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

General Basic Catalysis of Ester Hydrolysis and Its Relationship to Enzymatic Hydrolysis[1]

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The hydrolyses of *p*-nitrophenyl acetate, 2,4-dinitrophenyl acetate, phenyl acetate and ethyl thiolacetate are catalyzed by a number of bases including pyridine, 3- and 4-picoline, trimethylamine, imidazole, N-methylimidazole, quinoline and acetate ion. The hydrolysis of *p*-nitrophenyl acetate meets the requirements of general basic catalysis since the rate is proportional to the summation of catalytic constants times catalyst concentrations at constant *p*H and ionic strength. The catalytic rate constants are related to the basicity of the catalyzing amines of constant steric requirement in a manner similar to the Brønsted catalysis law. The slope of this linear free energy relationship is 1.62, the highest recorded for a general basic catalysis. The mechanism of this general basic catalysis, involving not a rate-determining proton transfer but rather the addition of the base to the substrate producing an unstable intermediate, offers an explanation for the deviation of the slope from its usual limitation. Imidazole catalysis of the hydrolysis of various esters demonstrates that the rate of hydrolysis increases with the acidity of the alcoholic residue of the ester. It appears that general basic catalysis of ester hydrolysis is of importance only for esters containing an alcohol that is a reasonably strong acid ($pK_a < 11$). Comparison of the imidazole- and α-chymotrypsin-catalyzed hydrolyses of *p*-nitrophenyl acetate shows that the two types of hydrolysis differ by a factor of *ca.* 10^5 in the rate of formation of *p*-nitrophenol but are similar in the rate of formation of acetate from the acylimidazole and acyl-enzyme intermediates. The action of imidazole as a general basic catalyst offers only a partial explanation of the mechanism of enzymatic hydrolysis.

Introduction

The hydrogen and hydroxyl ion-catalyzed hydrolyses of esters have received a great deal of attention and the mechanisms of these reactions have been worked out in great detail.[3] Investigators have also considered the possibility of catalysis by acids and bases other than these two aquo species. General acid or basic catalysis of ester hydrolysis is of particular interest from the viewpoint of enzymatic hydrolysis; it is likely that, for catalytic purposes, an enzyme uses general acids or bases on its surface rather than hydronium and/or hydroxyl ions in solution since the latter ions are present only in small concentration at the *p*H's at which enzymatic hydrolyses occur. It has been shown recently in this Laboratory that enzymatic hydrolysis does not resemble acidic or basic hydrolysis with respect to carbonyl oxygen exchange during ester hydrolysis[4] or with respect to the effect of substituents on the rate of hydrolysis.[5] On the other hand, it has been suggested that a Brønsted base, namely, imidazole, is a part of the catalytically active site of α-chymotrypsin.[6] In confirmation of this hypothesis, it was shown that thiol esters such as acetylglutathione are cleaved by imidazole[7] and that *p*-nitrophenyl acetate is hydrolyzed by imidazole[6] and by N-benzoyl-L-histidine methyl ester.[8]

Early investigations of general basic catalysis of ester hydrolysis were inconclusive. Dawson and co-workers studied the catalytic effects of acetic acid, acetate ion, chloroacetic acid and bisulfate ion on the hydrolysis of ethyl acetate.[9] However, the reported catalytic rate constants are minute (the catalytic constant for acetate ion was reported to be 0.25×10^{-7} l./mole sec. while that for hydroxide ion is 1.08 l./mole sec.) and their reality is questionable. These data have been critically discussed from the viewpoint of the primary salt effect and the effect of the nature of the buffer on the rate.[10,11] General catalysis by acetic acid and acetate ion, although of small magnitude, has been reported in the esterification of acetic acid in methanol.[12]

A consideration of the mechanism of the hydrolysis of carboxylic acid derivatives by hydrogen and hydroxyl ions points to the direction in which general basic catalysis of ester hydrolysis may be sought.[13] Wiberg[14] states that for the reaction

$$Y + R{-}\overset{\overset{O}{\|}}{C}{-}X \rightleftharpoons \left[R{-}\overset{\overset{O^-}{|}}{\underset{\underset{Y^+}{|}}{C}}{-}X \right] \rightleftharpoons R{-}\overset{\overset{O}{\|}}{C}{-}Y + X$$

the initial addition depends on the nucleophilicity of Y but the partitioning of the addition compound to form reactant or product is related to the relative stabilities of Y and X. One would then expect general basic catalysis of ester hydrolysis when the anion formed from the ester is at least of comparable stability (basicity) to the attacking base. Accordingly, in order to demonstrate general basic catalysis, esters were chosen in which the alcoholic portion of the ester was a much stronger acid than the ethanol in Dawson's original work. Among such esters are phenyl, negatively substituted phenyl and thiol esters.

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

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(3) C. K. Ingold, "Structure and Mechanism in Organic Chemistry," Cornell Univ. Press, Ithaca, N. Y., 1953, pp. 752–782; J. Hine, "Physical Organic Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1956, pp. 266–282.

(4) M. L. Bender and K. C. Kemp, THIS JOURNAL, **79**, 116 (1957).

(5) M. L. Bender and B. W. Turnquest, *ibid.*, **77**, 4271 (1955).

(6) M. L. Bender and B. W. Turnquest, *ibid.*, **79**, 1652 (1957), and references therein.

(7) 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.

(8) B. S. Hartley, *Ann. Repts. on Progr. Chem. (Chem. Soc., London)*, **51**, 311 (1954).

(9) H. M. Dawson and W. Lowson, *J. Chem. Soc.*, 2444 (1927); H. M. Dawson and W. Lowson, *ibid.*, 393 (1929); H. M. Dawson, E. R. Pycock and E. Spivey, *ibid.*, 291 (1933).

(10) R. P. Bell, "Acid–Base Catalysis," Oxford Univ. Press, London, 1941, p. 80.

(11) G. Gorin, personal communication.

(12) A. C. Rolfe and C. N. Hinshelwood, *Trans. Faraday Soc.*, **30**, 935 (1934).

(13) M. L. Bender, THIS JOURNAL, **73**, 1626 (1951); M. L. Bender and R. D. Ginger, *ibid.*, **77**, 348 (1955).

(14) K. B. Wiberg, *ibid.*, **77**, 2519 (1955).

Experimental

Materials.—2,4-Dinitrophenyl acetate was prepared from 2,4-dinitrophenol and acetic anhydride.[15] Two recrystallizations from Skellysolve B gave an almost colorless product, m.p. 71–71.5° (lit. m.p. 72°). The preparation of *p*-nitrophenyl acetate was described previously.[6] Ethyl thiolacetate was prepared from ethanethiol and acetyl chloride, b.p. 115–115.5° (lit. b.p. 115°).[16] Phenyl acetate was an Eastman Kodak Co. white label product, $n^{20}D$ 1.5025. Imidazole was described previously.[6] In order to avoid turbidity (probably due to hydrocarbon contamination) of the solution when 4-picoline was mixed with water, the Eastman Kodak Co. white label product was distilled (b.p. 142–143°), converted to the hydrochloride and crystallized from ethanol–ether, reconverted to the free amine with alkali and redistilled, b.p. 143–144°, $n^{20}D$ 1.5035 (lit. b.p. 145°, $n^{20}D$ 1.5029).[17] 3-Picoline, a Reilly Coal Tar Co. product, was fractionated through a Todd distillation assembly packed with a monel spiral; the middle fraction was collected using a reflux ratio of seven-to-one, b.p. 144.1°, $n^{22.5}D$ 1.5049 (lit. b.p. 143.8°, $n^{25}D$ 1.5038).[17] Fisher reagent grade pyridine was redistilled, b.p. 115°. Fisher reagent grade quinoline was redistilled, b.p. 236°. Eastman Kodak Co. white label trimethylamine (as the hydrochloride) was used without further purification. N-Methylimidazole was prepared from imidazole and methyl iodide, b.p. 196–198° (lit. b.p. 198°).[18] The neutral equivalent was determined by titration of 40-mg. samples with 0.02 *N* HCl using a Beckman model H *p*H meter. Titration curves were drawn and the end-point taken as the inflection point of these curves; neut. equiv. found 80.7 and 81.0, calcd. 82.1. The ionization constant of N-methylimidazole was determined potentiometrically in the above manner. The *p*H of the half-neutralization point of the amine, used as the value of pK_a, was found to be 7.00 and 6.98 for solutions 0.02 *M* in total amine. Dedichen[19] has reported a pK_a of 7.34 for this amine at ionic strengths of the same order of magnitude as above. J. T. Baker reagent grade acetic acid was redistilled, b.p. 118°. Commercial 1,4-dioxane was purified as described previously.[6]

Kinetic Determinations.—The general procedure for the spectrophotometric determination of the kinetics using a Cary recording spectrophotometer has been given previously.[6] The hydrolysis of *p*-nitrophenyl acetate was followed by measurement of the appearance of *p*-nitrophenolate ion at 400 mμ; the hydrolysis of 2,4-dinitrophenyl acetate was followed by measurement of the appearance of 2,4-dinitrophenolate ion at 360 mμ; the hydrolysis of phenyl acetate was followed by measurement of the appearance of phenol at 272 mμ and the hydrolysis of ethyl thiolacetate was followed by measurement of the disappearance of ester at 245 mμ. All species were followed at or very near their absorption maxima except in the case of ethyl thiolacetate where the intense absorption of imidazole interfered with measurements at the absorption maximum of the ester. The specific rate constants were calculated from the spectrophotometric data by the method of Guggenheim[20] or by the use of the usual first-order equation. The average arithmetic deviation from the Guggenheim plots varied from less than 1% in most cases to 4 or 5% in the worst cases (with 3-picoline, 4-picoline and pyridine as catalysts—this was due to a slight decrease in rate after the reaction was about 70% complete). The rate constants for the imidazole-catalyzed hydrolysis of ethyl thiolacetate were always larger in the first 15% of the reaction, reasonably constant through the middle range and smaller after about 60–70% completion.

The attempted hydrolyses of ethyl acetate were performed in the following manner. The ester and catalyst were mixed at 25.04°. Aliquots of the mixture were withdrawn and titrated in the indicated manner. *m*-Aminobenzoic acid, anthranilic acid, benzoic acid, pyridine, manganous chloride and sodium thiosulfate solutions were titrated to *p*H 8 with standard alkali using phenolphthalein or a Beckman model H *p*H meter; nickelous chloride and cupric chloride solutions were titrated to *p*H 4.0 with standard acid using the *p*H meter.

The hydrolysis of acetylcholine bromide with imidazole was followed by periodic examination of the infrared spectrum in a Perkin-Elmer double beam infrared spectrophotometer. The solutions were prepared as follows: imidazole was dissolved in a solution of DCl in deuterium oxide (99.8%) to give a solution containing equal amounts of free amine and deuteronated amine; to this solution was added a solution of ester in deuterium oxide. The concentrations were adjusted so that the final solution was 0.10 *M* in ester and 0.43 *M* in each imidazole species. Calcium fluoride infrared cells were used for the analysis of the deuterium oxide solutions.

The hydrolysis of methyl *p*-nitrobenzoate with imidazole was followed by measurement of the rate of appearance of *p*-nitrobenzoate ion with the Cary recording spectrophotometer. The ester has a maximum at 266 mμ and *p*-nitrobenzoate ion has a maximum at 272 mμ. However, at 290 mμ the optical density of the latter is much greater than that of the former so that the increase in optical density at this wave length can be used as a measure of the hydrolysis reaction.

Results

Hydrolysis of *p*-Nitrophenyl Acetate by Tertiary Amines.—In Table I are listed the catalytic rate constants, k_c, for the hydrolysis of *p*-nitrophenyl acetate by several heterocyclic bases and one aliphatic tertiary amine. These constants were obtained from the slopes of lines of a plot of k_{obs} *versus* the free amine concentration, using the least squares method (Fig. 1). In Fig. 1 it is seen that for six catalysts the observed hydrolytic rate constants varied in a linear fashion with the buffer (catalyst) concentration at constant hydrogen ion concentration and at constant ionic strength. It has already been pointed out that the rate of hydrolysis of *p*-nitrophenyl acetate by imidazole was independent of primary salt effects[6]; doubling the ionic strength of the medium (from 0.117 to 0.245 *M*) in the hydrolysis of *p*-nitrophenyl acetate with 4-picoline also had no effect on the specific rate constant.

TABLE I

THE HYDROLYSIS OF *p*-NITROPHENYL ACETATE BY TERTIARY AMINES[a]

Catalyst	*p*H	$k_c \times 10^2$, l./mole sec.	$k_1 \times 10^4$, $sec.^{-1}$	log k_c	pK_a of amine[b]
Pyridine	6.35	0.0531		−3.275	5.23[c]
3-Picoline	6.37	.261		−2.583	5.66[d]
4-Picoline	6.08	.795	0.52	−2.100	6.05[c]
N-Methyl-imidazole	7.05	31.7		−0.499	7.00[e]
Imidazole	7.15	46.9	1.45	−0.323	7.04[f]
Trimethyl-amine	8.35	9.87	12.0	−1.006	9.72[g]
Hydroxide ion		510		2.699	15.7
Imidazole	7.85		3.64		

[a] 5% dioxane–water, 26.2°. [b] Aqueous solution, 25°. [c] A. Gero and J. J. Markham, *J. Org. Chem.*, **16**, 1835 (1951). [d] E. F. G. Herington, *Disc. Faraday Soc.*, **9**, 26 (1950). [e] Determined in this investigation; Dedichen[19] reported 7.34. [f] A. H. M. Kirby and A. Neuberger, *Biochem. J.*, **32**, 1146 (1938). [g] H. S. Harned and R. A. Robinson, THIS JOURNAL, **50**, 3174 (1928).

The rate of alkaline hydrolysis of *p*-nitrophenyl acetate was estimated from the intercepts of Fig. 1. If it is assumed that $k_H(H^+) = 0$, the intercepts, k_i, are equal to $k_{H_2O} + k_{OH}(OH^-)$. A plot of k_i *versus* (OH^-) then yields k_{OH} as the slope and

(15) J. J. Blanksma, *Chem. Weekblad*, **6**, 725 (1909).

(16) P. N. Rylander and D. S. Tarbell, THIS JOURNAL, **72**, 3021 (1950).

(17) T. Eguchi, *Bull. Chem. Soc. Japan*, **3**, 228 (1928).

(18) O. Wallach, *Ber.*, **15**, 645 (1882).

(19) G. Dedichen, *ibid.*, **39**, 1840 (1906).

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

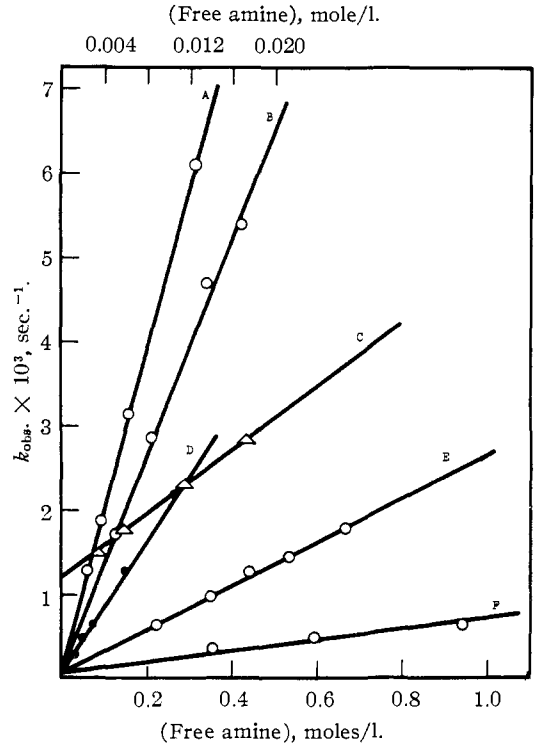


Fig. 1.—Hydrolysis of *p*-nitrophenyl acetate with various amines at 26.2°: A, imidazole; B, N-methylimidazole; C, trimethylamine; D, 4-picoline; E, 3-picoline; F, pyridine. Top scale A, B and C; bottom scale D, E and F.

k_{H_2O} as the intercept. The data in Table I were used for the plot illustrated in Fig. 2. The second-order rate constant for hydroxide ion was estimated to be 510 l./mole sec. from least squared data of Table I and k_{H_2O} was estimated at 5×10^{-5} sec.$^{-1}$.

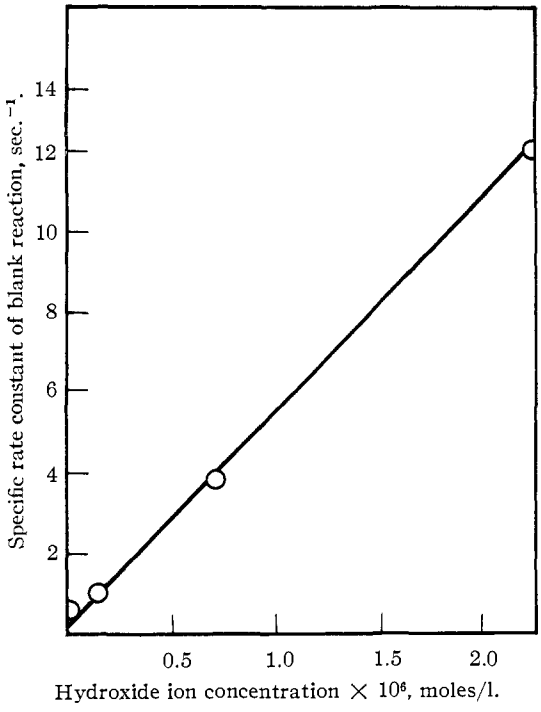


Fig. 2.—The alkaline hydrolysis of *p*-nitrophenyl acetate in 5% dioxane-water at 26.2°.

Hydrolysis of 2,4-Dinitrophenyl Acetate, Phenyl Acetate and Ethyl Thiolacetate by Various Bases.—The catalytic rate constants for the hydrolysis of these esters were obtained in the same manner as those for *p*-nitrophenyl acetate. The constants are listed in Table II and the plots from which they were obtained are given in Fig. 3.

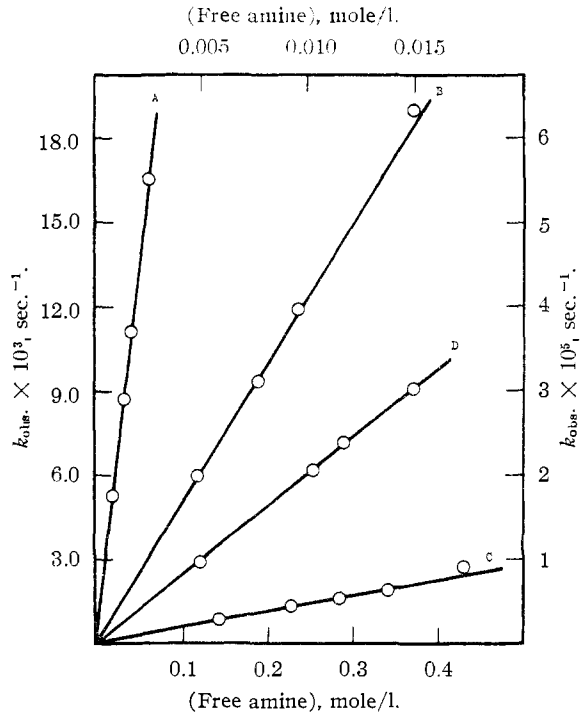


Fig. 3.—Hydrolysis of various esters with imidazole and acetate ion. Imidazole catalysis: A, 2,4-dinitrophenyl acetate (top and left scales); B, ethyl thiolacetate (bottom and right scales); C, phenyl acetate (bottom and left scales). Acetate catalysis: D, 2,4-dinitrophenyl acetate (top scale $\times 10^2$ and left scale $\times 10^{-1}$).

Attempted Hydrolysis of Ethyl Acetate, Acetylcholine and Methyl *p*-Nitrobenzoate with Various Acids and Bases.—A study has been made of the hydrolysis of ethyl acetate by various acids and bases. In all cases given in Table III there was no measurable amount of reaction at the indicated period of time.

The extent of hydrolysis of acetylcholine bromide by imidazole in deuterium oxide solution was estimated from the diminution of the carbonyl peak of the ester at 1730 cm.$^{-1}$. In twenty days at 25° the optical density fell from about 0.264 to 0.149 indicating approximately 40% hydrolysis. It was assumed that the deuterium oxide solution was near neutrality since pK_a was 7.04 for imidazole in water at 25°. The half-life calculated for the hydrolysis of acetylcholine in water at *p*H 7.0 is about 60 days (k_{OH} 1.20 l./mole sec. in water at 25°).[21] In view of the uncertainty in the true concentration of OD^- and in the value for the hydrolysis constant by deuteroxide ion, catalysis by

(21) J. Butterworth, D. D. Eley and G. S. Stone, *Biochem. J.*, **53**, 30 (1953).

TABLE II

THE HYDROLYSIS OF 2,4-DINITROPHENYL ACETATE, PHENYL ACETATE AND ETHYL THIOLACETATE BY VARIOUS BASES[a]

Ester	Catalyst	*p*H	$k_c \times 10^2$, l./mole sec.	log k_c	pK_a[c] of alcohol	log k_{OH}
2,4-Dinitrophenyl acetate	Imidazole	6.12	649	0.829	4.02	
	Quinoline	5.00	15.0[d]			
	Acetate ion	5.30	0.058[e]			
Phenyl acetate	Imidazole	7.49	.588[e]	−2.196	9.89	−0.240[b]
Ethyl thiolacetate	Imidazole	7.13	.0166[f]	−3.770	10.5	−1.386[g]
p-Nitrophenyl acetate	Imidazole			−0.323	7.16	0.906[b]

[a] 26.2°. [b] 56% acetone–water, 25°, ref. 22. [c] Aqueous solution, 25°. [d] Determined at only one concentration of quinoline, 6% dioxane–water. [e] 5% dioxane–water. [f] Aqueous solution. [g] 62% acetone–water, J. R. Schaefgen, THIS JOURNAL, **70**, 1308 (1948).

TABLE III

ATTEMPTED HYDROLYSIS OF ETHYL ACETATE WITH VARIOUS ACIDS AND BASES[d]

Catalyst	(Catalyst), *M*	(Ester), *M*	Time, months
m-Aminobenzoic acid	0.038	0.037	1.0
Anthranilic acid	.025	.024	1.0
Benzoic acid–sodium benzoate	.017 (0.017)	.017	1.0
Pyridine–pyridinium ion	.040 (.040)	.040	1.0
Manganese chloride	.033	.033	1.0
Nickelous chloride	.015	.015	0.25
Nickelous chloride[a]	.010	.025	.25
Sodium thiosulfate	.025	.025	.25
Cupric chloride[b]	.015	.013	.5
Cupric chloride[c]	.025	.025	.5

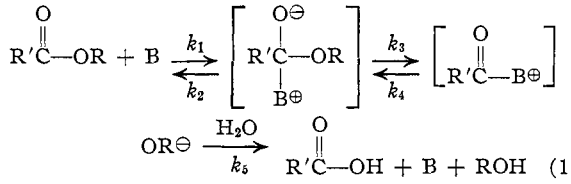
[a] *p*H 7.5, 0.060 *M* tris-(hydroxymethyl)-aminomethane buffer. [b] *p*H 6.5, 0.079 *M* tris buffer. [c] *p*H 7.7, 0.050 *M* tris buffer. [d] Aqueous solution, 25.04°.

imidazole must be considered to be unproven; if such hydrolysis does exist, it must be quite small.

The amount of hydrolysis of methyl *p*-nitrobenzoate in imidazole solution was determined by the increase in the optical density at 290 mμ. For a solution 1.82×10^{-4} *M* in ester, 0.601 *M* in total amine and 0.430 *M* in free amine at *p*H 7.50 in 5% dioxane–water at 25°, the optical density increased from 1.050 to 1.190 in six days; the increase found after this length of time represents about 25% hydrolysis. The half-life of the hydrolysis of this ester by hydroxide ion at 25° was approximated to be about 17 days at *p*H 7.5 by extrapolation of the known rate constant in 56% acetone–water to 5% dioxane–water.[22] It therefore appears that the small amount of hydrolysis found for methyl *p*-nitrobenzoate in imidazole solution is explainable on the basis of an alkaline reaction.

Discussion

Mechanism of the Reaction.—Catalysis of ester hydrolysis by bases other than hydroxide ion is believed to proceed according to the mechanism outlined for imidazole-catalyzed hydrolysis[6] involving attack of the ester by the base giving an acylammonium (or acid anhydride) intermediate which is subsequently hydrolyzed by water, yielding carboxylate ion and regenerating the catalyst. Only in the imidazole case is there direct evidence for intermediate formation.[6] In none of the other ammonium ion intermediates is there the possibility of stabilization of the intermediate by loss of a proton. The concentration of these intermediates would, therefore, not be expected to attain an experimentally measurable value. Catalysis of hydrolysis by acetate ion should give an anhydride



as intermediate by analogy with the acyl derivatives of the amines.[23] The rate constant for the uncatalyzed hydrolysis of acetic anhydride is given[24] as 2.5×10^{-3} sec.$^{-1}$ in aqueous solution at 25°; the similarity between this value and the value for N-acetylimidazole indicates that these two intermediates are of comparable stability. It is not surprising that the rates of hydrolytic cleavage are close since the latter is the nitrogen analog of the former. It was not possible, however, to obtain physical evidence for the existence of the anhydride. Since good first-order rate constants were obtained whether the intermediate is short or long-lived, the concentration of the intermediate must have no effect on the determination of these constants for our conditions of excess catalyst. This result substantiates the earlier kinetic arguments.[6]

General Basic Catalysis.—For general basic catalysis to be operative, the rate must be proportional to the summation of catalytic constants times catalyst concentrations at constant *p*H and ionic strength. It has been demonstrated that the hydrolysis of *p*-nitrophenyl acetate fulfills this requirement. A corollary of general basic catalysis is that a linear relationship exists between the log of the rate constant and the log of the equilibrium constant of the catalyzing base, the Brønsted relationship.[25] The data from the hydrolysis of *p*-nitrophenyl acetate with various tertiary amines given in Table I was used to make such a linear free energy plot according to the method of Brønsted, in which log k_c is plotted *versus* pK_a (Fig. 4).

It can be seen that the relationship is followed over three powers of ten in the rate. Trimethylamine shows a large negative deviation which is

(22) E. Tommila and C. N. Hinshelwood, *J. Chem. Soc.*, 1801 (1938).

(23) *Cf.* L. P. Hammett, "Physical Organic Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1940, p. 356.

(24) A. C. D. Rivett and N. V. Sidgwick, *J. Chem. Soc.*, **97**, 732 (1910).

(25) J. N. Brønsted and K. J. Pedersen, *Z. physik. Chem.*, **108**, 185 (1924).

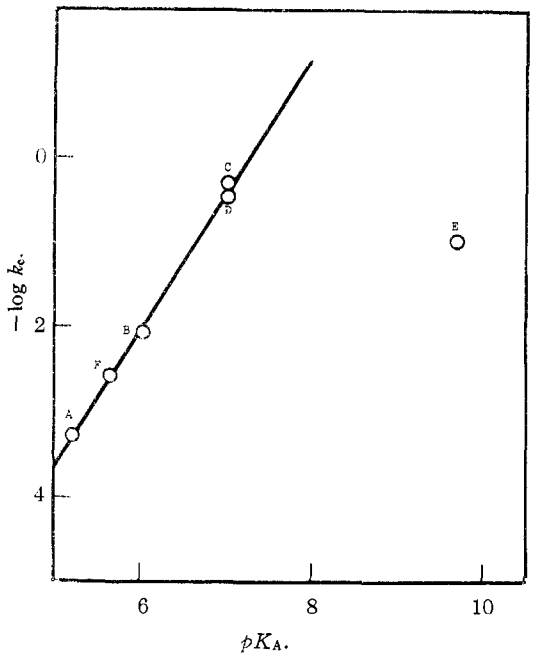


Fig. 4.—Linear free energy relationship in the hydrolysis of *p*-nitrophenyl acetate: A, pyridine; B, 4-picoline; C, imidazole; D, N-methylimidazole; E, trimethylamine; F, 3-picoline.

expected because of the much greater steric requirements of this amine compared to that of the heterocyclic bases. The slope of the line determined by the method of least squares is 1.62; this is the largest slope recorded for such a linear free energy relationship.[26,27]

Figure 4 gives information concerning the identity of the catalytic nitrogen atom of imidazole. *A priori*, one would expect the imino nitrogen to be more basic (and more nucleophilic) since imidazole is an analog of a vinylamine (containing nitrogen in place of carbon) and since vinylamines are protonated on carbon atom two of the double bond. N-Methylimidazole shows no deviation from the linear free energy relationship; a deviation would be expected if the attacking nitrogen contained a methyl group in place of hydrogen (compare trimethylamine). The nitrogen participating in the catalytic attack must then be the imino nitrogen. The intermediate obtained from this amine as catalyst, N-acetyl-N′-methylimidazolium ion, should be much less stable than N-acetylimidazole since the former cannot stabilize itself by loss of a proton while the latter can. The ready catalysis by imidazole (and N-methylimidazole) as well as their high basicities is due to the resonance stabilization of their respective quaternary salts.

Comparison of the rate constant for the alkaline hydrolysis of *p*-nitrophenyl acetate given in Table I with the data in Fig. 4 indicates that the hydroxide ion has an extremely large negative deviation in log k (*ca.* 11 powers of ten). The same type of negative deviation exists for the acetate ion-catalyzed hydrolysis of 2,4-dinitrophenyl acetate compared to that of quinoline. These two bases have pK_a values of 4.75 and 4.94, respectively; yet the ratio of their catalytic constants is 173. The present results appear to indicate that attack at a carbonyl carbon atom is governed in part by the charge on the attacking species, with uncharged reagents being more efficient than negatively charged reagents of the same basicity.

It has been shown by Pedersen for general basic catalysis involving proton transfer that if the measured rate constant, k_{obs}, is equal to the rate constant for proton transfer, the slope, α, of the Brønsted catalysis law plot must have the limits $0 < \alpha < 1$.[27,28] It has been pointed out that in such a situation as α approaches unity, general basic catalysis cannot be detected due to the much greater reactivity of hydroxyl ion as a catalyst.[27] The question might arise as to why general basic catalysis is observed in the present instance when the slope is 1.62. This phenomenon is explicable in terms of the large negative deviation in log k of the hydroxide ion catalysis. If the hydroxide ion catalysis were included in an approximate linear relationship, the slope would be in the range of 0.37–0.47 which is the region in which general basic catalysis is often observed.[27] If the relationship shown in Fig. 4 is a true Brønsted relationship, the slope of 1.62 indicates that in this reaction k_{obs} cannot be equal to the rate constant for proton transfer. It has been postulated that k_c in these ester hydrolyses does not involve proton transfer and is equal to the complex rate constant, $k_1k_3/(k_2 + k_3)$ (equation 1). The complexity of k_c makes it difficult to assess the limits of α in the present instance.

It can be seen that these ester hydrolyses conform to general basic catalysis if one extends the concept of basic catalysis to include not only a rate-determining proton transfer but also a rate-determining introduction of base into the substrate to form an unstable addition compound, the accepted mechanism for the hydroxide ion-catalyzed hydrolysis of esters and the favored mechanism for the amine- and acetate-catalyzed hydrolyses of esters.

The Brønsted relationship has been applied in the past, for the most part, to reactions in which proton transfer occurs in the catalytic step as originally set forth by Brønsted. There are two reports in which this relationship has been applied to reactions involving nucleophilic attack on carbon. One is the hydrolysis of anhydrides[29] in which the rate was proportional to the basicity of amines including pyridine, 3- and 4-picoline and isoquinoline with a slope of 0.925 but was not proportional to the basicity of sterically hindered amines such as quinoline, 2,6-lutidine and 2-picoline. The second example of such a relation is the catalysis of the hydrolysis of chloroacetate by general bases.[30,31] The rate was shown to follow the basicity of the catalyst over a large range of basicity involving 31 bases with a slope of 0.203. These examples

(26) Reference 23, p. 226.

(27) R. P. Bell, "Acid-Base Catalysis," Oxford Univ. Press, London, 1941, Chaps. 5, 7

(28) K. J. Pedersen, *J. Phys. Chem.*, **38**, 581 (1934).

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

(30) H. M. Dawson, E. R. Pycock and G. F. Smith, *ibid.*, 517 (1943).

(31) G. F. Smith, *ibid.*, 521 (1943).

together with the present results bring up the question of whether general basic catalysis must involve proton transfer in the rate-determining step. The function of a general basic catalyst appears to include not only a rate-determining proton transfer but also the introduction of a base into the substrate to form an unstable intermediate,[32,33] which is essentially the situation in the two cases cited above as well as in the present research.

Correlation of the Rate of Hydrolysis with the Acidity of the Alcoholic Residue.—An attempt was made to correlate the catalytic rate constants of imidazole-catalyzed hydrolysis with the pK_a's of the alcoholic residue of the various esters. The data in Table II were used for the plot illustrated in Fig. 5. Although a linear relationship does not exist between the rate constant and the acidity of the alcohol, it is evident that the rate increases with the acid strength of the alcohol derived from the ester in accord with the mechanistic arguments presented earlier. For a series of esters consisting of alcoholic and acyl portions of constant steric requirement, a linear free energy relationship would in all probability be applicable as has been found in the alkaline hydrolysis of substituted benzyl acetates where the rates followed the Hammett relation with a reaction constant of 0.760.[34] Figure 5 also shows a plot in which cancellation of the steric effects of the alcoholic group of the ester is attempted by plotting the log of the rate constants of the alkaline hydrolysis *versus* the log of the rate constants for imidazole catalysis. The data are given in Table II. For three esters the logarithms of alkaline and imidazole catalysis are linearly related. This relationship is similar to those obtained by Taft[35] who found that the linear free energy relationships in ester hydrolysis were independent of solvent and attacking base (hydroxide and methoxide ions). The relationship between alkaline and imidazole catalysis breaks down when an extension to methyl *p*-nitrobenzoate is attempted. The relationship predicts catalysis of this ester by imidazole equivalent to that for phenyl acetate; experimentally there is little if any imidazole-catalyzed hydrolysis of methyl *p*-nitrobenzoate. Changing steric requirements can explain part of this failure but it is probable that there are other factors.

TABLE IV

RATE CONSTANTS FOR THE FORMATION OF *p*-NITROPHENOL FROM *p*-NITROPHENYL ACETATE BY VARIOUS CATALYSTS

Catalyst	k, l./mole sec.	Solvent, 5% aqueous soln.	Temp., °C.	Ref.
Insulin	0.58	Isopropyl alc.	25	36
DFP-α-chymotrypsin	0.47	Isopropyl alc.	25	36
α-Chymotrypsin	>33	Isopropyl alc.	25	36
α-Chymotrypsin	3[a]	Isopropyl alc.	18	37
Imidazole	0.475	Dioxane	26.2	[b]
Hydroxide ion	511	Dioxane	26.2	[b]

[a] Sec.$^{-1}$. [b] This investigation.

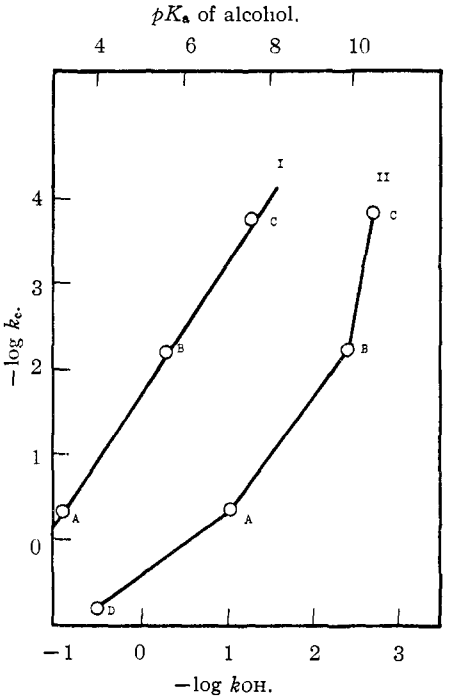


Fig. 5.—The imidazole-catalyzed hydrolysis of various esters in 5% dioxane-water at 26.2°: curve I, bottom scale; curve II, top scale; A, *p*-nitrophenyl acetate; B, phenyl acetate; C, ethyl thiolacetate; D, 2,4-dinitrophenyl acetate.

General Basic Catalysis of Ester Hydrolysis as a Model System for Enzymatic Hydrolysis.—Since it has been postulated that the active catalytic site of α-chymotrypsin consists of a general base, namely, imidazole, it is interesting to compare the kinetics of the α-chymotrypsin-catalyzed and imidazole-catalyzed hydrolyses of *p*-nitrophenyl acetate. These data together with other hydrolyses of *p*-nitrophenyl acetate are shown in Table IV.

In discussing the data in Table IV, the enzyme-catalyzed hydrolysis of this ester will be assumed to proceed through the mechanism[36–38]

$$CH_3COR + En \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (CH_3COR\ldots En) \xrightarrow{k_3} CH_3CEn + OR^- \quad (2)$$

$$CH_3CEn + H_2O \xrightarrow{k_4} CH_3COH + En + H^+ \quad (3)$$

The fact that the reactions involving insulin and DFP-α-chymotrypsin have the same rate constant

(32) J. W. Baker and E. Rothstein, "Handbuch der Katalyse," Vol. 2, G. M. Schwab, ed., J. Springer, Vienna, 1940, p. 51.

(33) E. L. King, "Catalysis," Vol. II, P. H. Emmett, ed., Reinhold Pub. Corp., New York, N. Y., 1955, pp. 389–397.

(34) Reference 23, p. 191.

(35) R. W. Taft, Jr., THIS JOURNAL, **74**, 2729 (1952).

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

(37) H. Gutfreund, *Disc. Faraday Soc.*, **20**, 167 (1955); H. Gutfreund, "Advances in Catalysis," Vol. IX, in press, Academic Press, Inc., New York, 1956; H. Gutfreund and J. M. Sturtevant, *Biochem. J.*, **63**, 656 (1956). In the last paper serine, and not imidazole, is postulated to participate in step k_4, thus liberating *p*-nitrophenol. I. B. Wilson and F. Bergmann, *J. Biol. Chem.*, **186**, 683 (1950).

(38) ADDED IN PROOF.—From the reported results of G. H. Dixon, W. J. Dreyer and H. Neurath, THIS JOURNAL, **78**, 4810 (1956), and of Gutfreund and Sturtevant[37] with respect to the *p*H dependence of steps k_2 and k_4, it appears that imidazole participates in step k_4 and not k_3. This implies that step k_4 may consist of a two-step process involving an acyl-imidazole intermediate.

as imidazole indicates that the catalytic species of these non-enzymatic proteins is the imidazole ring of a histidine residue.

Even though *p*-nitrophenyl acetate does not possess the structural requirements of a typical substrate of α-chymotrypsin, the data of Table IV indicate that the ester is hydrolyzed by the catalytically active site of this enzyme since diisopropyl fluorophosphate inhibits this reaction as well as the reactions of typical substrates. Confirmation of this hypothesis has come from the isolation of acetyl-α-chymotrypsin from the reaction of *o*-nitrophenyl acetate and α-chymotrypsin. This compound possesses one acetyl group per molecule of α-chymotrypsin and is enzymatically inactive; the acetyl group is rapidly hydrolyzed in neutral solution, regenerating the active enzyme.[39]

A large difference in rate exists between the rate of formation of *p*-nitrophenol by α-chymotrypsin on the one hand and by imidazole or DFP–α-chymotrypsin on the other hand.

The large difference in rate between active and DFP-inhibited enzyme is most easily explained by the reaction of the ester at the active site in the former case and at an inactive imidazole ring in the latter. It is significant that there are two histidine residues in α-chymotrypsin which can account for these two sites.[40] For the rate of the former reaction, the rapid spectrophotometric data of Gutfreund are preferred to those of Hartley and Kilby since Gutfreund's rate constant applies to k_3 of reaction 2 and is presumably analogous to the rate constant for the imidazole-catalyzed reaction.

The equivalence of the rate constants for imidazole and DFP–chymotrypsin catalysis and the much larger rate constant for α-chymotrypsin catalysis indicates that if the active site of α-chymotrypsin involves an imidazole ring[37] the mechanism is certainly more complex than a simple displacement on ester by imidazole to give an acyl–enzyme intermediate followed by hydrolytic cleavage of the intermediate. This conclusion is strengthened by the fact that esters of aliphatic alcohols are readily hydrolyzed by α-chymotrypsin, but there is little or no catalysis by imidazole. If the secondary imidazole in DFP–α-chymotrypsin is equivalent to imidazole alone and is thus not sterically hindered, the imidazole in α-chymotrypsin must contain a unique environment to cause such a large increase in rate.

In contrast to the large difference in the rate of liberation of *p*-nitrophenol with α-chymotrypsin and imidazole, the rate of formation of acetate from the acetyl-enzyme and N-acetylimidazole are reasonably close. The former was reported as 10^{-2} sec.$^{-1}$,[36] whereas the latter is found to be 1.5×10^{-3} sec.$^{-1}$. Since the rate of formation of acid is usually measured in enzymatic catalysis, differences in rate for various substrates would imply that specificity is important in the hydrolytic cleavage of the intermediate as well as in previous steps. It should be pointed out that N-acetylimidazole, while a relatively reactive intermediate, is still quite selective since it will react preferentially with more powerful nucleophiles such as phosphate ion and thiols rather than with water.

Catalysis of ester hydrolysis by imidazole by no means explains all the phases of enzymatic hydrolysis. However, there are some aspects of these reactions which appear to be similar (*i.e.*, formation of an acyl-catalyst intermediate and its rate of hydrolysis) so that it is at least reasonable to postulate imidazole as a part of the active center in α-chymotrypsin and to use the catalysis by imidazole as a starting point for the construction of a more complete model for α-chymotrypsin-catalyzed hydrolysis.

Hydrolysis of esters by imidazole may possibly serve as a model system for the enzyme papain. Smith[41] has presented evidence that the action of this enzyme is due to formation of a thiol ester of the enzyme (transesterification of the ester with the sulfhydryl group of the enzyme) and subsequent hydrolysis of the thiol ester. Since the hydrolysis of a thiol ester is more readily catalyzed by imidazole than the hydrolysis of the corresponding oxygenated ester, it is entirely feasible that papain catalyzes hydrolysis through such a mechanism. It should be pointed out that whereas the hydroxide ion-catalyzed hydrolysis of ethyl thiolacetate is only slightly faster than that of ethyl acetate,[16] the imidazole-catalyzed hydrolysis of the former is apparently infinitely faster than that of the latter.

The use of thiol esters in biological reactions is by no means limited to hydrolytic reactions. The important acyl transfer and oxidative decarboxylation reactions involve thiolesters such as acetylcoenzyme A and acetylglutathione; it is possible that these reactions occur through acylamine intermediates.

ADDED IN PROOF.—After submission of this and the preceding paper, it was found that many aspects of this research were carried out independently and simultaneously by T. C. Bruice and G. L. Schmir, *Arch. Biochem. Biophys.*, **63**, 484 (1956), and THIS JOURNAL, **79**, 1663 (1957). Substantial agreement exists between the results of these investigations. The authors gratefully acknowledge the opportunity of reading the manuscript of Bruice and Schmir prior to publication.

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