[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, ILLINOIS INSTITUTE OF TECHNOLOGY]

The Imidazole-catalyzed Hydrolysis of *p*-Nitrophenyl Acetate[1]

BY MYRON L. BENDER AND BYRON W. TURNQUEST

RECEIVED AUGUST 30, 1956

The kinetics of the imidazole-catalyzed hydrolysis of *p*-nitrophenyl acetate in 5% dioxane–water has been determined. The reaction was followed spectrophotometrically in three ways: by measurement of the disappearance of ester, the appearance of *p*-nitrophenolate ion and the change in N-acetylimidazole. The reaction products were established as acetate and *p*-nitrophenolate ion by direct infrared analysis of the reaction occurring in dioxane–deuterium oxide solution. The rate of disappearance of ester or of appearance of *p*-nitrophenolate ion is proportional to the ester concentration and varies in a linear fashion with the buffer (imidazole) concentration at constant hydrogen ion concentration. By variation of the *p*H, it is shown that the rate is proportional to the free imidazole concentration (k_c 0.47 l./mole sec. at 26.2°) and is independent of the imidazolium ion concentration. The catalytic rate constant is independent of ionic strength. The energy and entropy of activation of the first step of the imidazole-catalyzed hydrolysis are 7.1 ± 0.5 kcal./mole and −38.2 e.u., respectively. A mechanism of the hydrolysis is proposed, involving attack of the ester by imidazole giving N-acetylimidazole. N-Acetylimidazole is subsequently hydrolyzed by water, yielding acetate ion and regenerating the catalyst.

Introduction

In recent years there have been several suggestions that the imidazole ring of a histidine residue of several hydrolytic enzymes is responsible for the catalytic activity of these enzymes. In particular, imidazole has been proposed as an essential part of the active site of α-chymotrypsin, trypsin and acetylcholinesterase. The evidence in support of this hypothesis consists of thermodynamic studies,[3] the effect of *p*H on enzymatic activity,[4] studies on the photooxidation of the enzyme[5] and investigations of the chemistry of imidazole.[6] In order to test this hypothesis and in order to provide details of the mechanism of the catalytic action of imidazole in a non-enzymatic system, a kinetic investigation has been made of the imidazole-catalyzed hydrolysis of *p*-nitrophenyl acetate.

Experimental

Materials.—*p*-Nitrophenyl acetate was prepared from *p*-nitrophenol (recrystallized, m.p. 114–115.5°) and acetic anhydride.[7] After two recrystallizations from Skellysolve B, the product was nearly colorless, m.p. 77.5–78° (lit. m.p. 79.5–80°).[8] Imidazole was an Eastman Kodak Co. white label product, Lot. No. 15, m.p. 88–89°. Commercial 1,4-dioxane was purified according to the method of Fieser,[9] b.p. 99–100°, n^{22}D 1.4210.

Kinetics.—The rates of hydrolysis were followed in a Cary recording spectrophotometer at the following wave lengths: the rate of appearance of *p*-nitrophenolate ion was measured at 400 mμ, the rate of disappearance of *p*-nitrophenyl acetate was measured at 278 mμ and the rate of change of N-acetylimidazole was measured at 245 and 240 mμ. Each species was followed at or very near its absorption maximum. *p*-Nitrophenolate ion was shown to follow Beer's law over the concentration range used. In following the rate of reaction by the appearance of *p*-nitrophenolate ion, a constant hydrogen ion concentration must be maintained since the spectrophotometric method used may measure one or both of the equilibrium mixture of *p*-nitrophenol and *p*-nitrophenolate ion. If, however, constant *p*H is maintained, it can be shown that the kinetics can be satisfactorily determined whether one or both of these species is measured.

The stock solutions for all runs were equilibrated in a water-bath (±0.02°) prior to mixing. The temperature of the thermostated compartment of the Cary spectrophotometer was maintained to within 0.1° by circulating water from a constant temperature bath. For a portion of the kinetic determinations made at 26.2°, it was found that on mixing the ester in pure dioxane with the buffer in aqueous solution a temperature rise of one degree occurred. Since this value was always constant, the stock solutions were thermostated one degree below the temperature of the compartment of the spectrophotometer. For later determinations the ester, in dioxane solution, was mixed with the other aqueous components except buffer and allowed to come to thermal equilibrium. No temperature increase was noted when the aqueous dioxane was further diluted with water.

The following general procedure was used in the kinetic determinations. Water, sodium chloride solution (for the maintenance of constant ionic strength) and 1.0 ml. of ester in dioxane solution were added to one section of a two-cell flask; aliquots of buffer from a stock solution were added to the other side. The solutions were brought to thermal equilibrium, removed from the bath, quickly shaken and transferred to a previously thermostated cell and placed in the spectrophotometer. The time lapse between removal of the cell from the water thermostat and placement in the spectrophotometer was never longer than one minute for reactions with half-lives of the order of 3 to 30 minutes. The reactions were followed by continuous recording of the optical density at a suitable wave length.

The buffer (imidazole catalyst) was always in sufficient concentration to maintain constant hydrogen ion concentration as measured before and after reaction with a Beckman model G *p*H meter. The concentration of the free imidazole component of the buffer was determined by direct titration of the amine with acid. For these titrations, curves were constructed by plotting the *p*H *versus* the amount of added titrant; the end-point was selected as the inflection point of these curves. In the reactions run at 6.5 and 39.8° the titrations were carried out in a water-bath at these temperatures.

The specific rate constants were determined by the method of Guggenheim.[10] Typical Guggenheim plots from which the specific first-order rate constants, k_{obs}, were obtained are given in Fig. 1. The average arithmetic deviation from the Guggenheim plots was less than 1%; the reactions were usually followed from 10 to 90% completion.

Product Analysis.—For the infrared spectrophotometric analysis of the products of the reaction of *p*-nitrophenyl acetate with imidazole, the solution was composed of 0.11 *M* ester and 0.04 *M* imidazole in 70% dioxane–deuterium oxide. Spectra were taken immediately after mixing and after three days, when the reaction was complete. A Perkin–Elmer double beam spectrophotometer equipped with 0.10-mm. calcium fluoride cells was used.

Results

Infrared spectroscopic analysis has demonstrated that the reaction of *p*-nitrophenyl acetate and

(1) This investigation was supported by research grant H-2416 of the National Institutes of Health. Paper V of the series, "The Mechanism of Enzymatic Hydrolysis."

(2) Eastman Kodak Co. research fellow, 1955–1956.

(3) D. G. Doherty and F. Vaslow, THIS JOURNAL, **74**, 931 (1952).

(4) I. B. Wilson and F. Bergmann, *J. Biol. Chem.*, **186**, 683 (1950); H. Gutfreund, *Trans. Faraday Soc.*, **51**, 441 (1955); B. R. Hammond and H. Gutfreund, *Biochem. J.*, **61**, 187 (1955)

(5) L. Weil, S. James and A. R. Buchert, *Arch. Biochem. and Biophys.*, **46**, 266 (1953).

(6) T. Wagner-Jauregg and B. E. Hackley, Jr., THIS JOURNAL, **75**, 2125 (1953).

(7) F. Chattaway, *J. Chem. Soc.*, **134**, 2495 (1931).

(8) B. S. Hartley and B. A. Kilby, *Biochem. J.*, **56**, 288 (1954).

(9) L. F. Fieser, "Experiments in Organic Chemistry," D. C. Heath and Co., Boston, Mass., 1941, p. 368.

(10) E. A. Guggenheim, *Phil. Mag.*, [7] **2**, 538 (1926).

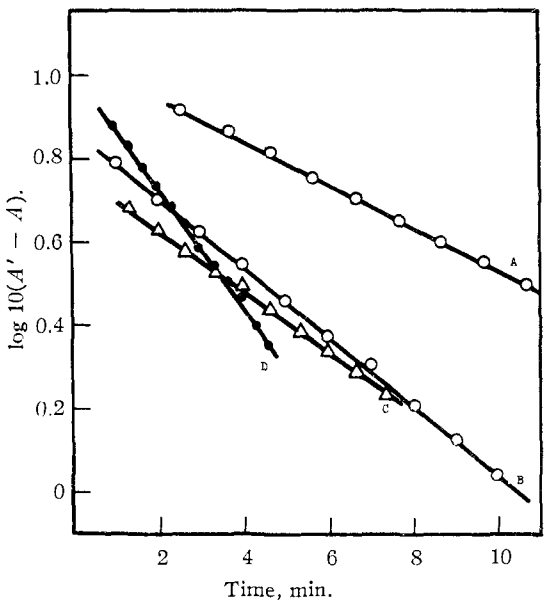


Fig. 1.—Guggenheim plots of the hydrolysis of *p*-nitrophenyl acetate with imidazole.

	10^4 ester	(Free amine)	*p*H	Temp., °C.
(A)	1.0	0.0105	7.15	6.5
(B)	1.1	.00627	7.15	26.2
(C)	1.1	.00610	7.15	26.2[a]
(D)	1.7	.00625	6.87	39.8

[a] Rate of disappearance of ester.

imidazole is a hydrolytic reaction. *p*-Nitrophenyl acetate has a strong absorption band at 1755 cm.$^{-1}$ due to the carbonyl function of the ester group. Imidazole at the concentration used (0.04 *M*) is almost transparent below 2000 cm.$^{-1}$. After completion of the reaction of *p*-nitrophenyl acetate (0.11 *M*) and imidazole (0.04 *M*) in 70% dioxane–deuterium oxide solution, a spectrum was obtained which could only be explained on the basis of a hydrolytic reaction. The carbonyl band at 1755 cm.$^{-1}$ was replaced by three new bands, free acetic acid at 1703 cm.$^{-1}$ and acetate ion at 1550 and 1415 cm.$^{-1}$.[11] Likewise, *p*-nitrophenol was identified by absorption bands at 1596, 1516, 1445 and 1373 cm.$^{-1}$. In confirmation of the latter data, the kinetics were generally followed by measurement of the optical density at 400 mμ, the absorption maximum for *p*-nitrophenolate ion, and the final optical density corresponded to the value calculated from a Beer's law curve.

In Table I are listed the catalytic rate constants for the hydrolysis of *p*-nitrophenyl acetate by imidazole at three different concentrations of hydrogen ion as determined by measurement of the rate of appearance of *p*-nitrophenolate ion at 400 mμ. The catalytic rate constants, k_c, obtained as average values of the individual points agree well with those obtained from the slopes of the lines of a plot of k_{obs} *versus* the free imidazole concentration, using the least squares method (Fig. 2). Table I and Fig. 2 indicate that the hydrolysis of *p*-nitrophenyl acetate is catalyzed by imidazole since the observed rate constant varies in a linear fashion

(11) G. Ehrlich, THIS JOURNAL, **76**, 5263 (1954).

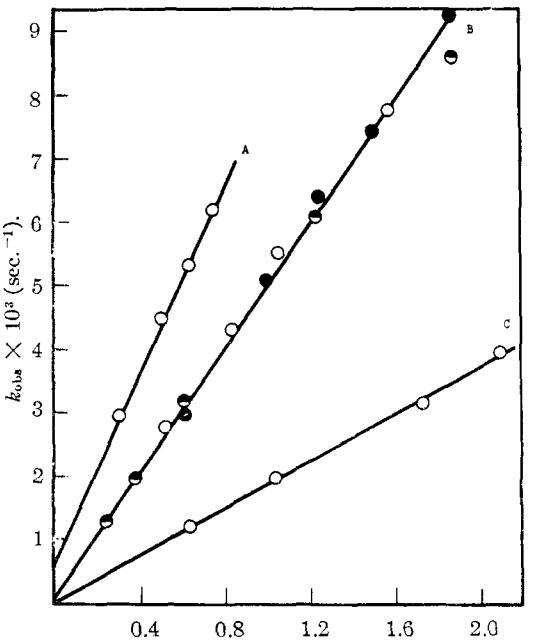


Fig. 2.—Hydrolysis of *p*-nitrophenyl acetate with imidazole: A, 39.8°; B, 26.2°: ○, *p*H 6.30; ◒, *p*H 7.15; ●, *p*H 7.80; (C), 6.5°.

with the buffer (imidazole) concentration at constant hydrogen ion concentration.

The data at various *p*H values, at which the relative concentrations of the free imidazole and imidazolium ion are changed, indicate that the observed rate constant is directly proportional to the free imidazole concentration and is independent of the imidazolium ion concentration. The increase in rate with increasing buffer concentration cannot be due to a primary salt effect. Even though the ionic strength varied by a factor of thirty-five between the higher and lower *p*H values given in Table I, the catalytic rate constant is the same within experimental error. The effect of variation in the ester concentration is also demonstrated by the data in Table I. A fivefold change in ester concentration between *p*H 7.80 and 6.30 produced no change in the observed rate constant indicating first-order dependence in ester. Doubling the ester concentration at constant *p*H and buffer concentration likewise produced no change. The reaction thus appears to be first order in ester and first order in imidazole.

In addition to the studies mentioned above, it was also possible to follow the kinetics by measurement of the rate of disappearance of the reactant, *p*-nitrophenyl acetate. Table II shows that within experimental error the rate of disappearance of ester is equal to the rate of appearance of *p*-nitrophenolate ion.

Figure 2 shows that the intercepts are almost identical for the imidazole-catalyzed hydrolysis of *p*-nitrophenyl acetate at *p*H 6.30 and 7.80. The intercept is due to the sum of the rate constants for the water reaction, the hydroxyl ion reaction and the hydronium ion reaction, the last being negligible under the present conditions. Since the blank

TABLE I

THE KINETICS OF THE HYDROLYSIS OF *p*-NITROPHENYL ACETATE BY IMIDAZOLE[a]

Imidazolium ion, $M \times 10^2$	Free imidazole, $M \times 10^2$	$k_{obs} \times 10^3$, sec.$^{-1}$	k_c, l./mole sec.
*p*H 7.80, ionic strength 0.00225 *M*, (ester) 1.1 × 10^{-4} *M*			
0.075	0.625	3.30	0.470
.120	1.00	5.08	.472
.150	1.25	6.42	.485
.180	1.50	7.44	.472
.225	1.87	9.25	.475
		Av.	0.475
*p*H 7.15, ionic strength 0.0112–0.0183 *M*, (ester) 1.1 × 10^{-4} *M*			
0.149	0.251	1.28	0.454
.224	.376	1.87	.460
.373	.627	3.15	.480
.373	.627	3.16	.482
.745	1.25	6.14	.480
.745	1.25	6.10	.476
.745	1.25	6.09[b]	.475
1.12	1.88	8.96	.469
1.12	1.88	8.89	.465
		Av.	0.471
*p*H 6.30, ionic strength 0.078 *M*, (ester) 5.5 × 10^{-4} *M*			
2.59	0.525	2.77	0.470
4.14	0.840	4.30	.476
5.18	1.05	5.54	.498
6.22	1.26	6.48	.490
7.78	1.58	7.79	.474
		Av.	0.482

[a] 5% Dioxane-water, 26.2°. [b] Ester concentration 2.2 × 10^{-4} *M*.

TABLE II

RATE CONSTANTS OF THE HYDROLYSIS OF *p*-NITROPHENYL ACETATE[a]

Free imidazole, $M \times 10^3$	$k_{obs} \times 10^3$, sec.$^{-1}$ Disappearance of ester, 278 mμ	Appearance of *p*-nitrophenolate ion, 400 mμ	Hydrolysis of N-acetylimidazole, 245 mμ
3.66	1.71	1.80	
6.10	2.84	3.00	
18.3			1.40
18.3			1.55[b]

[a] 5% Dioxane–water, *p*H 7.15, 26.2°, (ester) 1.1 × 10^{-4} *M*. [b] Rate followed at 240 mμ.

reaction contributes such a small amount to the total hydrolysis (1–10% depending on the imidazole concentration) the intercept is a somewhat crude measure of the blank reaction(s).

It was possible to demonstrate the existence of an intermediate in this hydrolysis and to measure its rate of hydrolytic cleavage. The intermediate is presumed to be N-acetylimidazole as will be discussed later. It was rapidly formed by using a large concentration of free imidazole (0.0183 *M*) so that the half-life of its formation was about 80 seconds and its formation was essentially complete in about eight to ten minutes; its slower rate of hydrolysis was then followed by measurement of the optical density at 245 mμ, the absorption maximum found by Stadtman for N-acetylimidazole.[12] Figure 3 shows the formation and decomposition of this intermediate as measured by the optical density at 245 mμ and Table II contains the rate constants for the hydrolysis of N-acetylimidazole derived from Fig. 3.

The effect of temperature on the hydrolysis as measured by the rate of appearance of *p*-nitrophenolate ion is given in Table III and Fig. 2. An Arrhenius plot was constructed from the data of Table III to determine the activation energies. Figure 4 shows that the activation energy for catalysis by imidazole is reasonably constant over the temperature range studied. The apparent activation energy for imidazole-catalyzed hydrolysis of *p*-nitrophenyl acetate is 7.1 ± 0.5 kcal./mole; the entropy of activation, calculated from the Eyring equation, is −38.2 e.u.

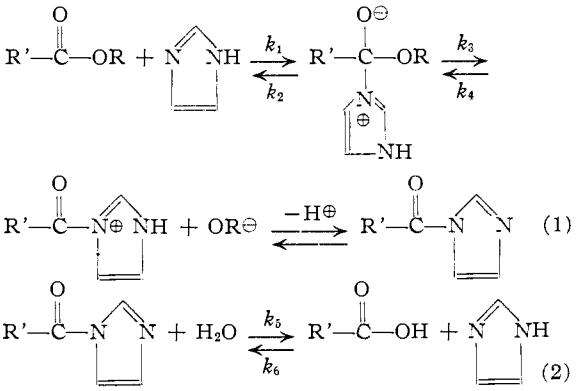
TABLE III

TEMPERATURE EFFECT ON THE IMIDAZOLE-CATALYZED HYDROLYSIS OF *p*-NITROPHENYL ACETATE

Temp., °C.	*p*H	$k_c \times 10^3$, l./mole sec.
6.5	7.15	190
26.2	7.15	471
39.8	6.87	749

Discussion

Catalysis of the hydrolysis of *p*-nitrophenyl acetate by imidazole is believed to proceed according to the mechanism



(1)

(2)

An alternative to equation 1 exists in which the proton loss occurs from the tetrahedral intermediate rather than from the N-acetylimidazolium ion. Either of these possibilities fits the following kinetic analysis.

The ultimate fate of products capable of proton transfer would, of course, depend on the *p*H of the solution. Treatment of the tetrahedral intermediate, $RC(O^-)(Im^+)OR$ (Im = imidazole), as a high energy intermediate by the method of the stationary state gives the following differential equation for the rate of appearance of *p*-nitrophenolate ion

$$d(OR^-)/dt = k_1k_3(\text{ester})(B) - k_2k_4(RCOIm^+)(OR^-)/(k_2 + k_3) \quad (3)$$

Since the reactions were followed spectrophotometrically (OR^-) was very small ($\sim 10^{-4}$ *M*) as was ($RCOIm^+$). If it may be assumed that $k_1k_3(\text{ester})(B) \gg k_2k_4(RCOIm^+)(OR^-)$, eq. 3 would then reduce to

$$d(OR^-)/dt = (k_1k_3/(k_2 + k_3))(\text{ester})(B) = k_c(\text{ester})(B) \quad (4)$$

(12) E. R. Stadtman, "The Mechanism of Enzyme Action," W. D. McElroy and B. Glass, eds., The Johns Hopkins Press, Baltimore, Md., 1954, pp. 581–598.

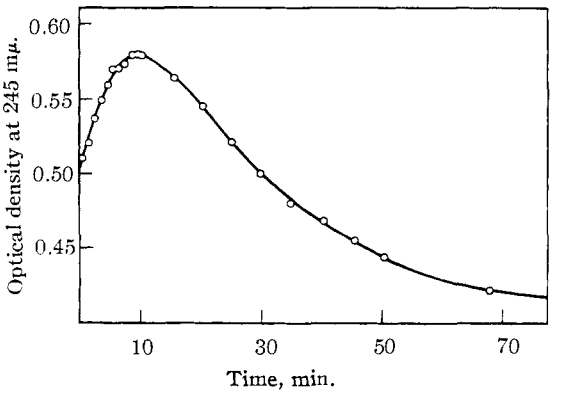


Fig. 3.—Formation and decomposition of N-acetylimidazole, (free imidazole) = 1.83×10^{-2} *M*, (*p*-nitrophenyl acetate) = 1.1×10^{-4} *M*, *p*H 7.15, 26.2°.

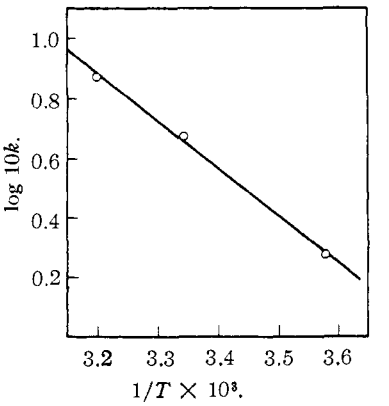


Fig. 4.—Arrhenius plot of the hydrolysis of *p*-nitrophenyl acetate with imidazole.

Equation 4 is in agreement with the experimental results for the appearance of *p*-nitrophenolate ion.

In the hydrolysis of esters by hydroxide ion, oxygen exchange evidence suggests that the reaction proceeds *via* a tetrahedral intermediate and not through a simple displacement reaction.[13] Such an intermediate would also be expected for imidazole-catalyzed hydrolysis. Since it was shown that the rate of disappearance of *p*-nitrophenyl acetate was equal to the rate of appearance of *p*-nitrophenolate ion, the tetrahedral intermediate must be an unstable one, so that the stationary state treatment is valid.

The existence of an acyl-catalyst intermediate such as $RCOIm^+$ in hydrolytic reactions has been postulated previously by several workers. Gold demonstrated that the hydrolysis of anhydrides is catalyzed by heterocyclic bases and suggested an acyl-catalyst intermediate.[14] Koshland[15] has shown that the hydrolysis of acetyl phosphate is catalyzed by pyridine and proposed an acylpyridinium ion intermediate. The latter author has prepared acetylpyridinium chloride and has estimated its half-life in aqueous solution as less than one second. Stadtman has demonstrated that acetylglutathione is cleaved by imidazole to give N-acetylimidazole and glutathione.[12] The presence of N-acetylimidazole was demonstrated by an increase in optical density at 245 mμ after addition of imidazole to a solution of acetylglutathione.

In the present investigation evidence for N-acetylimidazole as an intermediate in the reaction of *p*-nitrophenyl acetate with imidazole was obtained from the record given previously of the optical density at 245 mμ during the reaction of *p*-nitrophenyl acetate and imidazole. The initial rise and slow fall of the optical density in Fig. 3 are indicative of the formation and decomposition of an intermediate species. There is no obvious explanation for the large discrepancy between the rate constant reported here for the hydrolysis of N-acetylimidazole (1.47×10^{-3} sec.$^{-1}$) and that reported by Stadtman (1.5×10^{-4} sec.$^{-1}$) except for the difference in solvent.[16]

In any case the lifetime of N-acetylimidazole in aqueous solution is much longer than that of acetylpyridinium ion. The probable reason for the greater stability of the former is its ability to lose a proton to form an uncharged amide as shown in equation 1. No such possibility exists for acetylpyridinium ion. In the above respect imidazole is unique. The mechanism proposed for the imidazole-catalyzed hydrolysis of *p*-nitrophenyl acetate bears a marked resemblance to the mechanism of enzymatic hydrolysis of esters[4-6] as will be discussed in a subsequent paper.[17]

Acknowledgment.—The authors gratefully acknowledge valuable discussions with Drs. D. E. Koshland, Jr., and J. Hine.

CHICAGO 16, ILLINOIS

(13) M. L. Bender, THIS JOURNAL, **73**, 1626 (1951).

(14) V. Gold and E. G. Jefferson, *J. Chem. Soc.*, 1409 (1953).

(15) D. E. Koshland, Jr., THIS JOURNAL, **74**, 2286 (1952).

(16) Private communication from Dr. E. R. Stadtman indicates that the disparity in the rate constants for the hydrolysis of N-acetylimidazole is probably due to impurities in the dioxane used by us. He has found that 0.18 volume % of unpurified dioxane accelerated the hydrolysis of N-acetylimidazole by approximately 25-fold. While we have used so-called purified dioxane (see Experimental) we have found that the hydrolysis of N-acetylimidazole in 5% dioxane–water is indeed faster than in pure water. This unusual phenomenon will be investigated further.

(17) M. L. Bender and B. W. Turnquest, THIS JOURNAL, **78**, 1656 (1957).