An Unexpected Hydrolysis pH–Rate Profile, at pH Values Less than 7, of the Labile Imide, ICRF-187: (+)-1,2-Bis-(3,5-dioxopiperazin-1-yl)propane

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The hydrolysis of the cardioprotectant and anticancer agent, ICRF-187 (or ADR-529) and the structurally similar model compound, 4-methylpiperazine-2,6-dione (4-MP), was investigated in the acid to neutral pH range at 25°C and an ionic strength of 0.5 (sodium chloride). Their solution stability was shown to be compromised compared to 3-methylglutarimide (3-MG) and other imides. It appears that the tertiary piperazine nitrogens of ICRF-187 and 4-MP significantly contributed to the instability of these compounds over this pH range. Unexpectedly, bell-shaped curves were observed in the pH–rate profiles. A change in the rate-determining step from tetrahedral intermediate formation in the weakly acidic pH region to breakdown of the tetrahedral intermediate in the more acidic pH regions was proposed as an explanation for the bell-shaped curves. The piperazine nitrogen was implicated in the hydrolytic pathways that occur within these pH regions; the mechanism of involvement was dependent on the state of ionization of the parent molecule and the tetrahedral intermediate.

KEY WORDS: ICRF-187 (ADR-529); hydrolysis; pH–rate profile; imide; acidic degradation; bell-shaped profile; facilitation.

INTRODUCTION

The chemical stability of ICRF-187 (or ADR-529), an anticancer agent which has recently been shown to be effective in reducing the level of cumulative damage to the myocardium caused by doxorubicin (1–3), and two model compounds, 4-methylpiperazine-2,6-dione (4-MP) and 3-methylglutarimide (3-MG) (Fig. 1), was recently reported in the neutral to basic pH range (4). The ionization of all three imides was discussed in detail and the identification of the hydrolytic degradation products observed in these studies were confirmed (4). The degradation products were also recently reported by Burke *et al.* (5).

Although the basic pH hydrolysis of ICRF-187 has been reported by Hasinoff (6) and from this laboratory (4), the chemical stability of ICRF-187 at acidic pH values has not been extensively studied. One study on the stability of ICRF-187 between pH 1 and pH 5 at 25°C (7) suggested that ICRF-187 was significantly less stable in acidic solutions than previously reported for ICRF-159 (8), the *d,l* form of ICRF-187. These results, however, were based on thin-layer chromatographic (TLC) and polarimetric methods, respectively.

Chemical stability studies on structurally similar compounds have shown the imide moiety to be susceptible to base-catalyzed hydrolysis (5–15). However, imides appear to exhibit greater stability in the neutral and acidic pH range and, in the case of glutethimide, even at elevated temperatures (14).

In our earlier study (4), it was shown that the presence of the second nitrogen (the tertiary piperazine nitrogen in the 4 position) in ICRF-187 and 4-MP contributed to the base-catalyzed hydrolysis of these two compounds compared to 3-MG. Also, while no hydrolysis of 3-MG was observed at pH 2 and 7 over 6 weeks at 25°C (4), initial observations suggested that ICRF-187 and 4-MP were relatively unstable in the acidic to neutral pH range. Therefore, the objective of this paper is to report on the chemical stability of ICRF-187 and 4-MP in the acidic to neutral pH range. Additionally, an attempt is made to explain the very unusual pH–rate profile that was observed for these compounds.

EXPERIMENTAL

Materials and methods of analysis for the present study were identical to those reported earlier (4). The temperature for most of the kinetic studies was controlled at 25.0 ± 0.1°C with a circulating water bath (either a Forma Scientific Model 2059 or a Haake Model D-2) unless otherwise indicated. Elevated temperatures for the determination of activation parameters were controlled with constant temperature ovens (Stable Therm, Blue M).

Nonlinear curve fitting was accomplished with the Simplex algorithm in the program MULTI [by Yamaoka *et al.* (16)] or RS/1 (BNN Software Products Corp.) for personal computers. Linear regression analysis was performed with the program StatWorks (Data Metrics, Inc.).

The hydrolysis at 25°C of ICRF-187 and 4-MP was studied over a pH range of 1.0 to 7.0. The various components, concentration ranges, and pH ranges of the buffer solutions used in the kinetic studies reported here were pH 1–2.2 (HCl), 2.5–3.5 (0.05–0.1 *M* formate), 3.5–5.0 (0.025–0.1 *M* acetate), and 6.0–7.0 (0.025–0.1 *M* phosphate). All buffers were adjusted to an ionic strength of 0.5 with sodium chloride. The effects of buffer concentration were examined by varying the concentration of buffers while maintaining a constant pH. Either three or four concentrations of buffer were used at each pH.

Activation parameters ($\Delta H‡$, $\Delta S‡$, and E_a) were determined for the hydrolysis of ICRF-187 and 4-MP at pH 5.0 in 0.05 *M* acetate buffer. The acetate buffer was adjusted to pH 5.0 at room temperature since the ionization constant of acetic acid does not change substantially at elevated temperature (17). Ampules containing ICRF-187 and 4-MP were maintained at 37, 50, and 70 or 80°C and allowed to equilibrate for 30 min before the first sample was taken. The samples were removed from the oven, quenched, and analyzed at appropriate time intervals.

The solvent deuterium isotope effect on the hydrolysis of ICRF-187 and 4-MP was performed in 0.01 *M* formate

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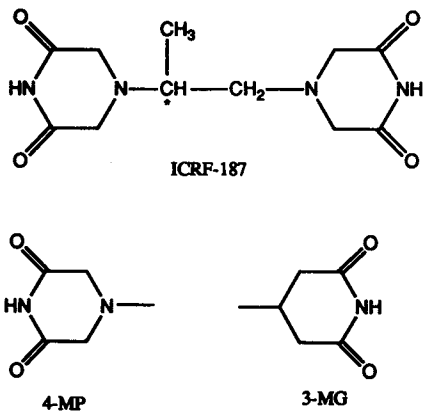


Fig. 1. Structures of ICRF-187, 4-methyl-2,6-piperazinedione (4-MP), and 3-methylglutarimide (3-MG).

buffer at pH or pD 4.0 for ICRF-187 and pH or pD 3.2 for 4-MP. It was also studied in 0.05 *M* acetate buffer at pH or pD 5.0 for ICRF-187 and pH or pD 5.5 for 4-MP.

RESULTS AND DISCUSSION

The pK_a values relevant to the degradation kinetics of ICRF-187 and 4-MP were determined under conditions identical to those for these kinetic studies and were reported earlier (4). The pK_{a1} value for 4-MP, corresponding to the protonation of the tertiary piperazine nitrogen, was determined to be 2.68 ± 0.05, while pK_{a2}, the imide functionality, was 9.62 ± 0.01, similar to the value of 10.03 reported for ICRF-187 by Hasinoff (6). ICRF-187 has four potential sites of ionization but only two measurable pK_a values. The pK_{a1} value of 2.47 ± 0.06, determined by potentiometric titration, was comparable to the pK_{a1} value for 4-MP. One equivalent of acid was required to reach the end point, an indication of the addition of one proton to the tertiary piperazine nitrogen in the molecule. The pK_{a2} value of ICRF-187 (9.70 ± 0.01) was in agreement with the pK_{a2} of 4-MP (9.62) and similar to that reported by Hasinoff (6). Since ICRF-187 has two ionizable imide nitrogens but only one observed pK_a, this indicates that pK_{a2} is a complex term that consists of two overlapping pK_a values (4). From the curve fit of the hydrolysis of ICRF-187 under basic pH conditions, estimates of the ionization constants, K_{a2}' and K_{a2}'', for the two imide groups were determined to be 2.29×10^{-10} and 1.34×10^{-10}, respectively, corresponding to a pK_{a2}' of 9.64 and a pK_{a2}'' of 9.87. These results are in reasonable agreement with the estimated macroscopic pK_{a2} value determined spectrophotometrically.

The kinetics of the hydrolytic degradation of ICRF-187 and 4-MP followed pseudo-first-order kinetics under all conditions studied. Buffer catalysis was observed for the hydrolysis of both compounds, although minimally in some instances, and depended on the compound, buffer system, and pH. For the instances where the rate of hydrolysis exhibited buffer dependence, reasonably linear plots were obtained when the observed pseudo-first-order rate constants were plotted against the total buffer concentration at a constant pH. The criterion used to indicate if the data exhibited buffer dependence was whether the slope was significantly different from zero.

The experimental pH–rate profile of 4-MP (Fig. 2) deviates significantly from what was expected in the acidic pH region based on earlier findings with other imides. A bell-shaped curve is evident between pH 1 and pH 6 and the reactivity was significantly greater than that of 3-MG (4), which showed no degradation over 6 weeks at 25°C and pH 2 and 7. There are two situations that generally give rise to a bell-shaped pH–rate profile. First, a bell-shaped curve has two inflection points, a reflection of two acid/base ionization equilibria, where the intermediate species is the most reactive (18–20). Alternatively, a bell-shaped pH–rate profile can be diagnostic of a change in the rate-determining step. Since a change in the rate-determining step cannot occur unless there are at least two consecutive steps and one intermediate in a reaction, the presence of the bell-shape may be a useful method for recognizing the existence of intermediates (21–32).

The bell-shaped curve for 4-MP cannot be attributed to the two ionizations since the pK_a values, 2.68 and 9.62, are well separated and are not consistent with the inflection points in the profile; instead, the shape represents an expression of the kinetics of the reaction. No intermediate was isolated or observed during the reaction, however, it is reasonable to postulate the presence of a tetrahedral intermediate based on extensive literature data on the existence of this type of species (21,22) for the hydrolysis of carbonyl compounds. The reaction scheme shown in Scheme I is proposed to explain the bell shape that occurs between pH 1 and pH 6.

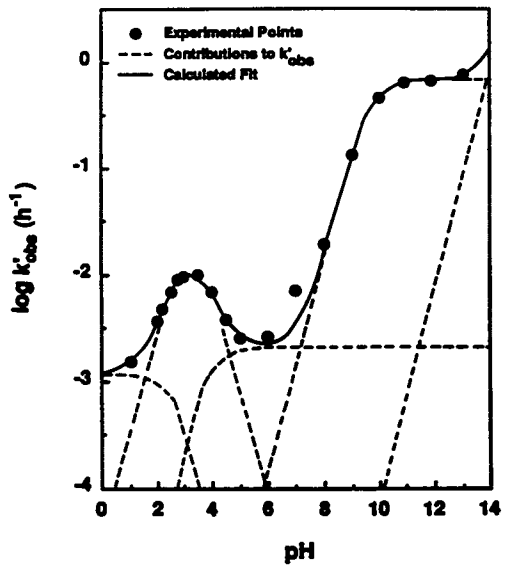


Fig. 2. The pH–rate profile for the hydrolysis of 4-MP at 25°C and ionic strength 0.5 (sodium chloride). The filled circles are the experimentally determined values of k'_{obs}, while the solid line is calculated from the fit of the data to Eq. (4) at pH values less than neutrality and Eq. 3 from Ref. 1 in the alkaline pH range. The dashed lines represent the individual contributions from each of the complex rate constants *a–g* and of both of the bimolecular rate constants, k'_{OH} and k''_{OH}, as defined by Eq. 3 of Ref. 1.

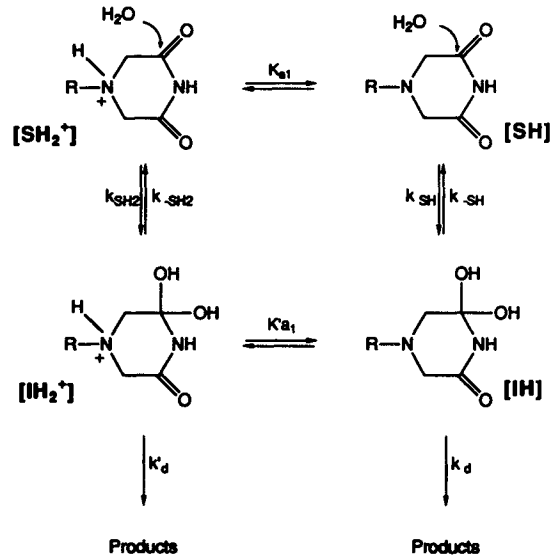
Mathematically, using Eq. (1), an expression for k'_{obs} can be written for any kinetic system provided that the steady-state assumption is applicable (33):

$$k'_{obs} = \text{(flux from reactants going to the intermediates)}\ \text{(fraction of intermediates that go to products)} \quad (1)$$

where k'_{obs} is the buffer-independent observed rate constant for the hydrolysis reaction. The steady-state approximation is applicable to the hydrolytic degradation of 4-MP, since no intermediate was observed to accumulate in the hydrolysis reaction. Therefore, employing Eq. (1), an expression for k'_{obs} can be written that is consistent with Scheme I and adequately describes the bell-shaped profile for the hydrolysis of 4-MP.

$$k'_{obs} = (k_{SH}f_{SH} + k_{SH2}f_{SH2})\left(\frac{k_d f_{IH} + k'_d f_{IH2}}{k_d f_{IH} + k'_d f_{IH2} + k_{-SH}f_{IH} + k_{-SH2}f_{IH2}}\right) \quad (2)$$

where k_{SH2} and k_{-SH2} represent the forward and reverse rate constants for the addition and loss of water to SH_2^+, respectively; k_{SH} and k_{-SH} represent the forward and reverse rate constants for the addition and loss of water to SH, respectively; k_d and k'_d represent the rate constants for the decomposition of the tetrahedral intermediates, IH_2^+ and IH, respectively; f_{SH2} and f_{SH} are the fractions of the monoprotonated and neutral species of 4-MP, respectively; and f_{IH2} and f_{IH} are the fractions of the monoprotonated and neutral tetrahedral intermediates of 4-MP, respectively. Since the pK_a values of 4-MP are well separated (pK_{a1} = 2.68 and pK_{a2} = 9.62), only one ionization need be considered within the pH range of 1 to 6. Substitution of the expressions for the fractions of the various species involved in the process, f_{SH2}, f_{SH}, f_{IH2}, and f_{IH}, yields Eq. (3):



Scheme I. The proposed reaction scheme for the hydrolysis of 4-MP and ICRF-187 over the pH range 1 to 6. This scheme is the basis for Eq. (4) used to fit the experimental data. SH_2^+ and SH represent the monoprotonated and neutral species of 4-MP or ICRF-187, respectively, and IH_2^+ and IH represent the monoprotonated and neutral tetrahedral intermediates of 4-MP and ICRF-187, respectively.

$$k'_{obs} = \left(\frac{k_{SH}K_{a1} + k_{SH2}[H^+]}{[H^+] + K_{a1}}\right)\left(\frac{k_d K'_{a1} + k'_d[H^+]}{\{k_d K'_{a1} + k'_d[H^+] + k_{-SH}K'_{a1} + k_{-SH2}[H^+]\}}\right) \quad (3)$$

where K_{a1} and K'_{a1} are the ionization constants of 4-MP and the tetrahedral intermediate, respectively. Multiplying, factoring, and collecting terms lead to Eq. (4):

$$k'_{obs} = \frac{a + b[H^+] + c[H^+]^2}{d + f[H^+] + g[H^+]^2} \quad (4)$$

where $[H^+]$ represents the hydrogen ion concentration, and a, b, c, d, f, and g are complex terms that are the composite of the sums and products of rate and dissociation constants shown in Table I:

From the dependence of Eq. (4) on $[H^+]$, this expression can be shown to account qualitatively for the bell-shaped curve in the pH–rate profile of 4-MP. At very low pH values where $[H^+] \gg K_{a1}$ and K'_{a1}, Eq. (4) collapses to a constant value defined by Eq. (5), which is consistent with the experimental observations.

$$k'_{obs} = \frac{c}{g} = \frac{k_{SH2}k'_d}{k_{-SH2} + k'_d} \quad (5)$$

Essentially c/g would represent the observed rate constant for the water or spontaneous degradation of monoprotonated 4-MP.

At higher pH's, where $[H^+]$ is very small, Eq. (4) simplifies to Eq. (6), also consistent with the experimental data.

$$k'_{obs} = \frac{a}{d} = \frac{k_{SH}k_d}{k_{-SH} + k_d} \quad (6)$$

Essentially a/d would represent the observed rate constant for the water or spontaneous degradation of monoprotonated 4-MP.

The data for k'_{obs} generated over the pH range 1 to 6 were fit to Eq. (4) (see Fig. 2) by a nonlinear curve-fitting program. The coefficients (i.e., the complex constants a–g) from the nonlinear analysis that are capable of defining the shape of the profile are reported in Table I. Included in the pH–rate profile is the hydrolysis data for 4-MP in the neutral to basic pH range.

Table I. The Values of the Coefficients (Complex Constants) a–g Generated from the Nonlinear Curve Fit of the Experimental Data to Eq. (4) for the Hydrolysis of 4-MP and ICRF-187 from pH 1 to pH 6

	4-MP	ICRF-187	Units
$a = k_{SH}k_dK_{a1}KK'_{a1}$	6.93×10^{-3}	1.12×10^{-2}	$hr^{-2}\,M^2$
$b = k_{SH}k'_dK_{a1} + k_{SH2}k_dK'_{a1}$	2.86×10^{2}	2.04×10^{3}	$hr^{-2}\,M$
$c = k_{SH2}k'_d$	9.78×10^{3}	1.76×10^{5}	hr^{-2}
$d = k_{-SH}K_{a1}K'_{a1} + k_dK_{a1}K'_{a1}$	3.38	2.82	$hr^{-1}\,M^2$
$f = k_{-SH2}K_{a1} + k'_dK_{a1} + k_{-SH}K'_{a1} + k_dK'_{a1}$	1.87×10^{4}	3.27×10^{5}	$hr^{-1}\,M$
$g = k_{-SH2} + k'_d$	8.38×10^{6}	1.20×10^{8}	hr^{-1}

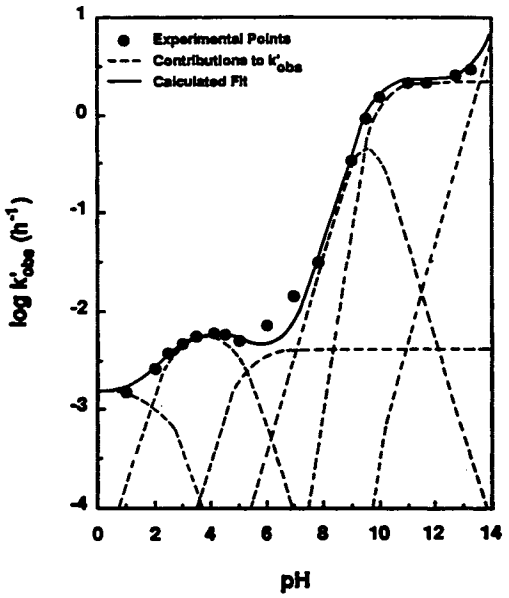
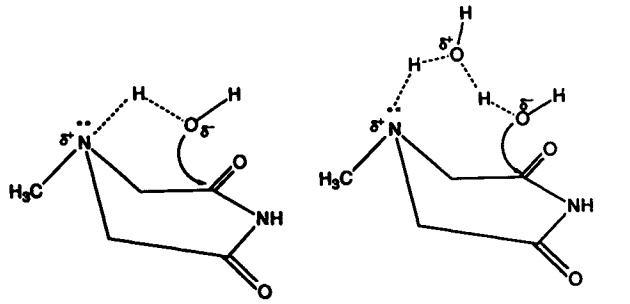


Fig. 3. The pH–rate profile for the hydrolysis of ICRF-187 at 25°C and ionic strength 0.5 (sodium chloride). The filled circles are the experimentally determined values of k'_{obs}, while the solid line is calculated from the fit of the data to Eq. (4) at pH values less than neutrality and Eq. 4 from Ref. 1 in the alkaline pH range. The dashed lines represent the individual contributions from each of the complex rate constants *a–g* and the bimolecular rate constants, k_{OH}, k'_{OH}, and k''_{OH}, as defined by Eq. 3 of Ref. 1.

Like 4-MP, the pH–rate profile for ICRF-187 is bell-shaped (see Fig. 3) in the acidic pH region and cannot be accounted for by the ionization of the compound. In the case of ICRF-187, the bell is less pronounced and could be easily missed if there was considerable scatter in the experimental data. The values for k'_{obs} for ICRF-187 generated over the pH range 1 to 6 were also fit to Eq. (4) and the coefficients (i.e., the complex constants *a–g*) from the nonlinear fit capable of defining the shape of the profile are also reported in Table I. Included in the pH–rate profile is the hydrolysis data for ICRF-187 in the neutral to basic pH range.

In the pH range of approximately 5 to 6 for 4-MP and 5 to 5.5 for ICRF-187, the proposed mechanism is water attack on the neutral species, a bimolecular mechanism. This is defined in Scheme I by the rate constant k_{SH} and assumes that $k_d > k_{SH}$ in Eq. (6). Possible transition states for this mechanism are illustrated in Scheme II. This scheme is consistent with the greater reactivity of ICRF-187 and 4-MP compared to 3-MG, where there is a carbon atom in place of the tertiary amine.

The activation parameters E_a, $\Delta S‡$, and $\Delta H‡$ were determined for the hydrolysis of ICRF-187 and 4-MP at pH 5.0 at four temperatures covering the range of 25 to 70°C for ICRF-187 and 25 to 80°C for 4-MP. An Eyring plot of the data for 4-MP given in Fig. 4 yielded a value for $\Delta S‡$ of -16.5 ± 0.8 eu. A similar plot of the data for ICRF-187 given in Fig. 5 yielded a value for $\Delta S‡$ of -10.6 ± 1.5 eu. The values of $\Delta H‡$ determined for these data were 16.4 ± 0.3 and 17.9 ± 0.5 kcal mol^{-1} for 4-MP and ICRF-187, respectively. Arrhenius treatment of the same data gave an E_a for 4-MP of 15.8 kcal mol^{-1} and an E_a for ICRF-187 of 17.2 kcal



Scheme II. The proposed anchimeric assistance of the piperazine nitrogen for the water attack on the neutral species of ICRF-187 and 4-MP.

mol^{-1}. In general, a bimolecular reaction will have a negative entropy of activation ($\Delta S‡$). Bimolecular A_2 reactions of acyl compounds have $\Delta S‡$ values which are usually more negative than -15 eu, while other bimolecular reactions have $\Delta S‡$ values in the range of -5 to -15 eu, with some overlap observed (34). The values of $\Delta S‡$ for 4-MP and ICRF-187 are consistent with these observations and support a bimolecular reaction.

The kinetic solvent isotope effect (KSIE) for the hydrolytic degradation of 4-MP and ICRF-187 was determined by measurement of the hydrolytic rate of these compounds at pH = pD 5.0 for ICRF-187 and at 5.5 for 4-MP in D_2O and H_2O at 25°C. The results are presented in Table II. The experimental values of 1.56 for ICRF-187 and 1.77 for 4-MP are hybrid values which represent contributions from at least three mechanisms that overlap at the pH values where the KSIE was studied. The experimental KSIE values obtained for ICRF-187 and 4-MP are consistent with the major contribution to the KSIE being the water attack on the neutral species (normal KSIE >2), with the effect partially offset by

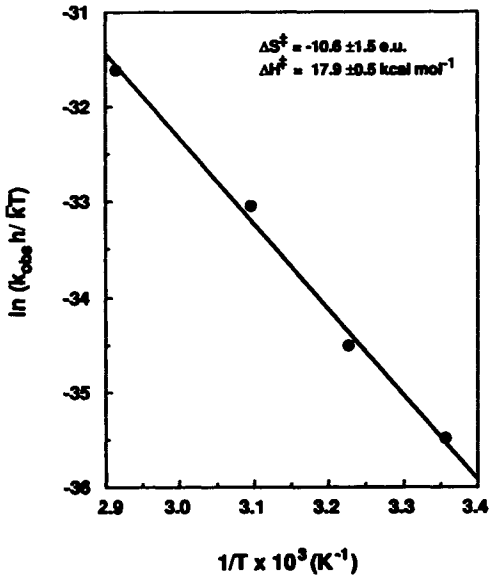


Fig. 4. An Eyring plot for the hydrolysis of 4-MP at 25, 37, 50, and 80°C in 0.05 *M* acetate buffer at pH 5.0 and a constant ionic strength of 0.5 (sodium chloride). The activation parameters $\Delta S‡$ and $\Delta H‡$ were determined from the zero intercept and the slope, respectively, and are weighted averages.

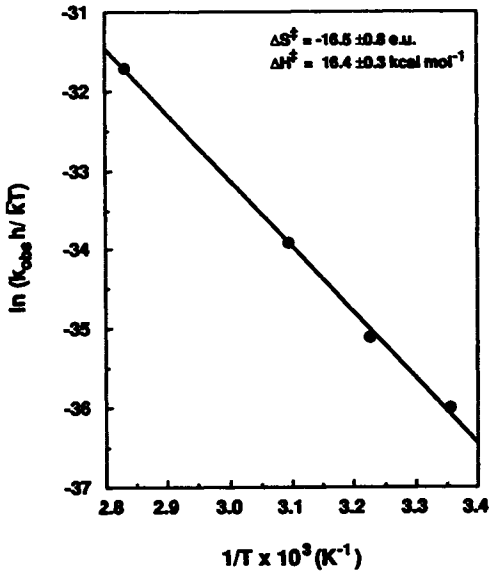


Fig. 5. An Eyring plot for the hydrolysis of ICRF-187 at 25, 37, 50, and 70°C in 0.05 *M* acetate buffer at pH 5.0 and a constant ionic strength of 0.5 (sodium chloride). The activation parameters $\Delta S‡$ and $\Delta H‡$ were determined from the zero intercept and the slope, respectively, and are weighted averages.

the inverse KSIE for the other overlapping mechanisms (35–38). The competing mechanisms are the residual effect of the base-catalyzed reaction which would result in a KSIE of 0.5 and the beginning of a contribution from the spontaneous solvolysis of the protonated species which would give an expected KSIE of 0.3 (35–38).

Water attack on the protonated species appears to be a reasonable hydrolytic mechanism between pH 3.2 and pH 5.0 for 4-MP and between pH 4.0 and pH 5.0 for ICRF-187. This mechanism is consistent with the pH–rate profiles in that the rate of hydrolysis increases as the pH decreases in the mildly acidic pH region. The piperazine nitrogens of both compounds begin to be protonated as the pH is decreased (pK_{a1} 2.68 for 4-MP and pK_{a1} 2.47 for ICRF-187). The protonation of the piperazine nitrogen should act in an electron withdrawing manner to increase the electrophilic character of the carbonyl carbon, with the result of that center becoming more susceptible to nucleophilic attack.

Table II. KSIE for the Hydrolysis of ICRF-187 at pH = pD 5.0 and 4-MP at pH = pD 5.5 at 25°C, in Sodium Acetate Buffer, and ICRF-187 at pH = pD 4.0, and 4-MP at pH = pD 3.2 at 25°C, in Sodium Formate Buffer, at a Constant Ionic Strength of 0.5 (Sodium Chloride) in H_2O and D_2O

Compound	pH/pD	Solvent	$k_{obs} \times 10^3$ (hr^{-1})	k_{H_2O}/k_{D_2O}
ICRF-187	5.0	H_2O	5.82	1.56
		D_2O	3.73	
	4.0	H_2O	4.96	1.41
		D_2O	3.53	
4-MP	5.5	H_2O	2.45	1.77
		D_2O	1.38	
	3.2	H_2O	10.52	1.42
		D_2O	7.39	

The rate of hydrolysis was expected, a priori, to continue to increase as the pH decreased until the substrate became fully protonated. Once the substrate became fully protonated, the rate of hydrolysis was expected to be constant. Instead, the hydrolysis rate decreased for both ICRF-187 and 4-MP as the pH decreased. The result was the bell-shaped pH–rate profiles for ICRF-187 and 4-MP, which may be explained by a change in the rate-determining step to account for the slower rate. Scheme I illustrates the proposed change in the slow step of the reaction going from the formation of the tetrahedral intermediate (water addition to the neutral species) to the rate-determining breakdown of the tetrahedral intermediate in the pH range of about 1.2 to 3.2 for 4-MP and 1.2 to 4.0 for ICRF-187. The breakdown of the neutral tetrahedral intermediate may be facilitated by the assistance of the piperazine nitrogen in the abstraction of a proton from one of the hydroxyl groups or possibly through a bridging water molecule. This facilitation by the piperazine nitrogen can occur only in the unprotonated intermediate, an indication of the existence of an equilibrium between the protonated and the neutral tetrahedral intermediates. As the pH is decreased, there is less of the neutral tetrahedral form available, which results in a decreased rate of tetrahedral intermediate breakdown. Essentially, $k_{SH2} \gg k'_d$ in Eq. (5).

A KSIE for the hydrolytic degradation of 4-MP and ICRF-187 was performed in an attempt to shed light on this proposed change in mechanism. The measurement of the hydrolytic rate of these compounds was performed at pH = pD 4.0 for ICRF-187 and at 3.2 for 4-MP in D_2O and H_2O at 25°C. The results are presented in Table II. The experimental KSIE values of 1.41 for ICRF-187 and 1.42 for 4-MP are hybrid values since there is not a single mechanism operating in this pH range. Because the experimental conditions were such that the KSIE was studied at the maximum of the bell-shaped curves, where overlap of a number of mechanisms and the presence of various ionic and nonionic species would be expected, it was not possible to interpret these results fully. It was surprising, however, that the KSIE was as small as ≈1.4. A complete pH–rate profile in D_2O and proton inventory studies would better probe the mechanism of ICRF-187 and 4-MP but was not attempted here.

At very low pH (<1.2), the equilibrium is such that the substrate and tetrahedral intermediate exist only in the protonated state. The nitrogen is no longer available to assist in the breakdown of the neutral intermediate and thus the breakdown of the protonated tetrahedral intermediate becomes rate-determining. This mechanism is consistent with the pH–rate profile in that the hydrolysis rate constant was observed to level off at pH 1 for both compounds.

In summary, the acid pH hydrolysis kinetics of ICRF-187 and the model compound, 4-MP, show them to be hyperreactive compared to imides without the tertiary piperazine nitrogen in the 4 position of the ring. Earlier studies (4) demonstrated that the nitrogen plays a role in the base hydrolysis of these two compounds. The pH–rate profiles for ICRF-187 and 4-MP show an unexpected bell shape under acid pH conditions. A change in rate-determining step from tetrahedral intermediate formation (slightly acidic pH values) to tetrahedral intermediate breakdown (more acidic pH

values) determined by the state of ionization of the nitrogen in the molecules and in the tetrahedral intermediates appear to account for the profiles.

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