JOC Article

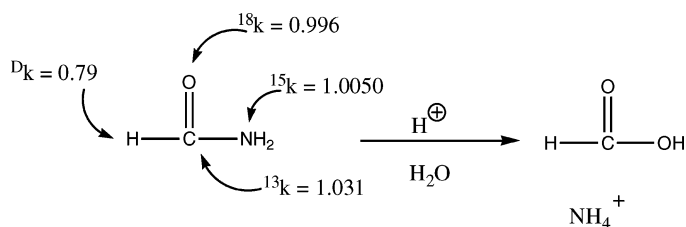# Multiple Isotope Effect Study of the Acid-Catalyzed Hydrolysis of Formamide

John F. Marlier,*,[†] Erica Campbell,[†] Catherine Lai,[†] Michael Weber,[†]
Laurie A. Reinhardt,[‡] and W. W. Cleland[‡]

*Department of Chemistry and Biochemistry, California Polytechnic State University,
San Luis Obispo, California 93407, and the Institute for Enzyme Research, Department of Biochemistry,
University of Wisconsin, Madison, Wisconsin 53726*

jmarlier@calpoly.edu

Multiple isotope effects were measured at the reactive center of formamide during acid-catalyzed hydrolysis in water at 25 °C. The mechanism involves a rapid pre-equilibrium protonation of the carbonyl oxygen, followed by the formation of at least one tetrahedral intermediate, which does not appreciably exchange its carbonyl oxygen with the solvent ($k_h/k_{ex} = 55$). The pK_a for formamide was determined by ^{15}N NMR and found to be about −2.0. The formyl-hydrogen kinetic isotope effect (KIE) is indicative of a transition state that is highly tetrahedral ($^Dk_{obs} = 0.79$); the carbonyl-carbon KIE ($^{13}k_{obs} = 1.031$) is in agreement with this conclusion. The small leaving-nitrogen KIE ($^{15}k_{obs} = 1.0050$) is consistent with some step prior to breaking the C−N bond as rate-determining. The carbonyl-oxygen KIE ($^{18}k_{obs} = 0.996$) points to attack of water as the rate-determining step. On the basis of these results, a mechanism is proposed in which attachment of the nucleophile to a protonated formamide molecule is rate determining.

Introduction

The hydrolysis of acyl groups is a reaction of central importance in chemistry and biology. Several reactions underscore this importance, such as the formation of organic polymers (polyesters and nylons) in chemistry and the hydrolysis of proteins, lipids, and acetyl choline in biology. In addition, esters and thioesters frequently serve as enzyme-bound intermediates. Consequently, these reactions have been investigated by most common physical organic methods,[1] including kinetics, stereochemistry, linear free-energy relationships, and solvent effects. Methods employing stable isotopes as isotopic tracers[2] and, most recently, in isotope effects have proved particularly useful.[3] Most

acyl groups with poor leaving groups react via a stepwise mechanism involving one or more tetrahedral intermediates (an example is shown later in eq 3). Considerable evidence supports the conclusion that acyl groups with very good leaving groups (such as halides and *p*-nitrophenol) can cause the tetrahedral intermediate to become unstable and result in either a dissociative mechanism (eq 1, Scheme 1) or a concerted mechanism (eq 2, Scheme 2).[4]

Amides are the least reactive of the common acyl groups as a result of the poor leaving ability of amines. Most alkyl amides appear to react via the stepwise mechanism involving a tetrahedral intermediate (or intermediates). The hydrolysis occurs under both acidic and basic conditions.[5a−c] The reaction under

[†] California Polytechnic State University.
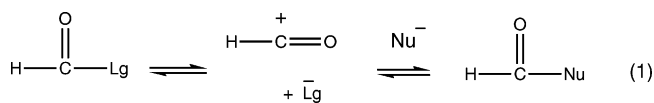[‡] University of Wisconsin.
(1) (a) Bender, M. L. *Chem. Rev.* **1960**, *60*, 53. (b) Johnson, S. L. *Adv. Phys. Org. Chem.* **1967**, *5*, 237. (c) O'Conner, C. J. *Q. Rev., Chem. Soc.* **1971**, *24*, 553. (d) Jencks, W. P. *Chem. Rev.* **1972**, *72*, 705.
(2) (a) Bender, M. L.; Heck, H. d'A. *J. Am. Chem. Soc.* **1967**, *89*, 1211. (b) Bender, M. L.; Thomas, R. J. *J. Am. Chem. Soc.* **1961**, *83*, 4189. (c) Shain, S. A.; Kirsch, J. F. *J. Am. Chem. Soc.* **1968**, *90*, 5848.
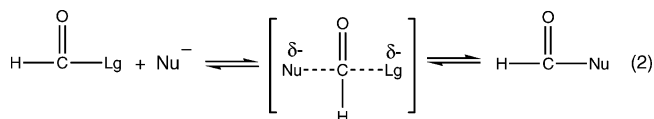
(3) Marlier, J. F. *Acc. Chem. Res.* **2001**, *34*, 283.
(4) (a) Ba-Saif, S.; Luthra, A. K.; Williams, A. *J. Am. Chem. Soc.* **1987**, *109*, 6362. (b) Ba-Saif, S.; Luthra, A. K.; Williams, A. *J. Am. Chem. Soc.* **1989**, *111*, 2647. (c) Ba-Saif, S.; Colthust, M.: Waring, M. A.; Williams, A. *J. Chem. Soc., Perkin Trans. 2* **1991**, 1901. (d) Guthrie, J. P. *J. Am. Chem. Soc.* **1991**, *113*, 3941. (e) Stefandis, D.; Jencks, W. P. *J. Am. Chem. Soc.* **1993**, *115*, 6045. (f) Hengge, A. C.; Hess, R. A. *J. Am. Chem. Soc.* **1994**, *116*, 11256.

SCHEME 1

$$H-\overset{\overset{O}{\parallel}}{C}-Lg \rightleftharpoons \left[H-\overset{+}{C}=O \quad + \quad \overset{-}{Lg}\right] \overset{Nu^-}{\rightleftharpoons} H-\overset{\overset{O}{\parallel}}{C}-Nu \quad (1)$$

SCHEME 2

$$H-\overset{\overset{O}{\parallel}}{C}-Lg + Nu^- \rightleftharpoons \left[\overset{\delta-}{Nu}----\overset{\overset{O}{\parallel}}{\underset{H}{C}}----\overset{\delta-}{Lg}\right] \rightleftharpoons H-\overset{\overset{O}{\parallel}}{C}-Nu \quad (2)$$

basic conditions has been extensively studied, and the details of this reaction are quite well-understood, except for some controversy concerning the nature of the attacking nucleophile.[6] The acid-catalyzed hydrolysis is more poorly understood. It is known that the kinetics of amide hydrolysis[5a] (including formamide[5b]) are usually first order with respect to acid at lower concentrations (1 M or less), becoming independent of acid at higher concentrations. PIX (positional isotope exchange) experiments have been used to determine the rate of the incorporation of an oxygen atom from the solvent into the carbonyl group of the starting material.[5a,c] The results of these experiments are quantitatively expressed as a ratio of the rate constant for hydrolysis over that for exchange, k_h/k_{ex}. For the acid-catalyzed hydrolysis of amides with alkylamine or $-NH_2$ leaving groups, this ratio is usually much greater than unity, indicating that formation of the tetrahedral species is rate-determining. In addition, PIX experiments on anilides show that k_h/k_{ex} ratios increase with the increasing pK_a of the leaving anilide.[5a] This is consistent with the hypothesis that more basic leaving-N atoms will more readily acquire a proton in the transition state (see step 3, eq 3, Scheme 3), resulting in an increase of the C−N/C−O bond cleavage ratio.

The case for the stepwise mechanism involving tetrahedral intermediates is based on empirical observations, which include the PIX experiments described above, as well as theoretical calculations. Brown et al. has summarized the experimental evidence;[5a,c] these include the observations that the hydronium ion dependency for hydrolysis and exchange are the same and that the solvent deuterium isotope effect on k_h/k_{ex} is unity. Together these findings argue that hydrolysis and exchange share a common pathway. Theoretical calculations agree with this assessment. Any concerted mechanism (eq 2) must involve proton transfer to the leaving-N during a rate-determining attack of the nucleophile on the carbonyl-C. Calculations in a recent paper by Kim et al.[5d] support the fact that the energy of the N-protonation pathway for formamide is higher than that of the O-protonation pathway. As a result of the above, the favored mechanism involves the rate-determining attack of water (assisted by a second water molecule) on the protonated amide, as shown in eq 3.[5a,c]

Although PIX experiments have been a part of the study of amide hydrolysis for about 50 years, the use of stable isotopes in kinetic isotope effect (KIE) studies is more recent. A complete

(5) (a) Brown, R. S.; Bennet, A. J.; Slebocka-Tilk, H. *Acc. Chem. Res.* **1992**, *25*, 481 and references therein. (b) Kribble, V. K.; Holst, K. A. *J. Am. Chem. Soc.* **1938**, *60*, 2976. (c) Bennet, A. J.; Slebocka-Tilk, H.; Brown, R. S.; Guthrie, J. P.; Jodhan, A. *J. Am. Chem. Soc.* **1990**, *112*, 8497. (d) Monojkumar, T. K.; Suh, S. B.; Oh, K. S.; Cho, S. J.; Cui, C.; Zhang, X.; Kim, K. S. **2005**, *70*, 2651.

(6) (a) Marlier, J. F.; Dopke, N. C.; Johnstone, K. R.; Wirdzig, T. J. *J. Am. Chem. Soc.* **1999**, *121*, 4356. (b) Slebocka-Tilk, H.; Neverov, A. A.; Brown, R. S. *J. Am. Chem. Soc.* **2003**, *125*, 1851.

multiple KIE study of the alkaline hydrolysis of formamide has been reported; this work includes the formyl-H ($^Dk = 0.80$), the carbonyl-C ($^{13}k = 1.0321$), the carbonyl-O ($^{18}k = 0.980$), the nucleophile-O ($^{18}k = 1.022$), and the leaving-N ($^{15}k = 1.0040$) isotope effects.[6] At relatively low hydroxide concentration (0.1−0.2 M), the tetrahedral intermediate has a charge of -1 (T^{-1}), and PIX experiments ($k_h/k_{ex} = 2.1$) show that the partitioning of T^{-1} back to amide and forward to formate occurs at nearly the same rate. Consequently, the energy of the transition state leading into T^{-1} is nearly the same as that for its breakdown. As the hydroxide concentration is raised, the tetrahedral intermediate loses a second proton and hydrolysis becomes favored over the return to amide (i.e. k_h/k_{ex} increases). The above KIEs, measured at low-hydroxide concentration, are consistent with transition states for the formation and breakdown of T^{-1}, which are both sp³-like and resemble the structure of T^{-1}. The nucleophile-O KIE was interpreted as arising from attack of a water molecule, which is coordinated to OH^- ($^{18}k = 1.022$).[6]

It is clear that considerable detail concerning the structure of the transition state has been gained by multiple KIE studies of the hydrolysis of esters and amides. This paper reports the results of a PIX experiment and multiple KIE experiments (the formyl-H, the carbonyl-C, the carbonyl-O, and the leaving-N) for the acid-catalyzed hydrolysis of formamide. These KIEs are interpreted in terms of the stepwise mechanism; the evidence for this mechanism has been summarized above. Unlike the recently reported multiple KIEs for the acid-catalyzed hydrolysis of methyl formate, a nucleophile-O KIE is not possible in this case because the acid-catalyzed hydrolysis of formamide is much slower than the exchange of the oxygen atoms of formic acid with water.

Results

The rate constants for the acid-catalyzed hydrolysis of formamide in aqueous acid at 25 °C were determined in various concentrations of H_2SO_4 and in 1.0 M HCl. The rate constants in H_2SO_4 were measured by a continuous UV spectrophotometric method; the rate constant in HCl was measured by a point assay using formate dehydrogenase. No salts were added to control ionic strength. The rate constants were 0.0068 min^{-1} in 1.0 M HCl and 0.0173 min^{-1} in 1.0 M H_2SO_4, consistent with the fact that HCl is monoprotic and H_2SO_4 is diprotic. Due to the ease of analysis, the subsequent PIX experiment and isotope effects were performed in 1.0 M HCl. An investigation of the effect of increasing [H_2SO_4] on the rate constant showed that rate constants level off between $H_0 = 0.5$ and 1.0, as has been observed previously.[5b]

The exchange of the carbonyl-O with the solvent was carried out in 1.0 M HCl containing water that was 23.5 atom % ^{18}O. The reaction was quenched after 53% hydrolysis. Unreacted formamide was isolated by extraction with ethyl acetate. The amount of exchange (k_h/k_{ex}) was calculated from the above information and the measured relative abundance ratio (m/z 47/45), as determined by GC-MS. There was a barely detectable amount of exchange into formamide ($k_h/k_{ex} = 55$). It is estimated from the precision of the GC-MS measurements that a k_h/k_{ex} of about 100 would be detectable.

The carbonyl-C and carbonyl-O KIEs were measured in a single experiment. Because the oxygen atoms of formic acid exchange rapidly with the solvent, these KIEs had to be measured by isolation of residual substrate. After quenching
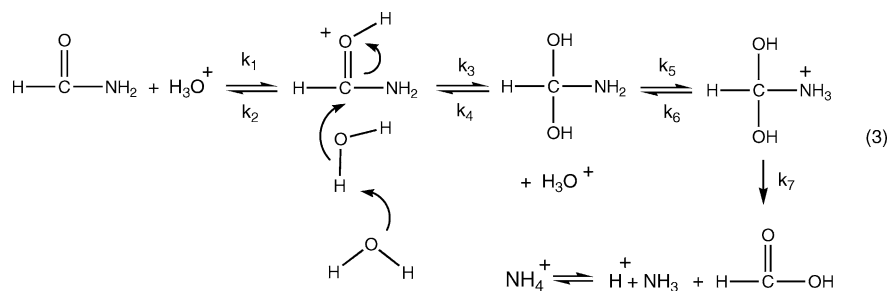
SCHEME 3

TABLE 1. Isotope Effects on the Acid-Catalyzed Hydrolysis of Formamide in 1.0 M HCl at 25 °C

atom	isotope effect, $k(\text{light})/k(\text{heavy})^{a,b}$
carbonyl-C	$1.031_3 \pm 0.001_3$ (13)
carbonyl-O	0.996 ± 0.002 (7)
formyl-H	0.79 ± 0.03 (5)
leaving-N	1.0050 ± 0.0007 (6)

[a] Corrected for percent reaction. [b] The standard deviation is given for each isotope effect; the number of determinations is given in parentheses.

by raising the pH to near neutrality, the unreacted formamide was isolated by ion exchange chromatography and then quantitatively hydrolyzed in alkaline solution. The % hydrolysis at quench was determined by assay with Nessler's reagent. The resulting formate was dried, oxidized by I_2 DMSO to CO_2, and analyzed by isotope ratio mass spectrometry, where the $^{13}\delta$ and $^{18}\delta$ are measured simultaneously.[6] One oxygen atom of formate is derived from the original carbonyl-O of formamide; the other is from the solvent. The KIEs were calculated from the above data and published equations.[7a] In past experiments, the carbonyl-O KIE was corrected for the amount of exchange with solvent. However, in the present case, no such correction was required because the amount of carbonyl-O exchange into formamide during acid-catalyzed hydrolysis was so small. The results of these experiments are summarized in Table 1.

The formyl-H KIE was measured by analysis of the residual substrate. After quenching, the unreacted formamide was isolated by extraction with ethyl acetate. The % reaction was determined via the formate dehydrogenase assay, and the $m/z = 46/45$ ratio was determined by GC-MS for the starting amide and that which was isolated at the quench. The KIE was calculated from the above information and published equations.[7b] These results are summarized in Table 1.

The leaving-N KIE was determined using the mass balance method. After quenching, both the unreacted formamide and the product, ammonium ion, were isolated by ion exchange chromatography. The % hydrolysis at quench was determined by assay with Nessler's reagent. The residual substrate was quantitatively hydrolyzed in alkali to formate plus ammonia, and the resultant ammonia was then steam distilled into an acidic solution. The ammonium ion product from the column was steam distilled directly into an acidic solution. The ammonium ion derived from the residual substrate and from the product was oxidized to N_2 by NaOBr prior to determination of the $^{15}\delta$ by isotope ratio mass spectrometry. The results are summarized in Table 1.

(7) (a) Bigeleisen, J.; Wolfsberg, M. *Adv. Chem. Phys.* **1958**, *1*, 15. (b) Bilkadi, Z.; de Lorimier, R.; Kirsch, J. F. *J. Am. Chem. Soc.* **1975**, *97*, 4317.
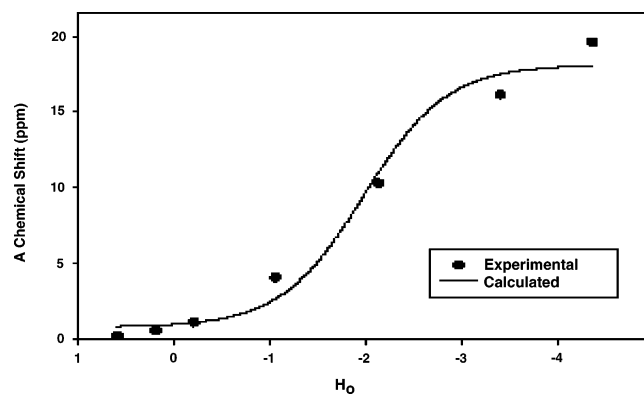

FIGURE 1. ^{15}N chemical shift for formamide as a function of H_0 in water at 25 °C. Chemical shifts are relative to the nitrogen of formamide in water at 25 °C.

The pK_a of formamide was estimated from changes in the ^{15}N chemical shift with increasing concentration of HCl (Figure 1). The chemical shifts are relative to formamide in water. Control experiments in high concentrations of NaCl (4.5 M) showed that there is a minimal effect of ionic strength on the ^{15}N chemical shifts for formamide.

Discussion

The acid-catalyzed hydrolysis of most amides has been shown to be first order in hydrogen ion and second order in water.[5a,c] Because the leaving group is poor, a mechanism involving a tetrahedral intermediate containing a positively charged nitrogen is considered likely. In addition, kinetic evidence suggests that any tetrahedral intermediates containing oxonium ions are too unstable to have a significant lifetime.[8] The mechanism shown in eq 3 fits this evidence. However, recent studies of the acid-catalyzed hydrolysis of methyl formate led to a proposed mechanism where water attack at the carbonyl-C and protonation of the carbonyl-O occur simultaneously.[9] This mechanism also provided a way to spread the developing positive charges to the solvent as a whole. This mechanism was then used to successfully explain why breakdown of the tetrahedral intermediate to the ester is faster than formation of formic acid ($k_h/k_{ex} = 11.3$) despite the fact that the pK_a for methanol (15.5) and water (15.7) are nearly identical. An analogous mechanism can be postulated for the acid-catalyzed hydrolysis of amides, but must take into account the evidence for a pre-equilibrium protonation step for amide hydrolysis.[5a,c] In this mechanism (eq

(8) (a) Yates, K.; MaClelland, R. A. *J. Am. Chem. Soc.* **1967**, *89*, 2686. (b) Yates, K. *Acc. Chem. Res.* **1971**, *4*, 136. (c) Cox, R. A. *Adv. Phys. Org. Chem.* **2000**, *35*, 1. (d) Jencks, W. P. *Acc. Chem. Res.* **1980**, *13*, 161.
(9) Marlier, J. F.; Frey, T. G.; Mallory, J. A.; Cleland, W. W. *J. Org. Chem.* **2005**, *70*, 1737.

SCHEME 4

$$(4)$$
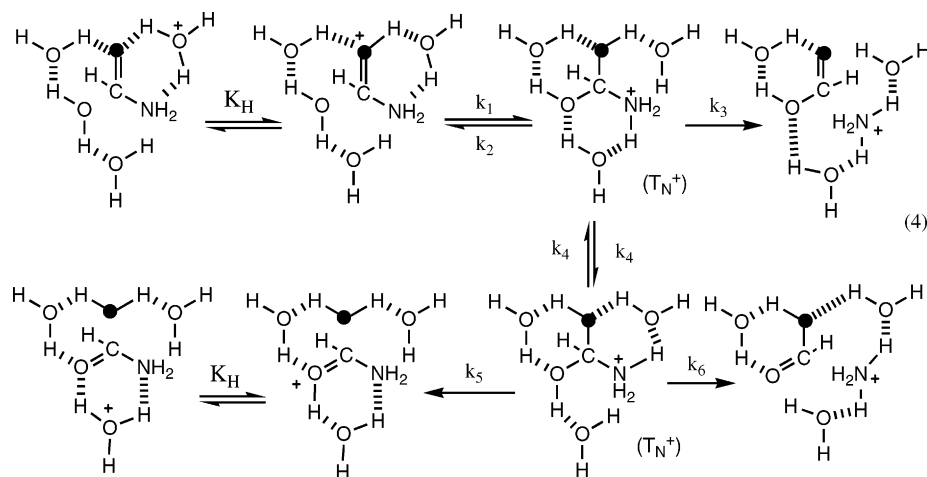
4, Scheme 4), this tetrahedral intermediate (T_N^+) differs from that in the acid-catalyzed ester hydrolysis because nitrogen is far more basic than oxygen and is likely to carry the bulk of the positive charge in both the transition state and the intermediate. T_N^+ may be considered a symmetrical tetrahedral intermediate in so much as the two forms of this intermediate differ only by a rapid proton transfer. In eq 4, the hashed lines represent hydrogen bonds; the symbol ● represents the oxygen from the original carbonyl of formamide.

Ignoring oxygen exchange (the bottom half of eq 4), the transition states for steps 2 and 3 are shown as structures **I** and **II**. Hydrating water molecules, which are not directly involved in bonding changes to atoms that experience isotope effects, have been omitted to simplify the structures. In these structures, the dashed lines represent bonds in transition; the symbol ● represents the oxygen from the original carbonyl of formamide.
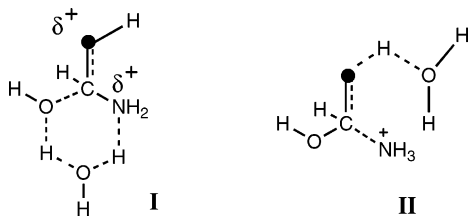
Carbonyl-O PIX Experiment. The lack of significant exchange of oxygen from the solvent into the carbonyl-O during hydrolysis ($k_h/k_{ex} = 55$) requires the breakdown of T_N^+ to formic acid and ammonium ion (k_3) to be much faster than the return to amide (k_2). In fact, because T_N^+ can return to amide via two pathways, one that leads to exchange (bottom half of eq 4) and one that does not (top half of eq 4), the ratio k_3/k_2 can be calculated to be 27.5 (k_3/k_2 is one-half of k_h/k_{ex}). An assumption in this calculation is that proton transfer between tetrahedral intermediates (k_4) is faster than the other chemical steps. It is clear that the lack of significant oxygen exchange is ultimately due to the large pK_a difference between the nitrogen leaving group and the oxygen leaving group of the tetrahedral intermediate. This result has three consequences. First, even the small amount of carbonyl-O exchange observed argues for the existence of a tetrahedral intermediate such as T_N^+. Second, the bottom portion of the mechanism given in eq 4 is relatively unimportant in interpreting the isotope effects. Third, the observed isotope effects arise largely from bonding changes on

going to the transition state for formation of T_N^+ (k_1). This can be shown mathematically as follows. Ignoring the bottom half of the mechanism in eq 4 and redefining k_1 to include the rapid pre-equilibrium protonation step, the relationship between the KIEs on the individual steps of the top portion of the mechanism and the observed KIE ($*k_{obs}$) for any isotope is given by eq 5

$$*k_{obs} = [*K_{eq}*k_3 + *k_1(k_3/k_2)]/[1 + (k_3/k_2)] \qquad (5)$$

In this equation, the asterisk refers to an isotope effect on equilibria (K) or on a rate constant (k). The term $*K_{eq}$ is the equilibrium isotope effect on the formation of T_N^+. Because the ratio k_3/k_2 is so large (27.5), the first numerator term of eq 5 is small compared to that of the second numerator term. As a result, $*k_{obs}$ reduces to the KIE on formation of T_N^+ (the redefined k_1) in the proposed mechanism, as shown in eq 6. All subsequent isotope effects will be interpreted using this assumption.

$$*k_{obs} \cong *k_1 \qquad (6)$$

The Formyl-H Isotope Effect. Secondary formyl-H KIEs have long been used to measure hybridization changes on going to the transition state for a host of organic reactions. In the hydrolysis of acyl groups formyl-H KIEs are inverse, reflecting a change in hybridization from sp^2 in the ground state to sp^3 in the transition state. For reactions involving tetrahedral intermediates, the transition state of highest energy can occur during formation or breakdown of the tetrahedral intermediate(s). The large inverse formyl-H KIE in the present case ($^Dk_{obs} = 0.79$) is near the calculated maximum magnitude expected for this type of acyl transfer reaction[10] and is interpreted as arising from a transition state with a high degree of sp^3 character that largely resembles the structure of a tetrahedral intermediate. Furthermore, the carbonyl-O PIX experiment fixes the formation of the tetrahedral intermediate as the rate-determining step. Taken together, these two results require a transition state that largely resembles T_N^+ of eq 4 and shown in structure **I**. A similar argument was presented for the acid-catalyzed hydrolysis of methyl formate,[9] where $^Dk_{obs} = 0.81$.

The degree of sp^3 character can be estimated from the published Wolfsberg–Stern-type calculations for the addition

---

(10) Hogg, J. L.; Rodgers, J.; Kovach, I.; Schowen, R. L. *J. Am. Chem. Soc.* **1980**, *102*, 79.

SCHEME 5
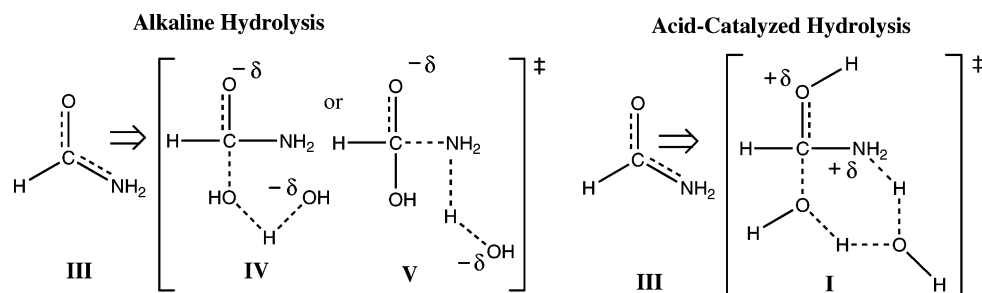
of hydroxide to acetaldehyde.[10] This is a reasonable model because the rate-determining step for formamide hydrolysis contains the addition of a heavy atom (oxygen) to a carbonyl-C containing a hydrogen atom and two heavy atoms (N and O), just like the model. The only significant difference is the fact that nitrogen is a much better electron donor than oxygen (by resonance) to the carbonyl-O. Using the results in Table 3 of ref 10 as a general guide, it is likely that the bond order to the nucleophile is approximately 0.8 in the transition state.

It is interesting to note that the mechanisms of the alkaline and acid-catalyzed hydrolyses of formamide are different even though they show very similar large inverse formyl-H KIEs ($^{D}k_{obs} = 0.80$ for alkaline; $^{D}k_{obs} = 0.79$ for acid). The alkaline hydrolysis mechanism, which contains terms that are first and second order in hydroxide, is far more complex than that for acid-catalyzed hydrolysis.[6] At low hydroxide concentration (where the first-order term dominates) there is considerable carbonyl-O exchange ($k_h/k_{ex} = 2.1$), indicating that the transition states for the formation and breakdown of the T^{-1} are of roughly equal energy in this case. At high hydroxide concentration, the mechanism branches through a second tetrahedral intermediate, T^{-2}, and the magnitude of k_h/k_{ex} increases as hydrolysis is favored. This is in striking contrast to the PIX and KIE experimental results just described for the acid-catalyzed hydrolysis.

The Carbonyl-C Isotope Effect. Both theoretical calculations and empirical evidence have established that the carbonyl-C KIE for addition of an oxygen nucleophile to a carbonyl-C is always normal and varies only slightly with the degree of hybridization change in the transition state. From a theoretical standpoint,[10] the calculated carbonyl-C KIE for the reaction of acetaldehyde with hydroxide varies only from $^{13}k_{obs} = 1.029$ to $^{13}k_{obs} = 1.015$ as the carbon-nucleophile bond order changes from 0.2 to 0.7. Empirical observations for the alkaline and acid-catalyzed hydrolyses of methyl formate agree closely with this prediction. The alkaline hydrolysis of methyl formate has an early, sp^2-like transition state (formyl-H: $^{D}k_{obs} = 0.95$) and exhibits a carbonyl-C KIE of $^{13}k_{obs} = 1.034$.[11] On the other hand, the acid-catalyzed hydrolysis of methyl formate has a late, sp^3-like transition state (formyl-H: $^{D}k_{obs} = 0.81$) but only a slightly smaller carbonyl-C KIE of $^{13}k_{obs} = 1.028$.[9] Similar results were reported for the alkaline hydrolysis of a series of aryl carbonates.[12] The only exception seems to be the carbonyl-C KIE for the hydrolysis of methyl benzoate. In this case, the carbonyl-C KIE is $^{13}k_{obs} = 1.0443$ for alkaline hydrolysis[13] but becomes significantly smaller ($^{13}k_{obs} = 1.026$) for acid-catalyzed hydrolysis.[14]

The similarity of the formyl-H KIEs for the acid-catalyzed and alkaline hydrolyses of formamide (acid, $^{D}k = 0.79$; alkaline, $^{D}k = 0.80$) and the above discussion lead to the expectation that the carbonyl-C KIEs for both reactions should be nearly equal in magnitude. This is exactly what is observed (alkaline, $^{13}k_{obs} = 1.032$; acid, $^{13}k_{obs} = 1.031$).

The Carbonyl-O Isotope Effect. In general, there are two components of the overall observed carbonyl-O KIE, namely, a normal KIE due to the breaking of the π-bond and an inverse KIE resulting from adding new stretching/bending modes in the transition state. It is believed that a combination of these two factors leads to the small magnitude of most-observed carbonyl-O KIEs.[3] However, interpretation of the carbonyl-O KIE for the acid-catalyzed hydrolysis of formamide ($^{18}k_{obs} = 0.996$) is complicated by comparison to that for alkaline hydrolysis ($^{18}k_{obs} = 0.980$).[6] The significantly *more inverse* KIE for alkaline hydrolysis is the opposite of what is expected. Why? To answer this question, one must consider the structure of the ground state-(s) and the structure of the transition state(s) for both reactions; these are given in structures **I** and **III** through **V** (Scheme 5; for simplicity, several waters of hydration have been omitted). In both cases, the large inverse formyl-H KIE points to a reaction where the sp$^2 \rightarrow$ sp^3 hybridization change at the carbonyl-C is similar and nearly complete in the transition state. If this is true, it follows that the extent of carbonyl π-bond cleavage on going from formamide to the sp^3-like transition state should be nearly the same in both cases. This should result in carbonyl-O KIEs of similar magnitude. The major difference is the formation of a new O–H σ bond to the carbonyl-O in the acid-catalyzed case. However, formation of this new σ bond in a very late transition state is expected to add an additional small inverse component to the observed KIE, which is not present in the alkaline case. So the expectation is for a *more inverse* KIE in the acid-catalyzed hydrolysis than in the alkaline case, as is observed in ester hydrolysis.[9,11,13,14] This is the opposite of what is observed.

How can this discrepancy be rationalized? Because the bonding to the carbonyl-C appears to be nearly tetrahedral in all transition states shown above, it seems likely that the differences could stem from three possibilities. First, the developing negative charge on the carbonyl-O during alkaline hydrolysis might be more strongly hydrated than the overall neutral charge to that atom in the transition state of acid-catalyzed hydrolysis.[13] This is undoubtedly true, but it is hard to see how stronger hydration to the carbonyl-O in the alkaline case could lead to such a significantly more inverse carbonyl-O KIE than formation of a formal O–H σ bond in the acid-catalyzed case. Clearly this is not a very likely explanation.
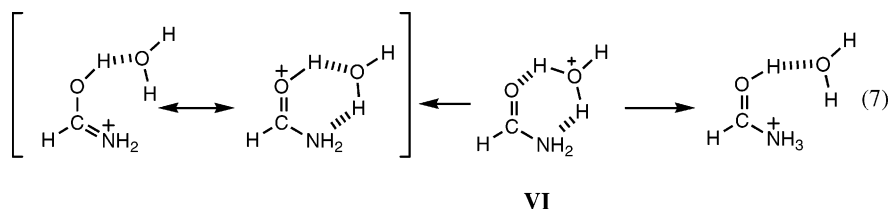
(11) Marlier, J. F. *J. Am. Chem. Soc.* **1993**, *115*, 5953.
(12) Marlier, J. F.; O'Leary, M. H. *J. Am. Chem. Soc.* **1990**, *112*, 5996.
(13) O'Leary, M. H.; Marlier, J. F. *J. Am. Chem. Soc.* **1979**, *101*, 3300.

(14) Marlier, J. F.; O'Leary, M. H. *J. Org. Chem.* **1981**, *46*, 2175.

SCHEME 6

Second, a significant fraction of leaving-group nitrogen atoms might be protonated in the ground state for acid-catalyzed hydrolysis. This would cause disruption of the resonance between the lone pair on nitrogen and the carbonyl-O and increased π-bond order in the ground state for the carbonyl-O. In turn, this would increase the factor that leads to a more normal isotope effect (i.e., breaking the π bond). The site of protonation of amides in acidic media has long been debated.[15] Although nitrogen is more electrophilic than oxygen, resonance theory dictates that the carbonyl-O of amides should be protonated more easily. This is illustrated in eq 7, where N-protonation is shown to the right of structure **VI** and the resulting resonance forms of O-protonation are shown to the left. The pK_a and the site of protonation of formamide have been studied by several methods, including proton NMR,[15a,b] IR spectroscopy,[15c] acid–base titration,[15d] theoretical calculation,[5d,15e] semiempirical thermodynamic cycle (a general method for many amides),[15f] and kinetic studies.[15g] Most estimated values for the pK_a of formamide range from -2 to -0.25 and pick oxygen as the site of protonation. Even though O-protonation is favored by most, some N-protonation is believed to occur. Perrin et al. have shown that proton exchange on the amide nitrogen proceeds via a protonated nitrogen.[15h,i] However, this is thought to occur through a minor pathway, which is not the same as that for hydrolysis. Recently, several carefully designed models with intramolecular proton donors have been shown to exchange amide protons rapidly at very low concentrations of weak acids via N-protonation.[15j] We revisited this issue via ^{15}N NMR in an attempt to see if specific protonation of the nitrogen atom of formamide could be observed. Unfortunately, it is still unclear from our results whether the observed change in chemical shift is due to N- or O-protonation, but the published evidence presented above and resonance theory clearly favor O-protonation. What is clear from the current study is that the pK_a of one of the atoms on formamide (N or O) appears to occur at about -2 (Figure 1) and is roughly within the range of the previously reported values. This means a significant proportion of formamide in 1 M HCl (the condition used in the current KIE study) is protonated. Because there is no good evidence for protonation occurring on nitrogen, this explanation for decreasing the π-bond character of the carbonyl does not seem likely.

The third possibility is loss of resonance in formamide because of strong hydrogen bonding in the acidic media (as seen in structure **VI**, Scheme 6). If the hydrogen bonds are strong enough to disrupt a significant amount of the resonance of the nitrogen lone pair (sp^2 character), this will increase the π-bond order in the ground state of the carbonyl-O in acid solution relative to what it is in alkali solution. This higher π-bond order must then be broken in the acid-catalyzed hydrolysis leading to a larger normal contribution to the observed KIE than is seen in the alkaline hydrolysis case. Of the three explanations proposed, this last one seems the most reasonable at the present time.

The Leaving-Nitrogen Isotope Effect. Because formation of the tetrahedral intermediate (k_1) is rate determining, it is clear that the transition state of the leaving-N must include the loss of any residual π-bond character and the formation of a new N–H bond. The loss of any π-bond character is apt to be small for reasons stated above and will lead to a very small normal KIE. The KIE on formation of an N–H bond is a primary KIE and will be controlled by two factors, the temperature-independent factor (TIF) and the temperature-dependent factor (TDF).[16] The TIF arises from motion of atoms along the reaction coordinate, and this term is always normal. Because hydrogen is lighter than nitrogen, it is likely that most of the motion will be due to the hydrogen. As a result, the TIF is expected to be a small, normal contribution. The TDF can be either normal or inverse; in this case, it will be inverse because of the introduction of the tighter bonding to the nitrogen in the transition state. Together these two contributions, plus the aforementioned loss of any residual resonance, point to a small normal or small inverse overall KIE, as is observed ($^{15}k = 1.0050$).

Conclusion. Results of a multiple KIE study of the acid-catalyzed hydrolysis of formamide are consistent with a stepwise mechanism involving at least one tetrahedral intermediate. In this mechanism, rapid protonation of the amide (p$K_a = -2$) is followed by the rate-determining attack of water, as evidenced by a leveling of the rate at high acid concentration and the lack of a sizable leaving-N KIE ($^{15}k = 1.0050$). The carbonyl-O was shown to exchange very little ^{18}O with the solvent, which is further evidence for the rate-determining formation of the tetrahedral intermediate, followed by a rapid breakdown to products. The formyl-H KIE is large and inverse ($^{D}k_{obs} = 0.79$), indicating that the transition state resembles the structure of the tetrahedral intermediate.

Experimental Section

Materials and Methods. Anhydrous DMSO, 1-d-formamide, 1-h-formamide, anhydrous ethyl acetate, anhydrous sodium carbonate, Nessler's reagent,[17] and MES. HEPES, NAD$^+$, formate dehydrogenase, and sublimed I$_2$ were obtained from chemical suppliers and were of the highest quality available. The isotopic composition of the carbonyl-O of the formamide used in the

(15) (a) Gillespie, R. J.; Birchall, T. *Can. J. Chem.* **1963**, *41*, 148. (b) Liler, M. *J. Chem. Soc. B* **1971**, 334. (c) Wu, C. C.; Jiang, J. C.; Hahndorf, I.; Chaudhuri, C.; Lee, Y. T.; Chang, H. C. *J. Phys. Chem. A* **2000**, *104*, 9556. (d) Wada, G.; Takenaka, T. *Bull. Chem. Soc. Jpn.* **1971**, *44*, 2877. (e) Fogarasi, G.; Szalay, P. G. *J. Phys. Chem. A* **1996**, *101*, 1400. (f) Fersht, A. R. *J. Am. Chem. Soc.* **1971**, *93*, 3504. (g) Mittal, S.; Gupta, K. S.; Gupta, Y. K. *Indian J. Chem.* **1982**, *21A*, 357. (h) Perrin, C. L. *Acc. Chem. Res.* **1989**, *22*, 268. (i) Perrin, C. A.; Dwyer, T. J.; Rebek, J.; Duff, R. J. *J. Am. Chem. Soc.* **1990**, *112*, 3122. (j) Cox, C.; Lectka, T. *Org. Lett.* **1999**, *1*, 749.

(16) Melander, L. *Reaction Rates of Isotopic Molecules*; Wiley: New York, 1980.

(17) Dawson, R. M. C.; Elliott, D. C.; Elliott, W. H.; Jones, K. M. *Data for Biochemical Research*; Clarendon Press: Oxford, 1969; p 619.

carbonyl-O and carbonyl-C KIEs ($^{13}\delta = -6.3$) was determined previously.[6] Isotope ratios for carbon, nitrogen, and oxygen in heavy-atom isotope effects were measured on an isotope ratio mass spectrometer. The $m/z = 46/45$ and $47/45$ of formamide for the measurement of the formyl-H KIE and carbonyl-O exchange were determined on a GC-MS with an XT-1 nonpolar column. Proton-decoupled ^{15}N NMR spectra (40 MHz) of formamide in water and in HCl were taken at 300 K; the D_2O lock solvent resided in a coaxial insert. The spectral width was 20.3 kHz in 32 K data points and 128 scans. The acquisition time was 0.40 s, and the relaxation delay was 3 s. Formamide (0.4 N) samples were prepared by the addition of ^{15}N formamide (8.1 uL, 0.2 mol) to H_2O and to seven different concentrations of HCl (aq; 0.2, 5, 1, 3, 6, 9, and 12 N). ^{15}N NMR spectra were taken immediately (<2 min) after the addition and mixing.

Determination of the Fraction of Reaction. The fraction of reaction for the formyl-H KIE was determined via a formate dehydrogenase assay. (a) Typically, a 50-μL aliquot of the quenched reaction mixture was added to 2000 μL of 0.10 M phosphate buffer at pH 7.6. A 50-μL aliquot of this diluted solution was added to a solution composed of 100 μL of 15 mM NAD^+ and 650 μL of the same phosphate buffer in a 1 mL quartz glass cuvette. The reaction was initiated by the addition of 200 μL of a formate dehydrogenase solution (~50 U/mL in the phosphate buffer), and the increase in absorbance at 340 nm was determined. Formamide was not reactive under these conditions. (b) To determine total formate plus formamide present at quench, a 50-μL aliquot of the quenched reaction mixture was mixed with 250 μL of 1.0 M NaOH and allowed to react for 1 h. A 150-μL aliquot of this reaction mixture was then added to 100 μL of 1.0 M HCl and 2000 μL of the phosphate buffer. A 50-μL aliquot was then assayed via formate dehydrogenase, as described above. The fraction of the reaction was the μmol of formate from (a) divided by the total μmol of formate plus formamide from (b).

The fraction of the reaction for the carbonyl-C, carbonyl-O, and leaving-N KIEs was determined using Nessler's reagent. (a) For the carbonyl-C and carbonyl-O KIEs, a 40-μL aliquot of each fraction obtained from the ion-exchange column containing only unreacted formamide (see below) was added to 160 μL of 1.0 M NaOH and reacted for 1 h. Next, a 100-μL aliquot of the above was added to 800 μL of water and 100 μL of Nessler's reagent. The absorbance at 425 nm was then determined for each fraction, and the total number of μmol of NH_3 was calculated from a standard curve. (b) For the leaving-N KIE, a 25-μL aliquot of fractions from the ion-exchange column that contained only NH_3 produced during hydrolysis was added to 875 μL of water and 100 μL of Nessler's reagent. (c) The total number of μmol of nitrogen in each reaction mixture (NH_3 plus unreacted formamide) was determined by alkaline hydrolysis of an aliquot obtained at quench but prior to addition to the ion-exchange column, followed by analysis with Nessler's reagent. For the carbonyl-O and carbonyl-C KIEs, the fraction of reaction was the μmol of NH_3 from (a) divided by the total μmol of NH_3 from (c). For the leaving-N KIE, the fraction of reaction was the ratio of the μmol of NH_3 from (b) divided by the total μmol of NH_3 from (c) subtracted from 1.

Carbonyl-Carbon and Carbonyl-Oxygen Isotope-Effect Procedures. A solution containing 500 μL of water and 16 μL of formamide (400 μmol) was incubated at 25 °C. Then a 500-μL aliquot of a 2.0 M HCl solution was added with stirring. At a designated time, the reaction was quenched with 1.0 M NaOH. The resulting pH was between 5 and 7. Control experiments show that there was negligible further reaction under these conditions. A 1.2-mL aliquot of the quenched solution was applied to a mixed bed resin containing 5 mL of strong cation-exchange resin (Na^+ form) plus 10 mL of strong anion-exchange resin (acetate form). The column was eluted first with water (8 fractions, 4 mL each). Formamide was shown to elute (via hydrolysis, followed by

Nessler's reagent) in fractions 1−6. Enough 10 M NaOH was added to the pooled fractions until the concentration was 1 M, and the hydrolysis to formate was allowed to proceed overnight. A 0.156-g sample of MES (H^+ form) was added to this hydrolysis mixture, followed by the addition of 2.0 M HCl until the pH was near 6.0. The neutralized solution was then reduced to a total volume of about 2 mL by rotary evaporation and placed in a 100-mL round-bottom flask equipped with two stopcocks. One stopcock was on a sidearm that was capped with a septum. The second stopcock was for attachment to the high vacuum line. The solution was dried under high vacuum at 70 °C overnight. While under vacuum, 2 mL of anhydrous DMSO containing 250 mg of I_2 was added via a syringe through the sidearm to the dried formate, and the resulting CO_2 was collected into a liquid nitrogen trap, as previously described.[6] Isotopic analysis gave the δ for both the oxygen atom and the carbon atom. There are two oxygen atoms in formate, one derived from the solvent and one from the nucleophile. The observed per mil isotopic abundance, $^{18}\delta_{(obs)}$, is related to that for the carbonyl, $^{18}\delta_{(CO)}$, and the nucleophile, $^{18}\delta_{(NU)}$, as shown in eq 8. The magnitude of $^{18}\delta_{(NU)}$ does not change during the course of the reaction, because the nucleophile (water, 56 M) is in huge excess over formamide. Thus $^{18}\delta_{(CO)}$ can be calculated from eq 9. Because the actual magnitude of $^{18}\delta_{(NU)}$ is not known, only an apparent $^{18}\delta_{(CO)}$ can be calculated from eq 10. This apparent $^{18}\delta_{(CO)}$ is displaced from the true value by the same constant (eq 9), where $C = (0.5)(^{18}\delta_{(NU)})/0.5$. The value of the constant is not important because the magnitude of the isotope effect depends only on the *difference* between the apparent $^{18}\delta_{(CO)}$ for the low and high conversion samples. As a result, the apparent $^{18}\delta_{(CO)}$ values are used in the KIE calculations.

$$^{18}\delta_{(obs)} = (0.5)^{18}\delta_{(CO)} + (0.5)(^{18}\delta_{(NU)}) \tag{8}$$

$$^{18}\delta_{(CO)} = [^{18}\delta_{(obs)} - (0.5)(^{18}\delta_{(NU)})]/0.5 = {}^{18}\delta_{(obs)}/0.5 - C \tag{9}$$

$$\text{apparent } ^{18}\delta_{(CO)} = {}^{18}\delta_{(obs)}/0.5 \tag{10}$$

Nitrogen Isotope-Effect Procedure. The acidic hydrolysis and quenching was carried out as described above. The quenched reaction mixture was applied to a column composed of 7 mL of strong cation-exchange resin (in the Na^+ form). The column was eluted first with water (8 fraction, 4 mL each), followed by 1 M NaCl (8 fractions, 8 mL each). The early fractions contained unreacted formamide; the later ones contained the product, ammonium ion. The unreacted formamide was hydrolyzed in 1 M NaOH for 2 h, then neutralized with H_2SO_4. This solution was then made basic with NaOH and steam distilled into 0.1 M H_2SO_4. The volume of the steam-distilled solution was reduced to about 2 mL by rotary evaporation and oxidized with NaOBr to N_2. The N_2 was analyzed by isotope ratio mass spectrometry. The ammonium ion isolated by ion exchange chromatography was treated in a similar manner, except no alkaline hydrolysis was needed.

Formyl-Hydrogen Isotope-Effect and PIX Procedures. The formyl-H KIE and PIX were measured by using a 1:1 mixture of 1-d-formamide to 1-h-formamide. The acidic hydrolysis and quenching was carried out as described above. The unreacted formamide was isolated by repeated extraction with ethyl acetate.[9] The ethyl acetate solution was dried over anhydrous sodium carbonate and filtered, and the ethyl acetate was removed by rotary evaporation. The isolated formamide was then dissolved in methanol (as a carrier), and the appropriate ratio ($m/z = 46/45$ or $47/45$) was directly measured by GC-MS without prior oxidation.

Determination of the $^{18}\delta$ for Water. A small sample of CO_2 (<100 μmol) was added to an evacuated round-bottom flask

equipped with a stopcock containing 20 mL of degassed water and 1 drop of concentrated H_2SO_4. The mixture was stirred overnight, and the CO_2 was isolated and analyzed by isotope ratio mass spectrometry. The $^{18}\delta$ for Madison, Wisconsin, water has not changed significantly over a ten-year period ($^{18}\delta = -39.3 \pm 0.3$).

JO060223T