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The "Neutral" Hydrolysis of Simple Carboxylic Esters in Water and the Rate Enhancements Produced by Acetylcholinesterase and Other Carboxylic Acid Esterases

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Supporting Information

ABSTRACT: Experiments at elevated temperatures permit the determination of rate constant and thermodynamic activation parameters for the neutral hydrolysis of the neurotransmitter acetylcholine in water. At 25 °C, the extrapolated rate constant for the uncatalyzed (or neutral) hydrolysis of acetylcholine is 3.9×10^{-7} s^{-1} at 25 °C ($\Delta H^{\ddagger}$ = 20.0 kcal/mol; $T\Delta S^{\ddagger}$ = −6.1 kcal/mol). Acetylcholine is more susceptible to neutral and base-catalyzed hydrolysis than ethyl acetate but less susceptible to acid-catalyzed hydrolysis. For acetylcholinesterase from the electric eel, the catalytic proficiency [$(k_{cat}/K_m)/k_{neutral}$] is 2×10^{16} M^{-1}, comparable in magnitude with the catalytic proficiencies of aminohydrolases that act on peptides and nucleosides.

Aliphatic carboxylic acid esters play central roles in lipid metabolism, in the action of excitable tissue, and in the activation of amino acids for protein biosynthesis. These oxyesters are rapidly cleaved by hydrolytic enzymes that catalyze either direct water attack on the ester linkage (such as phospholipase A2)[1] or a double-displacement reaction in which the enzyme and the substrate form an acyl-enzyme intermediate that subsequently undergoes hydrolysis (such as acetylcholinesterase).[2] At chemical nerve synapses and neuromuscular junctions, excitation is caused by release of the neurotransmitter acetylcholine ($AcCh^+$), and its enzymatic hydrolysis by acetylcholinesterase is required for recovery after excitation. To appreciate the power of these enzymes as catalysts, it would be useful to have information about the rates of carboxylic ester hydrolysis in water in the absence of a catalyst.

The kinetics of the specific acid- and base-catalyzed hydrolysis of oxyesters are relatively well-understood, but the rate constants of their neutral or "water" reactions ($k_{neutral}$ in eq 1) have remained somewhat obscure. Discussing earlier efforts[3,4] to describe the neutral reaction of ethyl acetate at 25 °C, Kirsch and Jencks pointed out that "the experimental difficulties involved in obtaining (a rate constant) which corresponds to a half-time of 89 years and represents only 36% of the observed hydrolysis rate under the most favorable conditions."[5] Thus, the terms in eq 1 that arise from specific acid and base catalysis [$k_{H+}(H^+)$ and $k_{OH-}(OH^-)$] are of sufficient magnitude to interfere with the direct determination of $k_{neutral}$ by kinetic measurements under ordinary conditions.

$$k_{obs} = k_{neutral} + k_{H+}(H^+) + k_{OH-}(OH^-) \quad (1)$$

With a few notable exceptions, catalyzed reactions tend to exhibit lower enthalpies of activation in aqueous solution than do their neutral or uncatalyzed counterparts.[6] We reasoned that if that were also true of ester hydrolysis, then the rate constant of the neutral reaction might increase with increasing temperature relative to the rate constants of the specific acid- and base-catalyzed reactions (Figure 1), allowing the neutral reaction to emerge as the predominant mechanism of hydrolysis at elevated temperatures. If rate constants for hydrolysis could be determined with reasonable precision at elevated temperatures, then it might be possible to use the Arrhenius relationship to arrive at the rate constant of the neutral reaction at ordinary temperatures by extrapolation.

We decided to test that possibility by examining the nonenzymatic hydrolysis of the excitatory neurotransmitter acetylcholine ($AcCh^+$) (Scheme 1).

To establish the value of k_{neut} and its dependence on temperature, it was necessary at the outset to have accurate information about the rate constants of the acid- and base-catalyzed reactions and their dependence on temperature. To determine the rate constant (k_{H+}) of the acid-catalyzed reaction in water, we used ^{1}H NMR to monitor the disappearance of $AcCh^+$ (0.02 M) in HCl (0.1 N) in experiments conducted over the temperature range from 10 to 60 °C. For the acid-catalyzed reaction, extrapolation of an Arrhenius plot of these results yielded $k_{H+} = 3.1 \times 10^{-5}$ M^{-1} s^{-1} at 25 °C ($\Delta H^{\ddagger}$ = 17.4 kcal/mol; $T\Delta S^{\ddagger}$ = −6.1 kcal/mol). To determine the rate constant (k_{OH-}) of the base-catalyzed reaction, we used tris(hydroxymethyl)aminomethane (THAM) buffers (0.1 M, pH 8.1 at 25 °C) over the range 18−60 °C. THAM was chosen because it is a relatively poor nucleophile[7] and because its enthalpy of ionization (11.35 kcal/mol) is sufficiently similar to that of water (13.34 kcal/mol)[8] to minimize the correction required for the effect of changing temperature on the activity of a hydroxide ion in THAM buffers at constant pH. At each temperature, rates were extrapolated to zero buffer concentration to obtain the rate of the base-catalyzed reaction. An Arrhenius plot of the results was linear and yielded k_{OH-} = 1.73 M^{-1} s^{-1} at 25 °C ($\Delta H^{\ddagger}$ = 8.6 kcal/mol; $T\Delta S^{\ddagger}$ = −8.5 kcal/mol). These values are consistent with individual rate constants reported earlier for $AcCh^+$ hydrolysis at various temperatures.[9−11] At 25 °C, the sum of the values for $k_{H+}(H^+)$ and $k_{OH-}(OH^-)$ in eq 1 reaches a minimum value (1.46×10^{-9} s^{-1}) at pH 4.6 at 25 °C.

To determine $k_{neutral}$ for $AcCh^+$ at 25 °C, rates of hydrolysis were determined over the temperature range 78−118 °C in potassium acetate buffer (pH 4.6). The heat of ionization of

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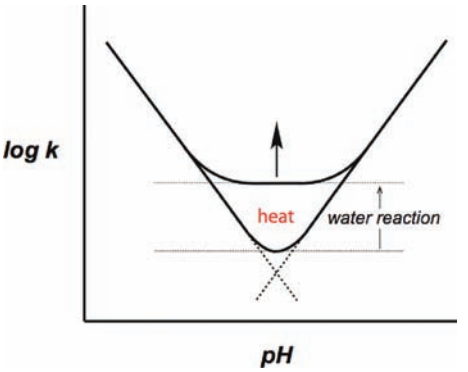
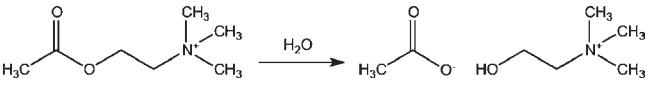


Figure 1. Influence of pH on an acid- and base-catalyzed reaction in water, when a neutral reaction is also present. The rate of the neutral reaction might be expected to increase, relative to the rates of the acid- and base-catalyzed reactions, with increasing temperature.

Scheme 1. Hydrolysis of Acetylcholine



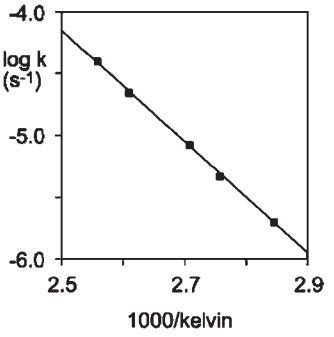


Figure 2. Influence of temperature on the rate of neutral hydrolysis of $AcCh^+$.

acetic acid is small enough (−0.10 kcal/mol) to render the pH practically invariant over the temperature range of these experiments. In separate experiments at 80 °C, using potassium acetate buffer (0.1 M, pH 4.6) with various concentrations of added KCl, the rate of hydrolysis was found to be insensitive to ionic strength, decreasing <15% when the ionic strength was raised from 0 to 3.0 M at 80 °C. At each temperature, the rate of hydrolysis of $AcCh^+$ was determined at buffer concentrations ranging from 0.1 to 0.8 M, and the results were extrapolated to zero buffer concentration. After subtraction of the values of $k_{H+}(H^+)$ and $k_{OH-}(OH^-)$ at that temperature, the resulting values were plotted as a logarithmic function of absolute temperature, yielding a linear Arrhenius plot (Figure 2). The extrapolated value of $k_{neutral}$ was 7.2×10^{-9} s^{-1} at 25 °C ($\Delta H^{\ddagger}$ = 21.0 kcal/mol; $T\Delta S^{\ddagger}$ = −7.5 kcal/mol at 25 °C).

These findings are summarized on the first 3 lines of Table 1. At pH 4.6, where the sum of the rates of the acid- and base-catalyzed reactions reaches a minimum value at 25 °C,[12] the neutral hydrolysis of $AcCh^+$ represents 70% of the observed rate of reaction, while the specific acid- and base-catalyzed reactions

Table 1. Rate Constants at 25 °C (s^{-1}) and Thermodynamics of Activation (kcal/mol) for the Hydrolysis of Acetic Acid Esters

Reactant	$k_{25\,°C}$	$\Delta G^{\ddagger}$	$\Delta H^{\ddagger}$	$T\Delta S^{\ddagger}$	T range (°C)
$AcCh^+$, neutral	$7.2\ (\pm 0.5) \times 10^{-9}$ s^{-1}	28.5	21.0	−7.5	78−118
" " (H^+)	$3.1\ (\pm 0.2) \times 10^{-5}$ M^{-1} s^{-1}	23.5	17.4	−6.1	10−60
" " (OH^-)	1.73 ($\pm$0.1) M^{-1} s^{-1}	17.1	8.6	−8.5	18−60
EtAc, neutral [a]	$(2.5-5.0) \times 10^{-10}$ s^{-1}	30.2	—	—	
" " (H^+) [b]	1.2×10^{-4} M^{-1} s^{-1}	22.7	15.5	−7.2	
" " (OH^-) [b]	1.1×10^{-1} M^{-1} s^{-1}	18.7	11.5	−7.2	

[a] The lesser value (2.5×10^{-10} M^{-1} s^{-1}) is taken from ref 1. The greater value (5×10^{-10} M^{-1} s^{-1}) is taken from ref 3 and was estimated from the intersection of Brønsted plots. [b] Taken from ref 3 (thermodynamics of activation not determined).

Table 2. Rate Enhancements Produced by Oxyesterases at 25 °C

Substrate (enzyme)	k_{non}, s^{-1}	k_{cat}, s^{-1}	k_{cat}/k_{non}
$AcCh^+$ (acetylcholinesterase, EC 3.1.1.7)[2]	7.2×10^{-9}	11 700	7.6×10^{13}
diC7PC [a] (phospholipase A2, EC 3.1.1.4)	4×10^{-10}	43	1.1×10^{11}
Hydroxybutyrate (hydroxybutyrate dimer hydrolase, EC 3.1.1.22)[16]	4×10^{-10}	1500	3.8×10^{12}
Tributyrin (triacylglycerol lipase, EC 3.1.1.3)[17]	4×10^{-10}	4300	1.1×10^{13}

[a] Micellar 1,2-dimyrisotylphosphatidylmethanol (ref 1). In the case of this micellar substrate, the value of k_{cat} may be limited by physical events such as lipid exchange between micelles.

each represent 15% of the rate observed. Because the heat of activation of the neutral hydrolysis of $AcCh^+$ (21.0 kcal/mol) is larger than the heats of activation of the specific acid- and base-catalyzed reactions (16.4 and 8.6 kcal/mol respectively), the modest predominance of the neutral reaction over the acid- and base-catalyzed reactions at 25 °C becomes more pronounced with increasing temperature, increasing to 97% of the observed reaction rate at 100 °C and pH 4.6. That tendency accords with the hypothesis on which these experiments were based.

The influence of the positively charged guanidinium group of $AcCh^+$ on the rates of the neutral, acid-catalyzed, and base-catalyzed reactions can be evaluated by comparison with the behavior of ethyl acetate. Although the thermodynamics of activation for the neutral hydrolysis of ethyl acetate have not yet been established, its approximate rate constant has been estimated as 2.5×10^{-10} s^{-1} (refs 1 and 2) or 5×10^{-10} (ref 3) at 25 °C. Thus, the neutral hydrolysis of $AcCh^+$ ($k_{neutral} = 7.2 \times 10^{-9}$ s^{-1} at 25 °C) proceeds 15−30-fold more rapidly than the neutral hydrolysis of ethyl acetate ($k = 2.5 \times 10^{-9}$ s^{-1} at 25 °C). The acid- and base-catalyzed reactions of ethyl acetate have been investigated in greater detail.[3−5] Table 1 shows that $AcCh^+$ is 4-fold less susceptible to acid-catalyzed hydrolysis than ethyl acetate and 16-fold more susceptible to base-catalyzed hydrolysis. These differences seem understandable, at least qualitatively, in terms of the expected retarding influence of a positive charge on the approach of a nucleophile in the case of the neutral and base-catalyzed reactions, and on the equilibrium of substrate protonation in the case of the acid-catalyzed reaction.

It is of interest to compare the rate constants of these reactions with those of the corresponding enzymatic reactions. Table 2 shows that electric eel acetylcholinesterase (EC 3.1.1.7; k_{cat} = 11 700 s^{-1}; $K_m = 9 \times 10^{-5}$ M at pH 7, 25 °C)[2] accelerates the hydrolysis of $AcCh^+$ by a factor of 7.6×10^{13}. Under these conditions, the value of its catalytic proficiency $[(k_{cat}/K_m)/k_{neutral}]$ is 1.8×10^{16} M^{-1}, comparable in magnitude with the catalytic proficiencies of aminohydrolases that act on peptides and nucleosides.[13,14] Also included in Table 2 are two additional oxyesterases: phospholipase A2 (EC 3.1.1.4) and a hydroxybutyrate-dimer hydrolase (EC 3.1.1.22). If ethyl acetate is used as a basis for comparison, then phospholipase A2 enhances the rate of ester hydrolysis of a micellar substrate[15] by a factor of $\sim 10^{11}$, and 3-hydroxybutyrate-dimer hydrolase[16] achieves a rate enhancement of 4×10^{12}. In view of the magnitudes of the rate enhancements that they produce, it is evident that both the double displacement mechanism used by acetylcholinesterase and the mechanism employed by phospholipase A2 (involving direct water attack) furnish effective strategies for accelerating carboxylic ester hydrolysis.

■ ASSOCIATED CONTENT

Supporting Information. Arrhenius plots for the acidic, basic, and neutral hydrolysis of acetylcholine. This material is available free of charge via the Internet at http://pubs.acs.org.

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