.72 ± 0.02 for Co(II)-substituted carbonic anhydrase I, with a value of 0.77 ± 0.01 for isozyme II. We concluded that fractionation factors in these cases determined from T_1 and T_2 measured isotope preferences for different populations of ligand sites. We suggest that since T_2 has a large contribution from a chemical shift mechanism, the fractionation factor determined from T_2 has a large contribution of the fractionation of inner-shell ligands. The fractionation factors determined from T_1 are close to unity, the value of the fractionation factor of bulk water, and contain a larger contribution of the fractionation of outer-shell water. Although not yet helpful in interpreting the complex contributions to the isotope effects in the enzymatic catalysis, these hydrogen/deuterium fractionation factors for the water bound to cobalt in carbonic anhydrase can be significantly different from the fractionation factor for solvent water and appear not to be sensitive to the active site environment in these two isozymes of carbonic anhydrase.

The hydrogen/deuterium fractionation factor, ϕ, of a metal-bound water is the equilibrium constant for this isotope exchange reaction:

$$M(HOH) + (HOD) \rightarrow M(HOD) + (HOH)$$

$$\phi = \frac{[M(HOD)]/[M(HOH)]}{[HOD]/[HOH]}$$

This fractionation factor measures the tendency of deuterium to accumulate at the aqueous ligand of the metal relative to the deuterium content of bulk solvent. Fractionation factors are valuable in interpreting the effects of deuterium on kinetic and equilibrium constants because they represent individual contributions to isotope effects measured relative to the common reference of water. Values of the fractionation factor for various functional groups are known,[1,2] and the fractionation of hydrogen isotopes in water bound to metal ions has been considered.[1] However, there are few reports of the fractionation factor of the aqueous ligands of a metal. Using NMR methods, Silverman[3] has measured such a factor for the water ligands of cobalt in cobalt(II)-substituted carbonic anhydrase II ($\phi = 1.05 \pm 0.17$) and Melton and Pollack[4] for $Cr(H_2O)_6^{2+}$ ($\phi = 1.00 \pm 0.03$). They relied on the properties of a paramagnetic metal to enhance the

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