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A Mechanistic Study of Thioester Hydrolysis with Heavy Atom Kinetic Isotope Effects

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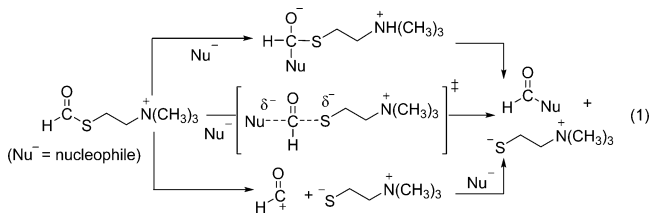
Supporting Information

ABSTRACT: The carbonyl-C, carbonyl-O, and leaving-S kinetic isotope effects (KIEs) were determined for the hydrolysis of formylthiocholine. Under acidic conditions, $^{13}k_{obs}$ = 1.0312, $^{18}k_{obs}$ = 0.997, and $^{34}k_{obs}$ = 0.995; for neutral conditions, $^{13}k_{obs}$ = 1.022, $^{18}k_{obs}$ = 1.010, and $^{34}k_{obs}$ = 0.996; and for alkaline conditions, $^{13}k_{obs}$ = 1.0263, $^{18}k_{obs}$ = 0.992, and $^{34}k_{obs}$ = 1.000. The observed KIEs provided helpful insights into a qualitative description of the bond orders in the transition state structure.

Thioesters are energy-rich compounds important in both organic chemistry and biochemistry. In both disciplines, thioesters commonly utilize this stored chemical energy as acyl group donors. The high reactivity of thioesters toward nucleophiles is partly due to lack of resonance stabilization in the ground state. Sulfur contains a 3p orbital, and this leads to very poor overlap with the 2p orbitals of the carbonyl and less resonance stabilization in the ground state.[1]

The mechanism of alkyl thioester hydrolysis has been investigated by both kinetic and solvent kinetic isotope effects (KIE) for over five decades.[2−5] Kinetic studies indicated that there were two distinct types of thioesters. The first type includes thioesters, which possess an activated carbonyl group due to the presence of electron-withdrawing substituents on the α-carbon.[6,7] These thioesters display three distinct regions in the pH-rate profile: hydroxide ion catalysis above pH 7, uncatalyzed hydrolysis between pH 2−7, and inhibition by hydronium ion below pH 2. The second type of thioester lacks an activated carbonyl (such as FTC) and shows a similar profile as those described above, except the hydrolysis is catalyzed by hydronium ion below pH 2.[8−10] See the Supporting Information for a full FTC pH-rate profile.

Three general mechanisms have been proposed for the hydrolysis of acyl groups, including thioesters.[11] These general mechanisms are shown in eq 1 for the reaction of a negatively charged nucleophile with FTC. The stepwise mechanism (top pathway) involves tetrahedral intermediates and is the most common mechanistic type. The concerted or associative mechanism (middle pathway) has been proposed for the alkaline hydrolysis of oxoesters with very good leaving groups.[12,13] The dissociative mechanism (bottom pathway) is proposed for the hydrolysis of acid chlorides.[14]



(1)

A recent multiple KIE and positional isotope exchange (PIX) investigation from our laboratories aided in assignment of the rate-determining step and establishment of a qualitative structure of the transition state for hydrolysis of FTC under acidic, neutral, and alkaline conditions.[10] Results for acidic and neutral hydrolyses were in agreement with the commonly accepted stepwise mechanism containing a tetrahedral intermediate. However, in alkaline conditions, a slightly larger than expected formyl-H KIE ($^{D}k_{obs}$ = 0.88) indicated that either the stepwise or the concerted mechanisms were possibilities. Nomenclature such as $^{D}k_{obs}$ is defined in the Supporting Information.

In the present KIE study, we report the carbonyl-C, carbonyl-O, and leaving-S KIEs on the acidic, neutral, and alkaline hydrolysis of FTC. Details for measuring isotopic composition and calculating the KIEs are given in the Supporting Information. The results of the KIE experiments are summarized in Table 1.

Acidic Hydrolysis. The acid-catalyzed hydrolysis of alkyl thioformates is first-order in hydronium ion.[8] A PIX experiment from our laboratories gave a ratio for the rate of hydrolysis to that for exchange of ^{18}O into the carbonyl of FTC of k_h/k_{ex} > 25.[10] The ratio may be higher than 25, but this is limited by the NMR methodology. Nevertheless, the result indicates that hydrolysis is much faster than exchange, assuming rapid proton transfers.[15,16] This further implies that formation of the

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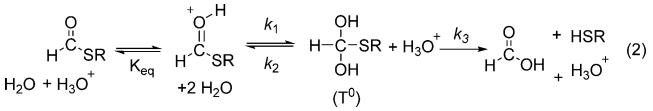
Table 1. Leaving-S, Carbonyl-C, and Carbonyl-O KIEs on the Hydrolysis of FTC under Acidic, Neutral, and Alkaline Conditions

reaction conditions	leaving-S KIE	carbonyl-C KIE	carbonyl-O KIE
0.20 M HCl (μ = 0.20)	0.995 ± 0.002	1.0312 ± 0.0009	0.997 ± 0.002
neutral H_2O (μ = 0.20)	0.996 ± 0.003	1.022 ± 0.001	1.010 ± 0.004
0.0050 M NaOH (μ = 0.20)	1.000 ± 0.001	1.0263 ± 0.0008	0.992 ± 0.003

tetrahedral intermediate is rate-determining. The formyl-H KIE is ${}^{D}k_{obs}$ = 0.80. The magnitude of this inverse KIE is in agreement with a transition state containing considerable sp^3 character.[17] These data are consistent with the mechanism shown in eq 2.

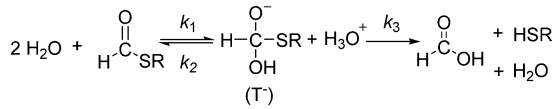


(2)

The carbonyl-C and carbonyl-O KIEs measured in the present study (Table 1) are similar to those for the acidic hydrolysis of methyl formate (${}^{13}k_{obs}$ = 1.028 and ${}^{18}k_{obs}$ = 0.9945).[18] Theoretical calculations for the carbonyl-C KIE predict a very gradual decrease in the KIE on going from early transition states (little sp^3 character) to late transition states (more sp^3 character).[17] This decrease is so gradual that the carbonyl-C has not been widely used in determination of transition state bonding. Published KIEs on aryl carbonate hydrolysis agree with this prediction.[19] The carbonyl-O KIE for acidic hydrolysis is essentially unity, and interpretation of this effect is complex. At least three factors affect the observed KIE. First, there is loss of the π bond to the carbonyl-O, resulting in a normal contribution to the observed KIE. Second, the carbonyl-O experiences formation of new bonds to solvent hydrogen atoms, which could introduce a small normal or small inverse contribution. Finally, new bending and torsional modes in the transition state will result in an inverse contribution. In the present case, the normal contribution from π-bond breaking appears to be balanced by the other factors.

The theoretical maximum sulfur KIE is calculated to be near 1.015, while observed KIEs for S_N1 and S_N2 reactions range from 1.009 to 1.018.[20−22] In the present case, the leaving-S KIE is ${}^{34}k_{obs}$ = 0.995. This measured value is statistically (>95% C.I.) different from unity. Initially, this is surprising since leaving heteroatom KIEs for oxoesters and amides are normal and in the range of ${}^{18}k_{obs}$ = 1.0009 to ${}^{15}k_{obs}$ = 1.0050, respectively.[23,24] In both amides and oxoesters, the breakdown of the tetrahedral intermediate is faster than its formation. Leaving group KIEs on formation of the tetrahedral intermediate are the result of several factors. First, there is loss of the partial π bond on going to the transition state, resulting in a normal contribution to the overall KIE. Thioesters are not expected to show this normal contribution due to the poor overlap between the 2p orbitals of the carbonyl-C and the 3p orbital of sulfur.[1] Second, another small normal contribution to the observed effect is possible when breakdown of the tetrahedral intermediate to products is slightly rate-determining.[23] PIX experiments indicate that this contribution will be minimal for FTC. If all the normal contributions to the leaving-heteroatom KIE are smaller for thioesters than for amides and oxoesters, then what accounts for the observed small inverse KIE? Formation of the transition state will create new bending modes to sulfur. This may lead to stiffer bonding to the sulfur and an inverse contribution to the observed KIE. Catrina and Hengge reached a similar conclusion in a study of phosphorothioate ester hydrolysis.[25] In this case, the nonbridging ${}^{18}k_{obs}$ trended toward the inverse direction as the transition state became tighter. Presumably, this was also due to the newly created bending modes to oxygen becoming dominant and to the large radius of the sulfur atom.

Neutral Hydrolysis. A stepwise mechanism involving at least two water molecules in the transition state has been proposed for neutral hydrolysis (eq 3).[26] Our previous PIX and KIE experiments are consistent with this prevailing mechanism.[10] The PIX experiment again indicated that formation of the tetrahedral intermediate is rate-determining (k_h/k_{ex} > 25). The formyl-H KIE (${}^{D}k_{obs}$ = 0.75) is a slightly more inverse KIE than in acid-catalyzed hydrolysis and is in agreement with a transition state containing even more sp^3 character than under acidic conditions. The solvent nucleophile-O KIE is ${}^{18}k_{obs}$ = 0.9917. Since k_3 is rapid, the observed KIE is due to formation of the tetrahedral intermediate (k_1). This can be explained when the inverse contribution to the observed KIE from the temperature-dependent factor (TDF) is only slightly larger than the normal contribution from the temperature-independent factor (TIF).[27,28]



(3)

The carbonyl-C and carbonyl-O KIEs change somewhat on going from acidic to neutral conditions (Table 1). It would be tempting to assign the smaller carbonyl-C KIE in neutral hydrolysis to a later transition state (more sp^3 character), since theoretical calculations predict that later transition states will correlate with slightly smaller KIEs.[17] However, to date, such correlations have proved difficult to establish for most acyl group transfer reactions.[11] The carbonyl-O KIE goes from essentially unity in acid-catalyzed hydrolysis to a normal KIE in neutral hydrolysis (Table 1). The interpretation is again complicated by transition state effects described in the acidic hydrolysis case. However, the sizable normal KIE indicates that breaking the carbonyl-O π bond plays the major role in determining the overall observed KIE.

The leaving-S KIE for neutral hydrolysis is ${}^{34}k_{obs}$ = 0.996, very close to that measured in the acid-catalyzed case above and is still statistically different than unity (>95%CI). The interpretation is similar. The normal contributions to the observed KIE are so small that what remains is a small inverse secondary KIE due to creation of new bending modes in the sp^3-transition state.

Alkaline Hydrolysis. Kinetic investigations indicated that alkaline hydrolysis is first-order in hydroxide, as is the case for oxoesters.[8,9,29] Our previous PIX results (k_h/k_{ex} > 25) argue that formation of the tetrahedral intermediate is rate-determining.[10] The formyl-H KIE for the alkaline hydrolysis of FTC (${}^{D}k_{obs}$ = 0.88) is considerably less inverse than that for acidic and neutral hydrolysis, consistent with an earlier transition state (less sp^3 character).[10] The formyl-H KIE for alkaline hydrolysis of FTC can also be compared to that of its oxoester counterpart, methyl formate.[29] The formyl-H KIE for

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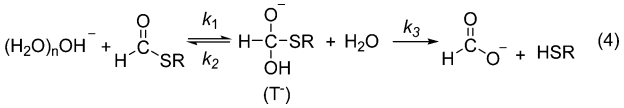
FTC is more inverse ($^{D}k_{obs}$ = 0.88 versus $^{D}k_{obs}$ = 0.95), arguing for a somewhat later transition state for FTC.[10,30] Up to this point, all of these results can be accommodated by the stepwise mechanism given in eq 4.

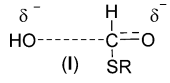


(4)

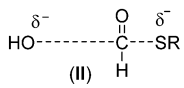
The solvent nucleophile-O KIE was measured in the same manner as for the neutral hydrolysis above.[10] In the alkaline hydrolysis case, there are two possible nucleophiles present, leading to two possible mechanisms. One mechanism involves water as the nucleophile with general base assistance by hydroxide; the other is hydroxide as the direct nucleophile. Because water and hydroxide have different δ values, the experiment gives two possible KIEs, depending on which nucleophile one chooses. For the general base mechanism with water as the nucleophile, the KIE is $^{18}k_{obs}$ = 1.029, whereas, for hydroxide as the actual nucleophile, the KIE becomes $^{18}k_{obs}$ = 0.989. The choice between these two possibilities rests on the magnitude of the formyl-H KIE. By theory, a very early transition state is expected to give rise to an observed normal solvent nucleophile-O KIE, whereas late transition states should result in an inverse solvent nucleophile-O KIE.[17] For the oxoester, methyl formate, the choice of the general base mechanism was clear because the formyl-H KIE was so small ($^{D}k_{obs}$ = 0.95), leaving little doubt that the transition state for formation of the tetrahedral intermediate was early.[29] However, in the alkaline hydrolysis of FTC, the formyl-H KIE is a slightly larger inverse effect ($^{D}k_{obs}$ = 0.88), forcing one to consider the alternative concerted mechanism (eq 1, middle pathway). Since the leaving group and nucleophile are both good, the conditions are right for this possible change in mechanism.[10]

The leaving-S KIE offers a possible way to distinguish between the stepwise and concerted mechanisms. If the stepwise mechanism is operating, the leaving-S KIE should be near unity (or slightly inverse) because breaking the C−S bond is fast in comparison to other mechanistic steps. If the concerted mechanism is operating, a measurable normal leaving-S KIE is possible when the transition state contains significant C−S bond breaking. This expectation is similar to empirical observations for *p*-nitrophenyl acetate hydrolysis, where sizable leaving-O KIEs indicated the presence of the concerted mechanism.[12,13] The observed leaving-S KIE is $^{34}k_{obs}$ = 1.000. This KIE is consistent with the stepwise mechanism when the transition state for formation of the tetrahedral intermediate is earlier for basic conditions than in the acidic and neutral cases. The resulting smaller amount of C−O bond formation will diminish the magnitude of the inverse contributions to the KIE. Combined with the lack of C−S bond breaking, the observed KIE for alkaline hydrolysis will trend toward unity.

However, the observed leaving-S KIE can also fit an asynchronous concerted mechanism. If the sulfur KIE for formation of the tetracoordinate transition state is small and inverse (as in acidic and neutral hydrolysis) and the C−S bond is only slightly broken (a small normal effect), the two KIEs may result in the observed KIE of unity. The transition states for these two possible mechanisms are given in structures I and II, respectively.



(I)



(II)

The carbonyl-C KIE for alkaline hydrolysis is slightly less inverse than that for neutral hydrolysis and slightly more inverse than that for acidic hydrolysis. This trend cannot be easily explained. On the basis of theoretical calculations, one would expect the earliest transition state structure (alkaline hydrolysis) to give the largest carbonyl-C KIE.[17] The observed carbonyl-O KIE is close to unity and similar to that for acidic hydrolysis. It is clear from the small formyl-H KIE that the transition state is somewhat early. As a result, the breaking of the π bond to the carbonyl-O and the resulting normal contribution to the KIE are minimized compared to acidic and neutral conditions. The remaining aforementioned factors appear to balance each other to create an overall observed effect of unity.

Conclusions. KIE and PIX experiments indicate that acidic and neutral hydrolysis of FTC follow a stepwise mechanism with rate-determining formation of a tetrahedral intermediate. The observed KIEs on alkaline hydrolysis of FTC can fit either a stepwise mechanism with an early transition state for formation of the tetrahedral intermediate or an asynchronous concerted mechanism with both a small amount of C−O bond formation and a small amount of C−S bond breaking. Leaving-S KIE experiments on thioester hydrolysis may prove useful in establishing the concerted mechanism in cases where one observes both no ^{18}O exchange (PIX) and a significant normal sulfur KIE.

EXPERIMENTAL SECTION

Synthesis of Formylthiocholine. Formylthiocholine was synthesized following our previously published synthetic scheme.[10] Briefly, 2-(dimethylamino)ethanethiol hydrochloride was added to the formyl−acetic mixed anhydride. After completion, excess acetic anhydride, acetic acid, and formic acid were removed under reduced pressure. Crude formyl 2-(dimethylamino)ethanethiol hydrochloride (FDC) was dissolved in acetone, deprotonated with PS-DIEA resin, and methylated with methyl iodide. The precipitate was recrystallized in absolute ethanol. Analytical data are as previously reported.[10]

Leaving-S KIE Procedures. These procedures were used to determine the S-leaving atom KIE in acidic, neutral, and alkaline conditions. (a) *Alkaline conditions:* FTC (14 mg, 51.1 μmol) was added to 875 μL of 0.2 M NaCl. To this solution was added 125 μL of 0.2 M NaOH. After 1 min, the reaction was quenched with 100 μL of 0.5 M MES buffer at pH 6.2. The reaction mixture was immediately added to a solution containing a slight excess of DTNB (22 mg, 55.5 μmol) in 10 mL of 50 mM MES buffer at pH 6.8.[31] The solution was immediately run through a 2 mL cation-exchange column with AG 50W resin. The column was washed with 20 mL of water and eluted with 0.5 M TEAB at pH 8.5. The fractions (total volume 50 mL) were assayed for free thiol using DTNB. The free thiol containing fractions were collected, and the buffer was removed by rotary evaporation. The samples were further dried by lyophilization and analyzed by IRMS. (b) *Neutral conditions:* FTC (14 mg, 51.1 μmol) was added to 1000 μL of 50 mM MES buffer at pH 6.8 containing 150 mM NaCl. The mixture was allowed to react until the reaction reached 20−80% hydrolysis, as determined by DTNB assays (~5 h).[31] The mixture was added to a solution of excess DTNB and subjected to cation-exchange chromatography as previously described. (c) *Acidic conditions:* FTC (14 mg, 51.1 μmol) was added to 875 μL of water. To this solution was added 125 μL of 1.6 M HCl. The mixture was allowed to react until the reaction reached 20−80% hydrolysis, as determined by DTNB assays (~2 h). The mixture was added to a solution of excess DTNB and subjected to cation-exchange chromatography as previously described. *Controls:* Recrystallized FTC was applied to

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the cation exchange column in the presence of DTNB. Elution with water was followed by elution with TEAB. FTC was present in TEAB eluted fractions. These fractions were concentrated and submitted for IRMS analysis, which showed the $^{34}\delta$ to be unchanged from original FTC within the precision of analysis. Next, completely hydrolyzed FTC plus DTNB were applied to the cation exchange column and eluted first with water, followed by TEAB. The fraction where FTC normally eluted was concentrated and submitted for IRMS analysis. After combustion, no SO_2 was detected.

Carbonyl-O and Carbonyl-C KIE Procedures. The carbonyl-C and carbonyl-O KIEs were measured in a single experiment. (a) *Basic conditions*: A solution containing 875 μL of 0.20 M NaCl and 16 mg of FTC (57 μmol) was prepared. To this solution was added 125 μL of a solution containing 40 μL of 1.0 M NaOH in 160 μL of water. The reaction was monitored by the DTNB assay described above, and the fraction of reaction at quenching was determined. At the appropriate time, the reaction was quenched by addition of 50 μL of MES buffer at pH 6.2. The final pH was always greater than 5.0. The reaction mixture was added to an anion exchange column (acetate form), washed with 30 mL of water. The product, formate was eluted with 0.1 M NaCl in 6 mL fractions. A 1.5 mL aliquot of 1.5 M MES buffer at pH 6.3 was added to the combined fractions containing formate. This solution was evaporated to a small volume (~1 mL) and transferred to a round-bottom flask that was equipped with two stopcocks. One stopcock was on a side arm 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 through the side arm to the dried formate, and the resulting CO_2 was collected into a liquid nitrogen trap as previously described.[10] Isotopic analysis gave the δ for both the oxygen and the carbon atoms. The above δ values and those for formate isolated after complete alkaline hydrolysis were used in the KIE calculation. (b) *Neutral conditions*: The reaction mixture contained 16 mg of FTC (57 μmol) in 1000 μL of 0.050 M MES buffer at pH 6.8. The final pH was not allowed to drop below pH 5.0. Assay, isolation, and isotopic analysis were performed in the same as for the alkaline hydrolysis. (c) *Acidic conditions*: The reaction solution contained 16 mg of FTC (57 μmol) in 1000 μL of 0.20 M HCl. Fraction of reaction was determined by the DTNB assay. The product of the reaction under acidic conditions is formic acid, which undergoes rapid ^{18}O exchange. To avoid this problem, the substrate, FTC, must be analyzed by an indirect method. To accomplish this, the reaction mixture after partial hydrolysis was first applied to an anion exchange column (acetate form) and the 30 mL water wash containing unreacted FTC was collected. The FTC (from partial hydrolysis) was then quantitatively hydrolyzed with NaOH and applied to a second anion exchange column identical to the first. This column was washed with water, followed by typical elution of formate with 0.10 M NaCl as described above. Oxidation of formate and isotopic analysis were as described above. Next, a sample of FTC was completely hydrolyzed with aqueous NaOH. For both the partial and the quantitatively hydrolyzed samples, formate contains one oxygen from the carbonyl-O of FTC and one oxygen from the nucleophile acquired during alkaline hydrolysis. The $^{18}\delta$ from the nucleophile is identical for both the partial and the quantitatively hydrolyzed samples because the solvent nucleophile is in great excess. This allows the carbonyl-O KIE to be calculated from the measured $^{18}\delta$ from both samples. *Controls:* Formate of known isotopic composition was eluted from the anion exchange column in the absence of FTC. The known $^{18}\delta$ of formate did not change. FTC was also passed through the anion exchange column. FTC was detected in the water wash. However, elution with 0.10 M NaCl did not produce formate, as expected.

■ ASSOCIATED CONTENT

Supporting Information

The equations for calculating the KIEs from substrate analysis and product analysis and the relationship between δ notation and isotope ratios are included. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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