(38) The pH-independent attack rate constant is 30-fold lower in dioxane–water than in water for the reaction of *p*-nitroaniline with formaldehyde to form the carbinolamine, while the equilibrium constant for this reaction is insensitive to solvent effects (K_1 = 14 M^{-1} in dioxane–water and 18 M^{-1} in water).[24a]
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(42) (a) J. Sayer, personal communication. (b) Estimates of K_0 = $[RN^+H_2CH_2O^-]a_{H^+}/[RNH_2CH_2OH]$ = $[T^-]a_{H^+}/[T^0]$ are obtained following the considerations of Sayer and Jencks.[35] Thus, from the estimated pK_a value of 9.98 for the alcoholic group of $CH_3N^+H_2CH_2OH$[42c] and correction for the phenyl substitution for methyl by ΔpK = −8.4 (0.10)/2 = −0.42 (employing an attenuation factor of 2 for the transmission of the substituent effect through an additional nitrogen atom[35,42d,42e] that is present in carbinolamines) and the considerations of Fox and Jencks[42f] with a ρ_i = +8.4 for the dissociation constants of substituted ammonium ions and alcohols, the calculated pK_0 value for $PhN^+H_2CH_2OH$ is 9.56. Based upon this same attenuation factor, a ρ = +1.11 value for the ionization of trifluoroacetophenone hydrates, and σ^- values from the literature,[42g] the values of ΔpK = −1.11σ^-/2 are 0.15, 0.0, −0.19, and −0.69 for *p*-CH_3O-, H-, *m*-F-, and *p*-NO_2-DPED, respectively (ignoring the effects of the $-CH_2CH_2NHPhX$ groups). The solvent effect on −OH → $-O^-$ + H^+ proton dissociations, based upon the pK'_a values for carboxylic acids (see Results) and K_{WD} (Table III) values in dioxane–water, is $10^{1.7}$. The pK_N values for K_N = $[>NCH_2O-H]a_{H^+}/[>^+NHCH_2OH]$ are obtained from the pK'_{a_2} value of the parent amine (Table I) and an acid strengthening effect of $10^{1.9}$ due to *N*-hydroxymethyl substitution.[24a] The pK_0, pK_N, and log K_n values are estimated from the following relationships: pK_0 = 9.56 + 1.7 + ΔpK, pK_N = pK'_{a_2} − 1.9, and log K_n = log K_1 − pK_0 + pK_N where K_n = $[T^{\pm}]/[R_2NH][F]$ = K_1K_0/K_N. The pK_0, pK_N, and log K_n values based upon the above and K_1 values from Table V for this series in dioxane–water are *p*-CH_3O-, 11.41, 2.61, −8.26; H-, 11.26, 1.74, −8.92; *m*-F-, 11.07, −0.21, −10.80; and *p*-NO_2-DPED, 10.57, −4.24, −14.31. The calculated rate constants, k_b, according to eq 12a are obtained from k_b = k''_1/K_n with k''_1 values from Table III and the K_n values from above. (c) J. Hine, J. C. Craig, J. G. Underwood, II, and F. A. Via, *J. Am. Chem. Soc.*, **92**, 5194 (1970); (d) A. Fisher, D. A. R. Happer, and J. Vaughan, *J. Chem. Soc.*, 4060 (1964); (e) R. Pollet and H. Vanden Eynde, *Bull. Soc. Chim. Belg.*, **77**, 341 (1968); (f) J. P. Fox and W. P. Jencks, *J. Am. Chem. Soc.*, **96**, 1436 (1974); (g) A. I. Biggs and R. A. Robinson, *J. Chem. Soc.*, 388 (1961).
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Mechanisms of Isoalloxazine (Flavine) Hydrolysis

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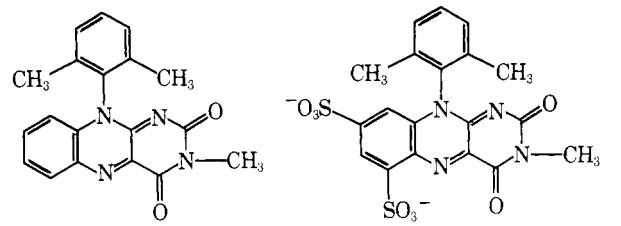
Abstract: The hydrolysis of 3-methyl-10-(2′,6′-dimethylphenyl)isoalloxazine (I) (30°, μ = 1.0, k = 3.16 × 10^2 M^{-2} sec^{-1}) exhibits no buffer catalysis, has a deuterium solvent kinetic isotope effect ($k_{obsd}^{H_2O}/k_{obsd}^{D_2O}$) of 0.6, and obeys the rate law $d[I]/dt = k[F][OH^-]^2$. The product, a ureido carboxylate (VII), arises from hydrolytic scission between positions 3 and 4 of the isoalloxazine ring. VII cyclizes in acid under anaerobic conditions to regenerate I. In the presence of oxygen, VII decarboxylates then cyclizes to form the ring contracted 3-methyl-9-(2′,6′-dimethylphenyl)isoimidazolone[4,5-*b*]quinoxaline (IX) (Scheme I). 3-Methyl-10-phenylisoalloxazine (III) hydrolyzes (k = 2.24 × 10^2 M^{-2} sec^{-1}) by the same mechanism as does I. The hydrolysis of 10-phenylisoalloxazine (VI) is first order in $[HO^-]$, k = 1.07 × 10^{-4} M^{-1} sec^{-1}. The change in kinetic dependence from $[HO^-]^2$ to $[HO^-]^1$ must be related to formation of the anion of VI (pK_a = 9.47) which is not susceptible to hydrolysis. 3,10-Dimethylisoalloxazine (IV) is hydrolytically reactive at the C-4 and C-10a positions. The kinetics of base hydrolysis of **IV** are biphasic as the result of two reaction paths each composed of two reactions with different pH dependence (Scheme II). 1,5-Dihydro-3-methyl-5-acetyl-10-phenylisoalloxazine (X) hydrolyzes in acid with a rate constant of 1.71 × 10^{-4} M^{-1} sec^{-1} giving 1,5-dihydro-3-methyl-10-phenylisoalloxazine which oxidizes to III in the presence of oxygen. The hydrolysis of 1,5-dihydro-3-methyl-5-acetyllumiflavine (XI) in acid has a rate constant of k = 8.67 × 10^{-5} M^{-1} sec^{-1}. In base, the hydrolysis of X is biphasic with the first step not involving removal of the 5-acetyl group. The largest rate constant of this biphasic reaction is only ca. 1 × 10^{-1} M^{-1} sec^{-1}. The rate constants for the acid and base hydrolysis of X and XI establish that neither the neutral nor anionic 1,5-dihydroisoalloxazine moieties are good leaving groups. For redox reactions which postulate facile expulsion of these species in dark (nonenzymatic) processes, this feature should be taken into account.

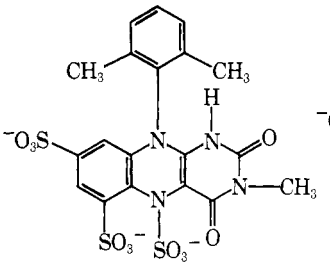
It is assumed that at least some flavine-catalyzed oxidation reactions proceed through covalent addition of the substrate to the isoalloxazine ring system.[1a,b] Many hypothetical but possible examples are provided in a recent review.[2] With this consideration in mind, it is of obvious importance to determine which positions of the isoalloxazine ring system are susceptible to nucleophilic (dark) addition reactions. Addition of SO_3^{2-} to the 5 position of flavine and flavinium compounds was established by Massey and Müller,[3a] and similar additions of phosphines have been established by Müller.[3b] The reaction of sulfite ion with 3-methyl-10-(2′,6′-dimethylphenyl)isoalloxazine (I) and 3-methyl-10-(2′,6′-dimethylphenyl)isoalloxazine-6,8-disulfonic acid (II) has been investigated in some detail.[4,5] Sulfite was found to add to the N(5) position of I and to the N(5) and C(4a) positions (by general-acid catalysis) of II to provide N-5 and **4a**, respectively. The objective of the present investigation has been to determine the mechanism of HO^--catalyzed hydrolysis of I and isoalloxazines III to VI in order to gain more insight into the mechanisms of nucleophilic additions to the isoalloxazine (flavine) nucleus. In addition, the hydrolyses of two N(5) acetyl-1,5-dihydroisoalloxazines have been examined in order to determine the susceptibility to displacement of substituents at the N(5) position of reduced flavine. The leaving abilities of the 4a and 5 positions are of some interest[6a] since metastable additions at these positions have been postulated to be involved in flavine catalysis.[1,6b,c]

The hydrolyses of isoalloxazine compounds were investigated as early as 1932.[7] Previous studies have been directed toward the hydrolysis of riboflavine,[7,8] lumiflavine,[9,10,12] and 10-methylisoalloxazine.[11a,b,c,12] The studies involving

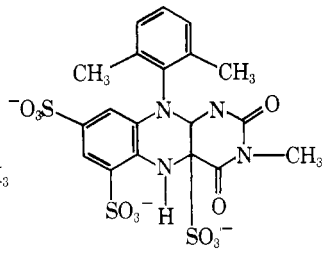


I

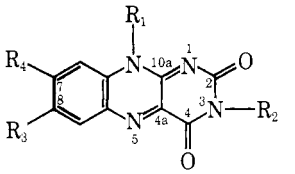
II



N-5



4a



III, $R_1 = -C_6H_5$; $R_2 = -CH_3$; $R_3 = R_4 = -H$
IV, $R_1 = -CH_3$; $R_2 = -CH_3$; $R_3 = R_4 = -H$
V, $R_1 = -CH_3$; $R_2 = R_3 = R_4 = -H$
VI, $R_1 = -C_6H_5$; $R_2 = R_3 = R_4 = -H$

lumiflavine and riboflavine present only fragmentary results. Many other studies have shown urea to be a product of flavine hydrolysis.[9]

Experimental Section

Materials. **3-Methyl-10-(2′,6′-dimethylphenyl)isoalloxazine** (I), mp 297–299°, **3-methyl-10-phenylisoalloxazine** (III), decomposing near 400°, and **3,10-dimethylisoalloxazine** (IV), mp 302–304°, were synthesized by published procedures.[13]

10-Methylisoalloxazine (V), mp 337–338°, and **10-phenylisoalloxazine** (VI), mp 335–336°, were prepared with the following modifications to published procedures.[13] After condensation of the appropriately substituted phenylenediamine with alloxan, the resulting solid was collected by filtration and recrystallized three times from a 4:1 formic acid:acetone mixture.

3-Methyl-9-(2′,6′-dimethylphenyl)isoimidazolone[4,5-*b*]quinoxaline (IX) was prepared by hydrolysis of I at pH 12.0 followed by chloroform extraction of the acidified reaction mixture. The chloroform extract was dried ($CaCl_2$) and vacuum evaporated, the resulting solid was chromatographed on silica gel using a 3:2 benzene:chloroform solvent mixture as eluent. The eluent was evaporated, and the yellow-white solid was chromatographed on an alumina column using chloroform : hexanes as the solvent system, varying the solvent ratio from 1:4 to 1:1 during elution. The resulting white solid was recrystallized twice from a 1:6 chloroform:hexane mixture. The spectral characteristics of the product, IX, are as follows: NMR singlets δ 1.97 (6 H) and 3.51 (3 H) and a multiplet between δ 6.8 and 8.1 (7 H); ultraviolet λ_{max} at 367, 349, and 334 nm in chloroform; and the infrared spectrum exhibited a carbonyl absorption at about 1720 cm^{-1} which is indicative of five-membered cyclic urea compounds; mass spectrum parent peak *m/e* (calculated 304) 304. Anal. Calcd C, 71.05; H, 5.26; N, 18.24. Found: C, 70.83; H, 5.05; N, 18.37.

1-(2′,6′-Dimethylphenyl)-2-(*N*-methyluredine)quinoxaline-3-carboxylic acid (VII) was prepared by anaerobic hydrolysis of I in situ just prior to use. The ultraviolet spectra of VII exhibited λ_{max} 252 and 370 nm in water at pH 11.0.

3-Methyl-1,5-dihydro-5-acetyl-10-phenylisoalloxazine (X) was synthesized using published procedures[14] by M. Brüstlein of this laboratory.

3-Methyl-1,5-dihydro-5-acetyllumiflavine (XI) was synthesized using published procedures.[14]

Kinetic Measurements. All kinetic experiments reported herein, unless otherwise stated, were carried out in aqueous solution (0.3% acetonitrile or dimethylformamide) at 30 ± 0.2°, μ = 1.0 with KCl. All water used was doubly glass distilled. For the anaerobic experiments, the water was boiled and saturated with high purity nitrogen at room temperature. Kinetic studies in the absence of buffers were carried out in a pH-stat cell[15] designed for the Cary 15 spectrophotometer or by use of standard acid solutions in the H_0 range.[16] Rapid hydrolytic experiments in the high pH range were completed using a thermostated Durrum stopped flow spectrophotometer. The anaerobic kinetic experiments were performed in Thunberg cuvettes or in the Cary 15 pH-stat cell employing a nitrogen atmosphere and an "O" ring seal between the cell and the cell head. While kinetic experiments were in progress, the sealed pH-stat cell was kept under positive nitrogen pressure.

Kinetic data for the hydrolysis of I were collected at 265 and 435 nm, while the hydrolytic reactions for the remaining flavines were followed at their respective λ_{max} near 435 nm. Except in the cases of IV and the base hydrolysis of X, all absorbance vs. time data collected were strictly pseudo first order.

The hydrolysis of IV gave absorbance vs. time plots that were biphasic, indicating the accumulation and decomposition of one or more intermediate species. The data analysis was completed using an EAI-20 analog computer equipped with an EAI-1133 *x–y* variplotter and an EAI repetitive operation unit which were used for data display. A schematic representation of the hydrolysis mechanism is shown in Scheme II, and the associated wiring diagram for the analog computer is shown in Figure 5.

The recyclization of VII to give I was studied in anaerobic acidic solutions. Two acid systems were used in the pH region, HCl with constant μ maintained with KCl and $HClO_4$ using $LiClO_4$. At acid concentrations of 1.0 *M* or greater, no salts were added.

pH measurements were taken using a Radiometer Model 26 pH meter equipped with a standardized Model EA-125 Metrohm or GK-2302c Radiometer thermostated electrode. The pK_a's of flavines V and VI were determined spectrophotometrically under kinetic conditions at 435 nm. Values were obtained from absorbance vs. pH plots by calculation of the best fit of a theoretical sigmoid curve to the experimental points. The pK_a of compound V was determined to be 9.61 and of VI 9.47.

The D_2O solvent kinetic isotope-effect experiments were carried out at pOH = pOD. The pOH and pOD values were calculated from the published values of K_{H_2O} (1.45×10^{-14}) and K_{D_2O} (2.0×10^{-15})[17] knowing the pH's and pD's. Values for pH and pD were obtained using a combination glass electrode, and the meter pD readings were corrected (pD = pH meter reading − 0.39).[18]

The $E_{1/2}$ of compound IX was determined to be −1.018 ± 0.004 V relative to the saturated calomel electrode on a Sargent Model XV polarograph equipped with a dropping mercury electrode at pH 8.49, μ = 0.1 Na_3BO_3 + 0.1 KCl, and T = 30 ± 0.2°.

Data Analysis. All calculations were carried out with the aid of an Olivetti-Underwood Programma 101 computer using programs written in this laboratory or an on-line terminal connected to the UCSB IBM 360-75 computer.

Results

All hydrolytic reactions, unless stated otherwise, were carried out at 30°, μ = 1.0 with KCl. When the log of the observed pseudo-first-order rate constants (log k_{obsd}) for the hydrolysis of I and III are plotted vs. pH, linear relationships are obtained with slopes 1.93 and 1.96, respectively. The alkaline hydrolysis of I and III are, therefore, second order in hydroxide ion (eq 1). From the best line of

$$\frac{d[\text{Flav}]}{dt} = k[\text{I or III}][HO^-]^2 \qquad (1)$$

$$k_{obsd} = k'[HO^-]^2$$

slope 2.0 for the log k_{obsd} vs. pH profile (Figure 1), the values of k' for I and III are calculated to be 3.16×10^2 M^{-2} sec^{-1} and 2.24×10^2 M^{-2} sec^{-1}, respectively. Employing the following amines (30°) at pH values within 1.5 pH units of their pK_a values, it could be established via

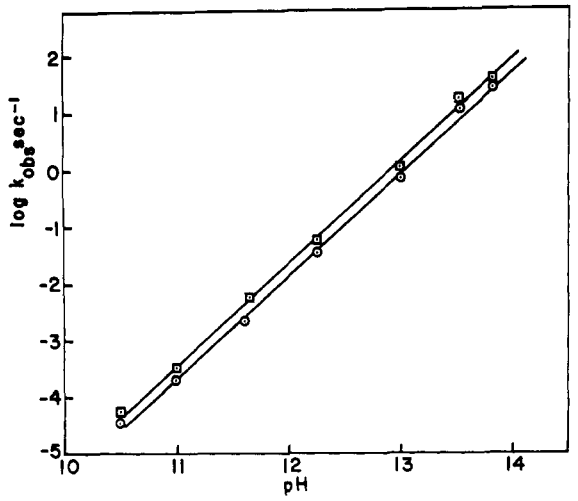


Figure 1. Log k_{obsd} vs. pH for the specific base catalyzed hydrolysis of I ≡ ⊡ and III = ⊙ at 30°, μ = 1.0 with KCl.

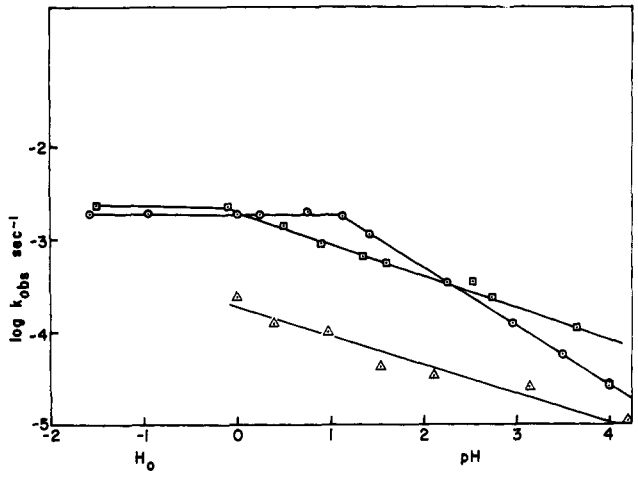


Figure 2. Log k_{obsd} vs. H_0 or pH for the anaerobic formation of I from VII at 30°, μ = 1.0 with KCl, ⊙; and μ = 1.0 with $LiClO_4$, □. Log k_{obsd} vs. pH for the cyclization of *o*-ureidobenzoic acid, μ = 1.0 with KCl is shown by ▲.

buffer dilution experiments (total amine buffer 5.0×10^{-4} to 1.0 *M*) that aminolysis or buffer catalysis of the hydrolysis of I does not occur: ethylamine (pK_a = 10.69), methylamine (pK_a = 10.63), diethylamine (pK_a = 10.98). The deuterium kinetic solvent isotope effect for hydrolysis of I ($k_{obsd}^{H_2O}/k_{obsd}^{D_2O}$) determined at pOD = pOH = 2.04 was found to be $(1.0 \times 10^{-2}/1.62 \times 10^{-2})$ = 0.62 and at pOD = pOH = 3.31 to be $(4.18 \times 10^{-5}/7.42 \times 10^{-5})$ = 0.56. The hydrolysis of I, under anaerobic conditions, provides a product which recyclizes to I on acidification. The log k_{obsd} vs. pH profile and a repetitive scan of the recyclization reaction are presented in Figures 2 and 3, respectively. Compound IX was isolated from the hydrolysis of I at pH 12.0 and identified (see Experimental Section). The aerobic contraction of the pyrimidine ring portion of the isoalloxazine molecule to give IX and the second-order dependence of hydrolysis on hydroxide suggest the intermediate to possess structure VII. A repetitive scan of the aerobic hydrolysis of I is presented in Figure 4. On hydrolysis of III, a quinoxaline-2-carboxylic acid [3-(*N*-methyluredine)-4-phenylquinoxaline-2-carboxylic acid] is also presumably obtained since the spectrum of the reaction solution corresponds to that of VII. Continued incubation under aerobic hydrolytic conditions converts VII to IX by oxidative decarboxylation of the intermediate VII to give VIII which cyclizes to IX. The overall reaction path is presented in Scheme

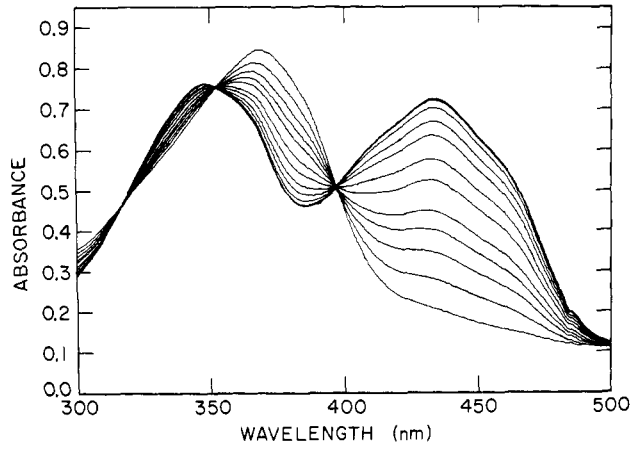


Figure 3. Repetitive scan of the formation of I from VII, pH 1.1, μ = 1.0 with KCl.

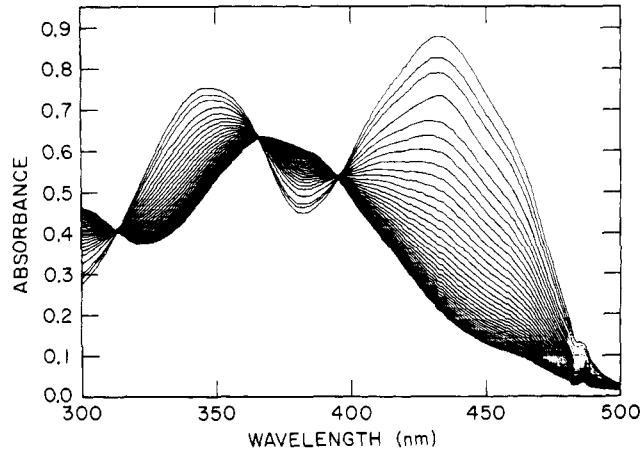
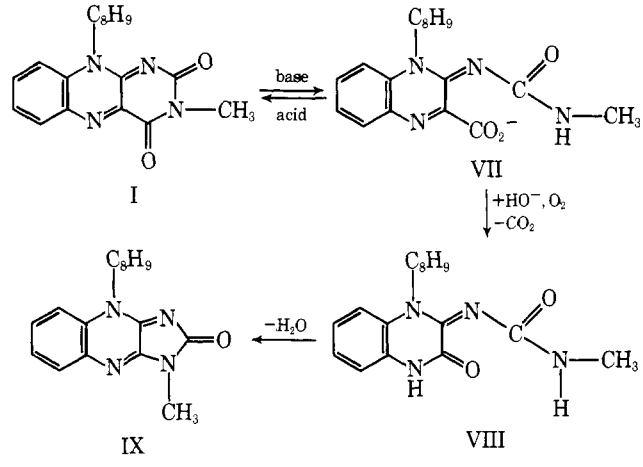


Figure 4. Repetitive scan of the hydrolysis of I, pH 11.2, μ = 1.0 with KCl.

Scheme I



I. For the individual steps associated with conversion of I to VII see the Discussion (Scheme IV). Although VII or VIII have not been isolated, quinoxaline-2-carboxylates have previously been shown to undergo oxidative decarboxylation[11c,19] to yield 2-oxoquinoxalines. The formation of IX will be considered in detail in the Discussion. At any point during or after the hydrolysis of I under anaerobic conditions, nearly 100% of I could be regenerated by adjusting the pH to 4.0 or less. At pH's greater than 4.0, the recyclization reaction was too slow to conveniently follow while maintaining anaerobic conditions. The extent of recyclization of VII and identification of I was accomplished by uv

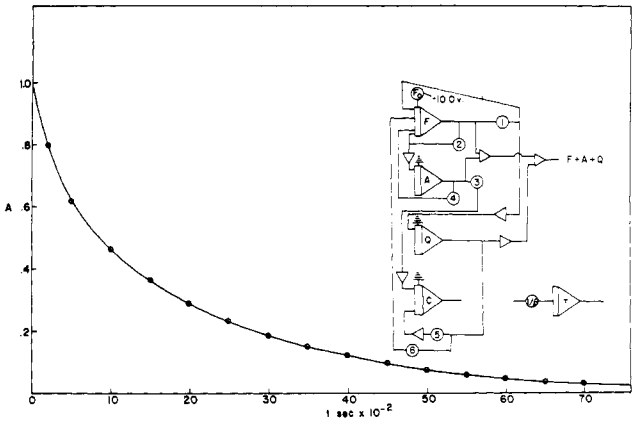


Figure 5. Absorbance vs. time plot for the hydrolysis of IV (30°, μ = 1.0 with KCl, pH 11.2). The points are experimental, and the line was fit by use of an analog computer, the wiring diagram of which is inset.

spectral analysis. Examination of Figure 2 reveals that log k_{rate}, for the recyclization of VII, increases almost linearly with increase in a_H plateauing just before the H_0 region. When maintaining constant ionic strength in the pH region with KCl, and employing HCl as a proton source, the pH profile has a slope of 0.62, while using $HClO_4/LiClO_4$, the slope is 0.35. In the H_0 region, where no salt was added, both profiles plateau at nearly the same value of k_{obsd}. Attempts were made to determine the pK_a's of VII by spectrophotometric and volumetric titrations. Difficulties were encountered with the limited solubility of VII in acid and from a rapid initial reaction in its recyclization to form I. It is, however, apparent that no functional group can be titrated between pH 2 and 7. The spectrophotometric titration of VII, at 260 nm, gave an absorbance vs. pH plot that can be described by eq 2. Replacement of the N-3 methyl group of

$$A = 0.065\ \text{pH} + 0.66 \qquad (2)$$

III with a proton provides an acid of pK_a 9.47 (VI). For VI in 1.0 and 0.1 M KOH, the determined values of k_{obsd} are 1.07×10^{-4} and 1.20×10^{-5} sec^{-1}, respectively. The line drawn through these points, when log k_{obsd} is plotted vs. pH, is 0.95. At completion, the spectrum of the hydrolytic reaction was essentially the same as that for the hydrolytic product of III. The product is then a substituted quinoxaline-2-carboxylic acid analogous to VII.

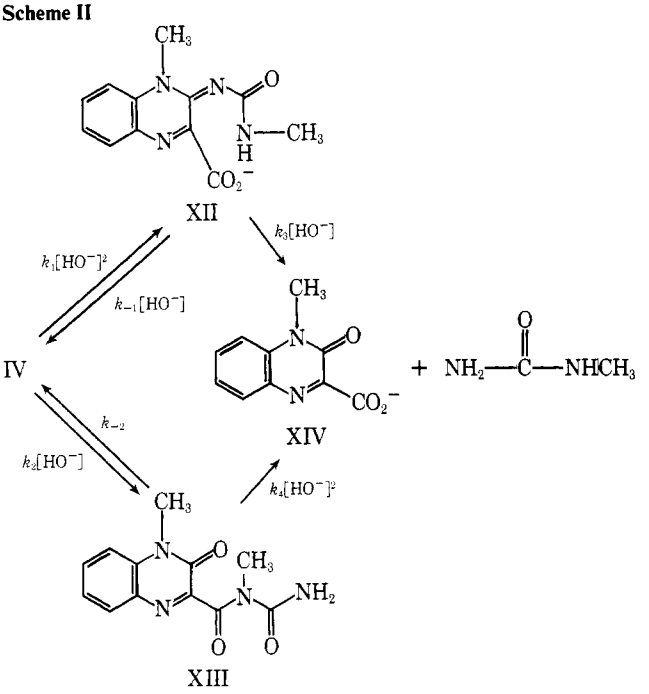
In the alkaline pH range, the hydrolysis of IV follows biphasic kinetics. The spectrum of the hydrolytic reaction at completion is identical with that of 1-methyl-2-ketoquinoxaline-3-carboxylic acid.[11a] Since 10-methylisoalloxazine has been shown to undergo hydrolytic scission initially at the 10a position,[11a,b,c] and we have shown I to undergo HO^- attack at the 4 position, the modes of hydrolysis of IV, as presented in Scheme II, are not surprising. It is also well known that isoalloxazines liberate urea upon hydrolysis.[9] An analog computer fit of the absorbance vs. time data for hydrolysis of IV is shown in Figure 5. Equally good fits were achieved for four reactions between pH 10.5 and 12.5. Table I shows the measured and analog computer-solved values of the rate constants of Scheme II. From Table I, it can be seen that the measured values of k_1 and k_3 at high pH show good agreement with analog-solved values at lower pH. At high hydroxide concentrations, it is possible to measure directly and independently k_1 and k_3. For example, in 1 M KOH, the spectra of XII can be obtained since $k_1[HO^-]^2$ is at least 3×10^3 greater than $k_3[HO^-]$, $k_{-1}[HO^-]$, and $k_2[HO^-]$. The spectra of XII exhibit λ_{max} at 268 and 380 nm. These values are very similar to those reported for VII (i.e., 252 and 370 nm) but different from

Table I. Second-Order Rate Constants for the Hydrolysis of IV (30°, H_2O, μ = 1.0 with KCl)

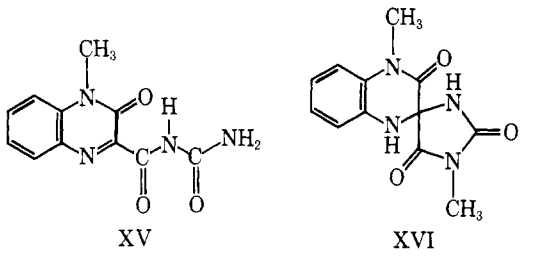
		Analog[a]	Determined separately[b]
k_1	7.78	$\pm 0.05\ M^{-2}$ sec^{-1}	$7.84 \pm 0.03\ M^{-2}$ sec^{-1}
k_{-1}	2.50×10^{-3}	$\pm 0.15\ M^{-1}$ sec^{-1}	
k_2	2.41×10^{-3}	$\pm 0.30\ M^{-1}$ sec^{-1}	
k_{-2}	2.11×10^{-2}	± 0.20 sec^{-1}	
k_3	2.31×10^{-3}	$\pm 0.09\ M^{-1}$ sec^{-1}	$2.29 \times 10^{-3} \pm 0.02\ M^{-1}$ sec^{-1}
k_4	1.51	$\pm 0.11\ M^{-2}$ sec^{-1}	

[a] Values of rate constants are averaged values determined at pH values of 10.50, 11.20, 11.65, and 12.50. [b] Determined at 1.0 M KOH where k_1 and k_3 steps are separated in time.

Scheme II



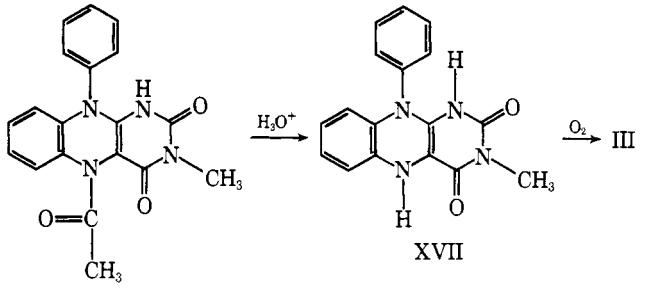
the λ_{max} values for 3,4-dihydro-3-keto-4-methylquinoxaline-2-carboxyureide (XV)[20] which are 236, 317, and 387 nm in EtOH. The formation of the spirohydantoin XVI from the ureido ketoquinoxaline XIII is not observed under



the conditions and on the time scale of the overall hydrolytic reaction.[21a,b]

Under acid conditions, X undergoes hydronium ion-catalyzed hydrolysis of the N-5 acetyl group to provide 3-methyl-1,5-dihydro-10-phenylisoalloxazine (XVII). In the presence of oxygen, XVII spontaneously oxidizes to give III, as shown in Scheme III. The product, III, was identified by analysis of the uv spectrum. In acidic media, the slope of the plot of log k_{obsd} vs. pH for hydrolysis of X was found to be −1.0 between 0.1 and 1.0 M HCl. From this plot, the value of the hydronium ion rate constant was calculated to be $k_2 = 1.71 \times 10^{-4}\ M^{-1}$ sec^{-1}. Hydrolysis of X in the basic pH range is associated with biphasic absorbance vs. time plots which dictate a process of consecutive reactions. In 1.0 M KOH, the two reactions are nearly separated in time. The second-order rate constant for the faster reaction was determined to be ca. $1 \times 10^{-1}\ M^{-1}$ sec^{-1}. The

Scheme III



X XVII III

uv spectrum of the compound formed by the first reaction does not correspond to that of 1,5-dihydro-III, III, or the product of basic hydrolysis of III. Therefore, the initial reaction cannot be hydrolytic removal of the N-5 acetyl group of X. 3-Methyl-1,5-dihydro-5-acetyllumiflavine (XI) undergoes acid hydrolysis with a rate constant (k_2 = 8.67 × 10^{-5} M^{-1} sec^{-1}) comparable to that of X. The second-order rate constant for XI was determined in 1.0, 0.5, and 0.1 *M* HCl.

Discussion

The results of this study show, for the first time, that the C-4 carbonyl position of isoalloxazines can be very reactive toward specific base hydrolysis. It has previously been shown, at high pH, that N-3-protio flavines undergo initial hydrolytic scission of the N1–C10a bond.[11a,b,c] This is due to ionization of the N-3 proton causing the C-4 position to be relatively unreactive toward nucleophilic attack. When the N-3 position is methylated as in 3,10-dimethylisoalloxazine (IV), both the C-4 and C-10a positions become susceptible to base hydrolysis. If the C-10a position is sterically blocked as in 3-methyl-10-phenyl-(III) or 3-methyl-10-(2′,6′-dimethylphenyl)isoalloxazine (I), specific-base hydrolysis is directed to the C-4 position. The initial hydrolytic product of I (i.e., VII) is amazingly unreactive to further alkaline hydrolysis though it is an imine of a ureido nitrogen. Acidification of an anaerobic solution of the initial hydrolytic product of I leads to ring closure and the regeneration of I, whereas aerobic hydrolysis provides the ring contracted product IX. The mechanisms of these reactions will be further elaborated, and the significance of the hydrolytic constants for N-5 acetyl-1,5-dihydroflavines will be discussed.

Mechanism of Hydrolysis Initiated at the 4 Position. Steric blocking of the 10a position of the isoalloxazine nucleus to nucleophilic attack,[22] as in I, results in preferential HO^- attack at the 4 position. On the basis of amine buffer dilution studies, the hydrolysis of I is not subject to amine general-acid or -base catalysis, nor does isoalloxazine aminolysis occur. The product analysis, the finding that the reaction is second order in [HO^-], and the solvent isotope effect $k_{obsd}^{H_2O}/k_{obsd}^{D_2O}$ of ca. 0.60 suggest Scheme IV where the rate-determining step is decomposition of the dianionic tetrahedral intermediate. Similar mechanisms have been offered for the hydrolysis of various amide substrates which have in common a greater than first-order dependence on [HO^-].[23a,b] Assuming steady state in the tetrahedral intermediates, the k_{obsd} based on Scheme IV can be expressed as eq 3.

$$k_{obsd} = \frac{k_1 k_3 K_{a_3} K_w}{a_H(k_{-1}a_H + k_3 K_{a_3})} \quad (3)$$

The D_2O solvent kinetic isotope effect may be explained by eq 4

$$\frac{k^H_{obsd}}{k^D_{obsd}} = \frac{k_1^H}{k_1^D}\frac{k_3^H}{k_3^D}\frac{K_{a_3}^H}{K_{a_3}^D}\frac{K_{H_2O}}{K_{D_2O}}\frac{a_D(k_{-1}^D a_D + k_3^D K_{a_3}^D)}{a_H(k_{-1}^H a_H + k_3^H K_{a_3}^H)} \quad (4)$$

assuming

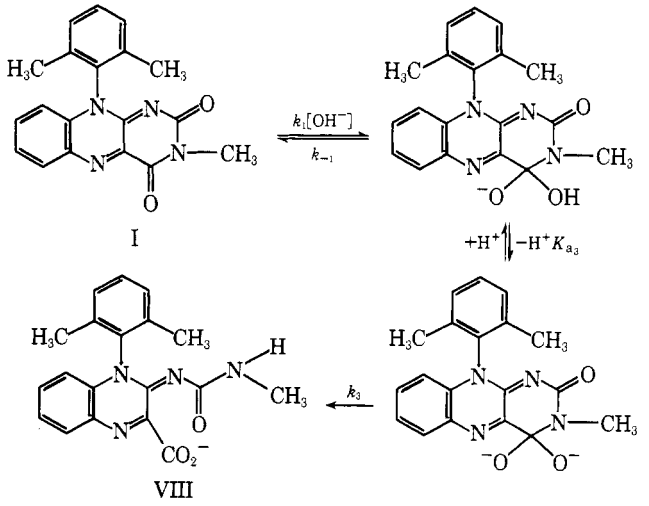
$$\frac{k_1^H}{k_1^D} \cong 0.8^{24},\ \frac{k_{-1}^H}{k_{-1}^D} \cong 1,\ \frac{k_3^H}{k_3^D} \cong 1,$$

$$\frac{K_{H_2O}}{K_{D_2O}} = 7.25^{17} \text{ and } \frac{K_{a_3}^H}{K_{a_3}^D} \cong 5^{25}$$

The calculated values of $k^H{}_{obsd}/k^D{}_{obsd}$ are 0.52 and 0.55 which are in good agreement with experimental values of 0.56 and 0.62. The $a_H k_3 K_{a_3}$ term in the denominator of eq 3 is not considered in the above calculation since the log k_{obsd} vs. pH profile has a slope of 2 ($a_H^2 k_{-1} \gg a_H k_3 K_{a_3}$).

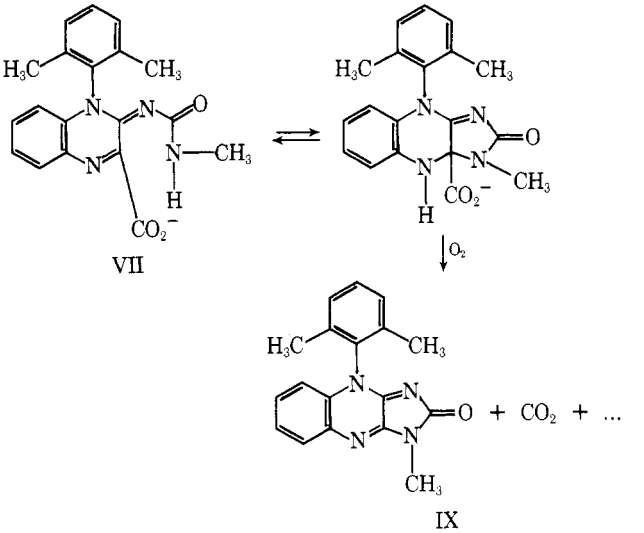
The oxidative decarboxylation and cyclization of VII to form IX (Scheme I) could conceivably occur via the alternate pathway of Scheme V. To differentiate between the mechanisms of Scheme I and V, the hydrolysis of I was performed under both anaerobic and aerobic conditions. Under anaerobic conditions, IX is not formed even after an extended period of time, while the aerobic reaction did produce IX. After a period of time, saturation of the anaerobic reaction with air led to the appearance of IX with approximately the same rate constant as that associated with appearance of IX in the aerobic reaction. These experiments are in accord with the mechanism of Scheme I. They do not rule out the mechanism shown in Scheme V if it is assumed that

Scheme IV



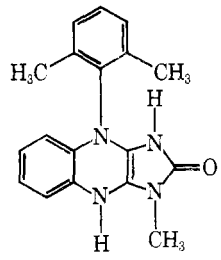
I VIII

Scheme V



VII IX

the intermediate is only present in an unfavorable equilibrium with VII. The fact that quinoxaline-2-carboxylic acids have been established[11c,19] to undergo oxidative decarboxylation provides preferential support of Scheme I. Importantly, decarboxylation of the intermediate shown in Scheme V should yield XIX without oxygen being present. While the



XIX

oxidation of XIX to give IX would require oxygen, the anaerobic hydrolysis of I is reversible when the pH is adjusted to pH 4 or less. The reversibility of the hydrolysis on acidification, even after extended periods of time, rules out decarboxylation of any intermediate such as shown in Scheme V. These findings strongly disfavor the reaction path shown in Scheme V.

The recyclization of VII in acidic solution to give I produced a log k_{obsd} vs. pH plot (Figure 2) which could not be explained by involving specific acid and or water catalysis on the various ionic species of VII. Precedence for intramolecular nucleophilic attack of an ureido group upon a carboxyl group exists in the literature.[26] The results presented by Hegarty and Bruice show the log k_{obsd} vs. pH profile for the cyclization of 2-ureidobenzoic acid to be of fractional slope as are our data for the recyclization VII. Figure 2 shows an extension of the log k_{obsd} vs. pH profile for 2-ureidobenzoic acid taken from the work of Hegarty in this laboratory. Although the interpretation of the log k_{obsd} vs. pH profile of both *o*-ureidobenzoic acid and VII in acid is obscure, the near identity suggests a similarity in mechanisms.

10-Phenylisoalloxazine (VI) hydrolysis is found to be first order in [HO^-], while the hydrolysis of III is second order in [HO^-]. This comparison is made in the pH range 13–14. The difference in hydroxide order for the hydrolysis of III and VI could be explained by the hydrolysis of neutral VI being second order in hydroxide, while its anion is unreactive. At pH above the pK_a of VI (9.47), the concentration of the reactive species, neutral VI, would be inversely proportional to [HO^-] which would bring about an apparent change in the order of hydroxide from second to first. This assumption may be used to calculate a second-order rate constant for neutral VI based on eq 1 and the mole fraction of neutral VI (eq 5). Solving eq 5, a value of

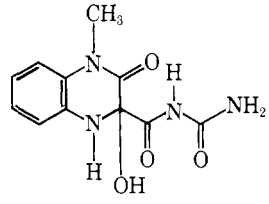
$$k_{obsd\,VI} = k_{VI}[HO^-]^2 a_H/K_a + a_H \cong k_{VI}[HO^-]K_w/K_a \quad (5)$$

ca. 2.5 is obtained for k_{VI} which is ca. 100-fold smaller than k_{III} (2.24×10^2). From this comparison, it seems that mere replacement of the proton on the N-3 position by a methyl group provides sufficient electronic and steric perturbations to increase the rate of hydrolysis at the C-4 position by ca. 100 fold.

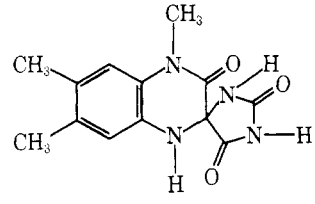
The difference in rate constants for III and VI is expected since examples exist in the literature which indicate as a general rule that tertiary amides hydrolyze faster than secondary amines.[27-30] However, this difference in the rate of hydrolysis of tertiary vs. secondary amides amounts to only a factor of 2–6, but it holds true for a wide variety of amides such as aliphatic, aromatic, and cyclic. A possible explanation for this observed rate change is that the tetrahedral intermediate of tertiary amides should be less stable than secondary amides because of increased steric interactions; hence, explusion of a secondary amine should be facilitated relative to a primary amine. The rate difference for III vs. VI is only ca. 15–50-fold greater than found for analogous amides.

3,10-Dimethylisoalloxazine (IV). The hydrolysis of 10-methylisoalloxazine (V) has been previously reported.[11a,b,c] At high pH, the initial reaction of V with hydroxide involves hydrolysis of the 1–10a imine bond, the C-4 position being comparatively unreactive because of ionization of the proton from the N-3 position. In the case of IV, both the 4 and 10a positions are electrophilic centers for hydrolysis. The best analog computer fit (Table I, Figure 5) of the biphasic hydrolysis data for IV utilized the reactions shown in Scheme II. The constants k_1 and k_4 are associated with hydroxide attack at the 4-carbonyl carbon, as was shown for I, while constants k_2 and k_3 refer to imine hydrolysis at the 10a position. Both the initial steps of the two reaction pathways are required to be reversible in order to fit Scheme II to the kinetic data. Bruice and Hegarty[31] have shown the feasibility of the reaction denoted by k_{-1} with the base-catalyzed cyclization of *o*-ureidobenzoic acid anion. As can be seen in Scheme II, the hydroxide dependence of the k_1 step is second order and the k_3 step first order so that, at hydroxide concentrations approaching 1.0 *M*, k_1 is the major hydrolytic pathway; hence, both k_1 and k_3 can be determined independently. Theoretically, at low hydroxide concentrations, one could determine k_2 and k_4 independently; however, the rates of the reactions would be too slow to allow determination on a practical time scale ($k_{2_{obsd}} \cong 2.4 \times 10^{-8}$ sec^{-1} at pH 9.00).

Guttman and Platek[11c] have reported a product study for the hydrolysis of 10-methylisoalloxazine (V) in which they claim to have isolated the carbinol amine (XIX) of XV. The authors assigned structure XIX on the basis of elemental analysis, nonaqueous titration, and ultraviolet spectral data. Dudley and Hemmerich have prepared a compound with the same uv spectra as XIX, which has been shown



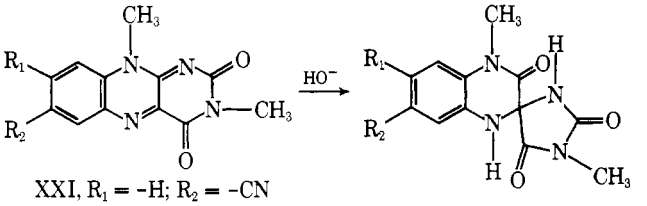
to be 1′,4′-dihydro-4′,6′,7′-trimethylspiro[imidazolidine-4,2′(3*H*)-quinoxaline]2,3′,5-trione[21b] (XX). Dudley and



XX

Hemmerich based the assignment of structure XX on infrared and ultraviolet spectra as well as elemental analysis, assuming one water of crystallization. The structures of similar spirohydantoins have been confirmed by NMR,[21a] and these have ultraviolet spectra very similar to compound XX and the product of Guttman and Platek. Treatment of XX with hot 50% acetic acid provides the carboxyureide analogous to XV. A similar reaction was observed for the supposed compound XIX and was presumed to be a dehydration to the imine. It would appear that Guttman and Platek have incorrectly assigned structure XIX, mistaking one water of crystallization for a hydrated imine bond;

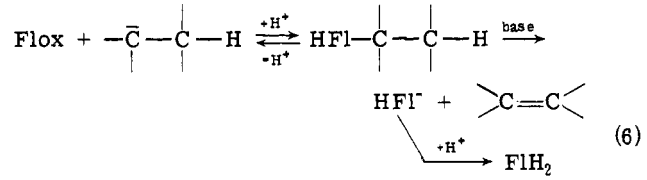
hence, upon hydrolysis of V, they actually obtained a spirohydantoin as one product. Interestingly, the hydrolysis of the electron deficient 7- and 8-cyano-3,10-dimethylisoalloxazines (XXI and XXII, respectively) is first order in [HO^-]



XXI, $R_1 = -H$; $R_2 = -CN$
XXII, $R_1 = -CN$; $R_2 = -H$

and attack occurs at the 10a position to provide the corresponding spirohydantoins.[32]

Many of the redox reactions which flavines participate in have been suggested to take place by way of covalent flavine–substrate adducts.[2,33] The positions of covalent-bond attachment to the flavine have been suggested to be C-4a, N-5, C-8, or C-10a. For dark reactions, the C-10a position has been effectively ruled out,[22] but the C-4a, N-5, and C-8 positions have been shown to be susceptible to nucleophilic attack.[3,4,5] 1,5-Dihydroisoalloxazines have been envisioned as arising from a covalent intermediate via processes requiring the stabilization of an isoalloxazine anion or incipient anion, as shown in eq 6 (for a kinetic study see ref 6a).



(6)

A characteristic of model flavine (dark) redox reactions is the general lack of observable intermediates. This observation obligates any covalent intermediate to be of high free energy so that the rate-determining step is intermediate formation. If the N-5 position is the seat of initial nucleophilic addition, it follows that the 1,5-dihydroflavine moiety must be an extraordinarily facile leaving group. The rate of specific acid-catalyzed hydrolysis of X is only 18 times greater than that of acetamide. Thus, 1,5-dihydroflavine is comparable to an ordinary amine as a leaving group in water. The acid-catalyzed hydrolysis of 3-methyl-1,5-dihydro-5-acetyllumiflavine (XI) in water is two times less reactive than X (Results). The similar hydrolytic reactivity of compounds X and XI indicates the 10-phenyl substituent, and the lack of the 7,8-methyl substituents in X has little effect on its rate of hydrolysis. The base-catalyzed hydrolysis of X is biphasic and, when carried out aerobically, does not yield the isoalloxazine III or the hydrolysis products of III (the hydrolytic products were not identified). The biphasic kinetics, therefore, cannot be explained as initial hydrolysis of the N-5 acetyl group followed by reoxidation of the 1,5-dihydroflavine to give III, which is then followed by attack of HO^- at the 4 position, as previously described for III. The first reaction might therefore be hydrolysis at the C-4 carbonyl group. The rate of base hydrolysis of the N-5 acetyl group is not therefore, a measure of the leaving ability of 1,5-dihydroflavine anion since the preceding hydrolytic reaction destroys the flavine nature of the molecule. The observed rate of hydrolysis is required to be greater than that for the hydrolysis of the N-5-acetyl group. Thus, the 5-N^- species is at best no better a leaving group than a substituted aniline. One might compare the rate of alkaline hydrolysis of *N*-acetylimidazole (an amide with an excellent leaving group) with the initial rate of hydrolysis of X. The second-order rate constant is $k_{HO^-} = 316.6\ M^{-1}\ sec^{-1}$ for *N*-acetylimidazole[34] and about $\sim 1 \times 10^{-1}\ M^{-1}\ sec^{-1}$ for X. The chemical inertness of X as expressed by its hydrolysis kinetics indicates that carbon N-5 adducts of 1,5-dihydroflavines, if formed, along the redox pathway should be easily observed. In dark model studies, to date, this is not so.

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