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Chymotrypsin-Catalyzed Phenyl Ester Hydrolysis. Evidence for Electrophilic Assistance on Carbonyl Oxygen*

A. Williams

ABSTRACT: The chymotrypsin-catalyzed hydrolysis of substituted aryl hippurates and *N*-methanesulfonyl-L-phenylalaninates has been measured at pH 6.91, 25°, and 0.1 M ionic concentration. The parameter k_{cat} was essentially constant but k_{cat}/K_m obeyed a Hammett relationship (ρ 0.5 for hippurates, 0.45 for *N*-methanesulfonyl-L-phenylalaninates). The low sensitivity of k_{cat}/K_m to leaving group structure is interpreted as evidence for electrophilic assistance at the *carbonyl oxygen* during the formation of a tetrahedral intermediate. The positive ρ values are reconciled with the negative ones observed in chymotrypsin-catalyzed anilide hydrolysis by a mechanism involving almost complete proton transfer from an imidazolium cation to the departing aniline anion in the transition state for the breakdown of the tetrahedral adduct; proton transfer has not occurred in the corresponding transition state for aryl ester hydrolysis and the rate-determining step in acylation of chymotrypsin by aryl esters is the formation of the tetrahedral intermediate. A mechanism involving protonation of the nitrogen of the anilide prior to tetrahedral adduct formation is demonstrated not to be consistent with the kinetics for chymotrypsin-catalyzed hydrolysis of anilides. Acylation of chymotrypsin by substituted phenyl acetates has a ρ value of +1.8 which could arise from the absence of electrophilic assistance at the carbonyl oxygen owing to the nonspecific esters orientating themselves differently from specific substrates on the enzyme surface.

Few systematic studies have been made on the effects of leaving group structure on reactivity in the acylation of chymotrypsin by esters. Bender and Nakamura (1962) have observed that k_{cat}/K_m for substituted phenyl acetates obeys a Hammett equation, being correlated with σ^- with a ρ value of +1.8. More work has been reported on substituent effects where the acyl group has a specific backbone and an aniline leaving group (Sager and Parks, 1963, 1964; Inagami *et al.*, 1965; Wang and Parker, 1967; Caplow, 1969; Parker and Wang, 1968; Wang, 1968; Inagami *et al.*, 1969). The acylation of chymotrypsin by substituted anilides in general obeys a Hammett relationship with a negative ρ value in contrast to the aryl ester results. The suggestion has been made that acylation by anilides requires electrophilic assistance in the form of a proton transfer to the nitrogen of the anilide from an imidazolium moiety. This catalysis is the microscopic reverse of the general base (imidazole) catalyzed attack of the nucleo-

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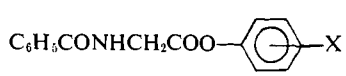
TABLE I: Analytical and Physical Properties of Substrates.[a]

Compound	Mp (°C)	Comments
N-Mesyl-L-phenylalaninate		
p-Nitrophenyl	127–130	*Anal.* Calcd: C, 52.7; H, 4.4; N, 7.7. Found: C, 53.2; H, 4.5; N, 7.8.
m-Nitrophenyl	85–88	*Anal.* Calcd: C, 52.7; H, 4.4; N, 7.7. Found: C, 52.4; H, 4.6; N, 7.5.
p-Acetophenyl	108–110	*Anal.* Calcd: C, 59.9; H, 5.3; N, 3.9. Found: C, 59.5; H, 4.8; N, 3.9.
p-Chlorophenyl	84–85	*Anal.* Calcd: C, 53.7; H, 4.5; N, 3.9. Found: C, 54.2; H, 4.8; N, 3.9.
Phenyl	105–108	*Anal.* Calcd: C, 60.1; H, 5.3; N, 4.4. Found: C, 60.3; H, 5.2; N, 4.4.
p-Cresyl	87–90	*Anal.* Calcd: C, 61.2; H, 5.7; N, 4.2. Found: C, 61.2; H, 4.9; N, 4.1.
p-Methoxyphenyl	99–103	*Anal.* Calcd: C, 58.5; H, 5.5; N, 4.0. Found: C, 58.6; H, 4.9; N, 4.1.
Hippurates		
p-Aminophenyl	133–136	Kindly supplied by Mr. E. C. Lucas.
p-Nitrophenyl	170–171	167–168° (Weiss, 1893; McDonald and Balls, 1957).
p-Acetophenyl	153–155	*Anal.* Calcd: C, 68.6; H, 5.05; N, 4.7. Found: C, 68.6; H, 5.04; N, 4.6.
m-Nitrophenyl	118–122	118–122° (Lowe and Williams, 1965).
Phenyl	104	104° (Weiss, 1893).
p-Cresyl	107–108	107–108.5° (Williams, 1964).
p-Methoxyphenyl	104–105	*Anal.* Calcd: C, 67.4; H, 5.3; N, 4.9. Found: C, 67.4; H, 5.4; N, 4.9.
p-Fluorophenyl	103	*Anal.* Calcd: C, 68.4; H, 4.6; N, 5.3. Found: C, 68.1; H, 4.2; N, 5.6.
p-Chlorophenyl	130–132	130–131.5° (Lowe and Williams, 1965).

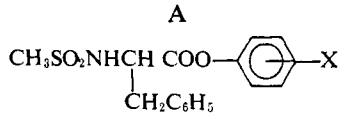
[a] Analyses were performed by Mrs. M. J. Clark of this laboratory using a Hewlett-Packard 185 analyzer. Melting points were determined using a Kofler "Thermospan" instrument.

phile on the acyl-enzyme. A report by Bundy and Moore (1966), however, shows that acylation by nitroanilides of *N*-benzoyl-L-tyrosine is *enhanced* by electron-withdrawing substituents.

The departure of the aniline moiety from the tetrahedral adduct in nonenzymatic anilide hydrolysis will not proceed without assistance because the $ArNH^-$ anion is a poor leaving group compared to the entering nucleophile; protonation of the nitrogen (in the tetrahedral adduct) yields a relatively good leaving group. It is reasonable that electrophilic catalysis will occur at the nitrogen in acylation of chymotrypsin by anilides and that substituents on the anilide moiety will yield a negative ρ. Any electrophilic assistance on the carbonyl oxygen prior to (and aiding) tetrahedral adduct formation will thus be masked. Such catalysis will only be revealed when the leaving group (such as aryloxy) needs no assistance in its expulsion from a tetrahedral adduct. We report here the results of a study of the acylation of chymotrypsin by substituted phenyl ester specific substrates (A and B) as evidence that acylation is aided by electrophilic catalysis at the carbonyl oxygen.



A



B

TABLE II: Spectral Data for Substrate Hydrolyses.[a]

Leaving Group	Wavelength (nm)	Extinction Change ($\Delta\epsilon$)
Phenyl	270	1300
m-Nitrophenyl	355	1270
p-Acetophenyl	325	2460
p-Nitrophenyl	350	4950
p-Chlorophenyl	275	897
p-Cresyl	285	636
p-Methoxyphenyl	290	2170
p-Fluorophenyl	280	1820
p-Aminophenyl	310	710

[a] These results apply to pH 6.91, 0.1 M ionic concentration, and 25° and apply to both series of esters used here.

Experimental Section

Materials. α-Chymotrypsin was obtained from Seravac Laboratories (PTY) Ltd., Maidenhead, U. K. Stock solutions were made up in water and their molarity was determined by spectrophotometric titration with *N-trans*-cinnamoylimidazole at 335 nm (Schonbaum *et al.*, 1961). The Seravac product gave a titration value of about 80% of that calculated on a weight basis, assuming a molecular weight of 24,800.

N-Mesyl-L-phenylalanine esters[1] were prepared from the acid chloride by mixing 0.01 mole of the latter, 0.01 mole of substituted phenol in 30 ml of dichloromethane, and 0.01 mole of dry pyridine, with stirring and cooling. The mixture

[1] Abbreviation used here is: mesyl, methanesulfonyl.

TABLE III: Kinetic Data for Hippurate Esters.[a]

Substrate	k_{cat}/K_m (M^{-1} sec^{-1} × 10^{-3})	k_{cat} (sec^{-1})	σ^{-}[b]	σ[b]
p-Aminophenyl (5)[c]	2.96 ± 0.03	0.50 ± 0.02	−0.15[d]	−0.66[d]
p-Nitrophenyl (6)[c]	17 ± 1	0.49 ± 0.03	1.27	0.78
p-Acetophenyl (8)[c]	17 ± 1	0.530 ± 0.03	0.874	0.50
m-Nitrophenyl (10)[c]	7.7 ± 0.5	0.52 ± 0.04	0.71	0.71
Phenyl (12)[c]	3.6 ± 0.3	0.49 ± 0.04	0	0
p-Cresyl (6)[c]	4.5 ± 0.3	0.61 ± 0.03	−0.17	−0.17
p-Methoxyphenyl (8)[c]	5.5 ± 0.3	0.56 ± 0.02	−0.27	−0.27
p-Fluorophenyl (8)[c]	3.7 ± 0.2	0.52 ± 0.03	0.06	0.06
p-Chlorophenyl (7)[c]	4.3 ± 0.2	0.49 ± 0.03	0.23	0.23

[a] Enzyme concentration 0.59 × 10^{-5} M, 25°, 0.1 M ionic concentration, pH 6.91, substrate concentration 0.5–2.10^{-4} M. [b] From Leffler and Grunwald (1963) p 173. [c] Number of initial rates measured. [d] Barlin and Perrin (1966).

was kept stirring overnight at room temperature, washed with dilute HCl, with sodium bicarbonate solution, dried (Na_2SO_4), and evaporated. The products were recrystallised from benzene–petroleum ether (bp 40–60°). Structures were confirmed by infrared, nuclear magnetic resonance, and by analysis (see Table I for physical constants). *N*-Mesyl-L-phenylalanine acid chloride was prepared by swirling 0.015 mole of phosphorus pentachloride (finely ground) with 0.01 mole of *N*-mesyl-L-phenylalanine in the minimum quantity of chloroform. The acid dissolved with effervescence of HCl gas and after keeping for 10 min in the cold the solution was filtered and the acid chloride precipitated as fine needles with petroleum ether (bp 40–60°), mp 82°. *Anal.* Calcd for $C_{10}H_{12}NSO_3Cl$: C, 46.0; H, 4.6; N, 5.36. Found: C, 46.2; H, 4.3; N, 5.3; infrared (Nujol) 1790 cm^{-1} (−COCl). *N*-Mesyl-L-phenylalanine was prepared by the method of Helferich and Grunert (1940); it had mp 108° (lit. for DL form 103°). *Anal.* Calcd: C, 49.5; H, 5.4; N, 5.8. Found: C, 50.0; H, 5.7; N, 5.9. Hippurate esters were prepared according to the method of Weiss (1893) or from the acid chloride by the method described above. Hippuryl chloride was prepared by a modification of Fischer's (1905) technique. Finely ground hippuric acid (0.01 mole) was suspended in chloroform (20 ml), cooled in ice, and 0.015 mole of phosphorus pentachloride (finely ground) was added during about 5 min with occasional stirring. Petroleum ether was then added (20 ml) and the suspension was swirled, filtered rapidly, washed once with dry petroleum ether, and transferred to a vacuum desiccator ($CaCl_2$). The material (used within 2-hr preparation) had infrared (Nujol) 1760 cm^{-1} (COCl).

Buffers were of analytical grade material; acetonitrile was purified by the method of Lewis and Smyth (1939).

Kinetic Methods. The hydrolysis of the substituted phenyl esters was followed spectrophotometrically at an appropriate ultraviolet wavelength (Table II). In a typical experiment the substrate (*ca.* 0.01 ml), dissolved in acetonitrile stock solution, was mixed with buffer solution (phosphate buffer, pH 6.91, 25°, 0.1 M ionic concentration) in a silica cell and placed in the thermostatted cell compartment of a Unicam SP800 spectrophotometer. After the temperature had equilibrated, enzyme (0.01 ml) was placed on the tip of a glass rod using a Lambda pipet. A continuous record of the absorbance was started to check blank rate (blank hydrolysis at pH 6.91 was less than 1% during the total time for any chymotrypsin catalysis); enzyme was added when the pen passed a marker and in this way an accurate zero was obtained. The experiments were repeated at different substrate concentrations and the initial rates were processed to obtain Michaelis–Menten parameters using a programmed version of Wilkinson's (1961) method (Williams, 1969). A minimum of five substrate concentrations was employed for each analysis and extinction coefficients were checked by allowing complete hydrolysis to occur (Table II shows average extinction coefficients).

Results

The hydrolysis of the aryl esters catalyzed by chymotrypsin obeyed the Michaelis–Menten rate law (eq 1) and the param-

$$\text{rate} = \frac{k_{cat}[E][S]}{[S] + K_m} \quad (1)$$

eters derived from initial rates are tabulated (Tables III and IV). The hydrolysis of the *p*-nitrophenyl ester of *N*-mesyl-L-phenylalanine deserves comment because the value of K_m was so small that at [S] ≈ K_m there was very little substrate available. The value of k_{cat}/K_m was calculated from the pseudo-first-order hydrolysis at low substrate concentration (10^{-5} M). Product inhibition was shown to be negligible at this concentration of the acid product. Thus the pseudo-first-order rate constant was equal to (k_{cat}/K_m)[E]. The value of k_{cat} for this substrate was measured by the conventional methods. Figure 1 illustrates Lineweaver–Burk regressions for some of the substrates (note that these regressions were not used to derive the kinetic parameters and that the solid lines represent the rates calculated from the data of Table III and IV). Figures 2 and 3 illustrate the dependence of k_{cat}/K_m for both sets of substrates on Hammett's σ. There is a slightly better σ^{-} dependency for both sets of substrate but this is not sufficient to warrent firm mechanistic conclusions. It is probably safe to compare values of k_{cat}/K_m for similar substrates measured at a constant pH because pK_a differences are small (Bender

TABLE IV: Kinetic Data for *N*-Mesyl-L-phenylalaninate Esters.[a]

Substrate	k_{cat}/K_m (M^{-1} sec^{-1} × 10^{-6})	k_{cat} (sec^{-1})	σ^{-}[b]	σ[b]
p-Nitrophenyl	8.65 ± 1.0[c]	36.5 ± 2.0	1.27	0.78
m-Nitrophenyl (10)[d]	3.54 ± 0.4	30.7 ± 1.9	0.71	0.71
p-Acetophenyl (9)[d]	2.69 ± 0.3	34.6 ± 1.6	0.874	0.50
p-Chlorophenyl (10)[d]	2.77 ± 0.09	36.5 ± 1.5	0.23	0.23
Phenyl (8)[d]	1.22 ± 0.08	32.7 ± 0.8	0	0
p-Cresyl (11)[d]	1.83 ± 0.09	36.5 ± 0.9	−0.17	−0.17
p-Methoxyphenyl (10)[d]	2.29 ± 0.08	34.6 ± 1.0	−0.27	−0.27

[a] Chymotrypsin concentration 0.312 × 10^{-7} M, 25°, 0.1 M ionic concentration, pH 6.91, substrate concentration 1–5 × 10^{-5} M. [b] From Leffler and Grunwald (1963), p 173. [c] This result was determined as described in the text. [d] Number of initial rates measured.

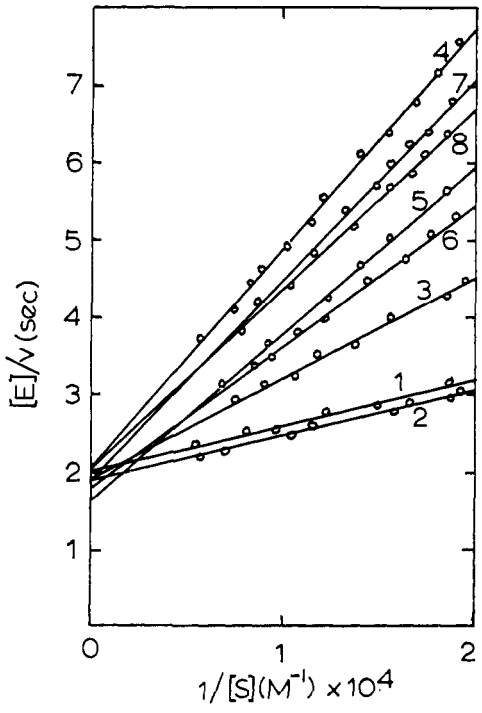


FIGURE 1: Lineweaver–Burk plots for phenyl hippurate substrates of chymotrypsin. Lines are calculated from the data of Table III: (1) *p*-nitrophenyl; (2) *p*-acetophenyl; (3) *m*-nitrophenyl; (4) phenyl; (5) *p*-cresyl; (6) *p*-methoxyphenyl; (7) *p*-fluorophenyl; (8) *p*-chlorophenyl. Conditions were pH 6.91, 25°, 0.1 M ionic concentration.

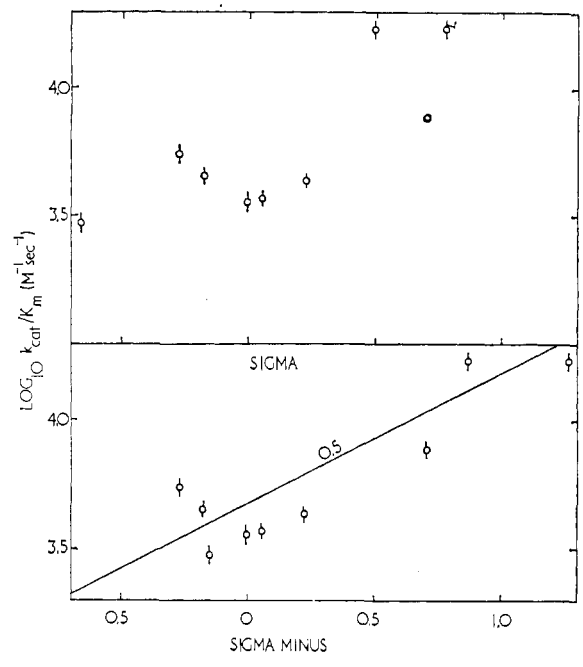


FIGURE 2: Dependence of k_{cat}/K_m on σ and σ^- for substituted phenyl hippurate substrates of chymotrypsin (see Table III for conditions).

et al., 1964) in acylation. We find that the pK_a's for acylation by phenyl and *p*-nitrophenyl hippurate are identical (Figure 4).

Discussion

For the purpose of this discussion we shall assume that the overall mechanism for chymotrypsin-catalyzed reactions can be formulated as

$$\text{E-OH} + \text{RCOX} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \text{EOH} - \text{RCOX} \xrightarrow{k_2} \text{EOCOR} + \text{HX}$$

$$\text{EO-COR} \xrightarrow{k_3(H_2O)} \text{EOH} + \text{RCO}_2\text{H} \qquad (2)$$

This mechanism (eq 2) has been thoroughly vindicated in a number of authoritative works as the "minimal" mechanism for catalysis (Bruice and Benkovic, 1966; Jencks, 1969; Bender and Kézdy, 1964). Moreover, the hydroxyl group of serine-195 is acylated (see, *e.g.*, Keizer and Bernhard, 1966) and a basic group with a pK_a of approximately 7 is involved in both deacylation (k_3) and in acylation (usually measured as the composite function k_2/K_s, where $K_s = (k_{-1} + k_2)/k_1$). The basic group is usually identified as the imidazole moiety of histidine-57 (Ong *et al.*, 1964; Smillie and Hartley, 1964).

The results of this study indicate that ester substrates with natural like structures show only a small sensitivity in acylation (k_{cat}/K_m)[2] to the leaving group ability ($\rho \approx 0.5$ of

[2] The ratio k_{cat}/K_m represents the acylation of free enzyme by free substrate.

TABLE V: Comparison of k_{cat}/K_m for *p*-Nitrophenyl and Ethyl Leaving Groups (25°).

Acyl Group	k_{cat}/K_m (M^{-1} sec^{-1})		
	p-Nitrophenyl	Ethyl	Ratio
Hippurate[a,h]	0.589×10^4	0.425×10^2	134
N-Acetyl-L-tryptophanate[b,h]	52×10^3	2.78×10^5	54.6
N-Acetyl-L-phenylalaninate[b,h]	32×10^5	0.715×10^5	44.8
N-Acetylglycinate[a,f]	4.75×10^3	0.135	35,000
Cinnamate[c]	1.13×10^4[e]	3.57[d,f]	3,170
Acetate	(k_{OH^-} 8.05)[g]	(k_{OH^-} 0.137)[g]	58.8

[a] Zerner and Bender (1964). [b] Zerner *et al.* (1964). [c] Bender and Zerner (1962). [d] Methyl ester. [e] pH 8.34. [f] pH 7.90. [g] From data in National Bureau of Standards Circular 510 "Tables of Chemical Kinetics." [h] pH 7.0.

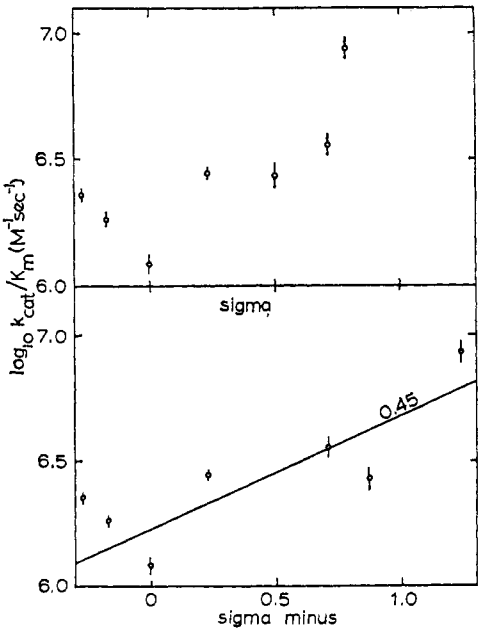


FIGURE 3: Dependence of k_{cat}/K_m on σ and σ^- for substituted phenyl *N*-mesyl-L-phenylalaninate substrates of chymotrypsin (see Table IV for conditions).

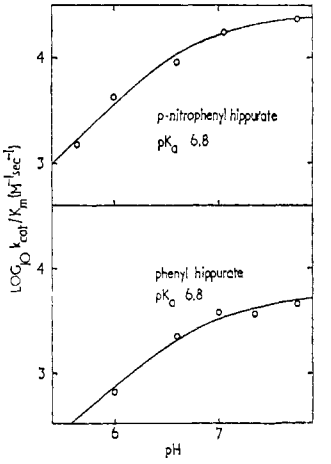
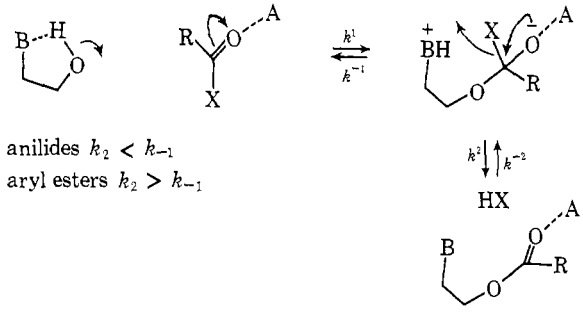


FIGURE 4: pH dependence of k_{cat}/K_m for *p*-nitrophenyl (A) and phenyl hippurate (B). Conditions were 25°, 0.1 M ionic concentration, 1% CH_3CN.

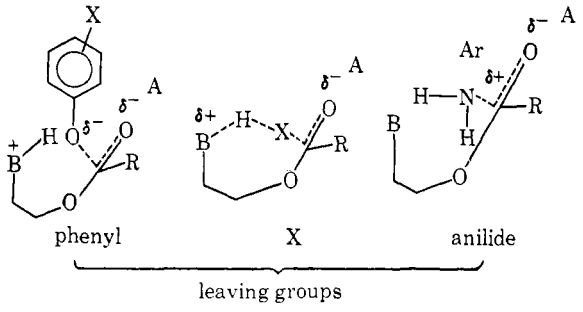
Figures 2 and 3) compared to the ρ value for acylation by substituted phenyl acetates and compared to the attack of nucleophiles on substituted phenyl esters ($\rho \approx 1.8$, see Bender and Nakamura, 1962, and references quoted therein). The low selectivity to leaving group also exists where the ratio of k_{cat}/K_m for *p*-nitrophenyl and ethyl ester substrates ranges as low as 44 (for the specific acyl group *N*-acetyl-L-phenylalanine). This low ratio (*cf.* Table V) does not agree with results for acylation by acetate esters (Bender and Nakamura, 1962) where we estimate the ratio to be greater than 10,000. As the specificity of the substrate decreases the selectivity increases, for example, cinnamate and *N*-acetylglycinate are generally supposed to be less specific than hippurates or *N*-acyl-L-phenylalaninates[8]—and this is reflected in the higher ratios (Table V). The low sensitivity toward acylation of chymotrypsin can be mimicked in a simple model where hydroxide reacts with acetate esters (Table V); whereas hydroxide has a pK_a of 15.7 the serine hydroxyl of chymotrypsin has an apparent pK_a of 7 (the attacking chymotrypsin nucleophile is a composite system whose half-protonation occurs at pH 7—Blow *et al.*, 1969) so that chymotrypsin should be much less reactive than hydroxide and consequently less selective. A possible explanation of this anomally is that electrophilic assistance occurs at the carbonyl oxygen in acylation reducing the selectivity of the enzyme; electrophilic assistance at the ether oxygen is probably not operative during acylation (~*ca.* 20 kcal/mole of resonance energy would be lost—see below). Expulsion of an aryl leaving group is probably not the rate-determining step in acylation by aryl ester substrates and hence no electrophilic assistance (see also Caplow, 1969) should be reflected in k_{cat}/K_m for this step. Hubbard and Kirsch (1970) have observed small ρ values in the acylation of chymotrypsin by substituted *p*-nitrophenyl

[8] We use here the objective test that specific substrates have larger k_{cat}/K_m values than nonspecific ones. This test incorporates a measure of both binding (essentially K_m) and catalytic (essentially k_{cat}) specificity See Table V for a comparison of k_{cat}/K_m for different substrate types.

SCHEME I



Transition state for k^2 step

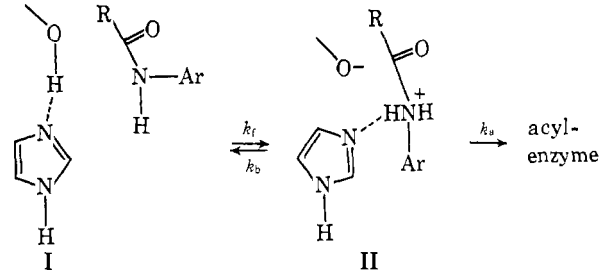


benzoates. These are ascribed to initial acylation on imidazole but could equally well be caused by electrophilic assistance.

Bender and Nakamura (1962) observed that the ρ value for k_{cat}/K_m for substituted phenyl acetates was as large as that expected from model reactions involving nucleophilic attack and did not discuss the possibility of electrophilic catalysis for this case. These workers also found that the *p*-aceto substituent deviated from their correlation by a factor of about 12-fold and this was traced to the acylation rate constant (k_2) rather than to the binding constant (K_s). Bender *et al.* (1964) subsequently pointed out that the aryloxy group of *p*-nitrophenyl acetate must bind approximately in the same position on chymotrypsin as the aryl group of *N*-acetyl-L-tryptophan ethyl ester and that comparison of the k_2 kinetic specificities of these two substrates is meaningless. Possibly, the aryloxy group of phenyl acetate substrates binds at the ρ_2 or nonpolar site[4] on chymotrypsin. The addition of aromatic compounds such as indole (A. Williams, 1968, unpublished data) and other nonpolar compounds such as dioxane (Faller and Sturtevant, 1966) causes a pronounced depression of the rate of acylation of chymotrypsin by *p*-nitrophenyl acetate. A recent crystallographic study (Steitz *et al.*, 1969) has shown that dioxane binds to α-chymotrypsin in a position coincident with that of the toluene ring of tosylchymotrypsin (Sigler *et al.*, 1968). If *p*-nitrophenyl acetate also binds in this position, then, provided the ester group is in the orientation of that of the sulphonyl (the ether oxygen replacing the sulfur) in the tosylchymotrypsin it will be in an excellent position for acylation. The absence of serious deviations from the correlations for

[4] The ρ_2 site defined by Hein and Niemann (1962) accepts nonpolar side chains and is probably itself nonpolar in nature. This site appears to be tacitly identified as the "tosyl hole" in the paper by Steitz *et al.* (1969).

SCHEME II



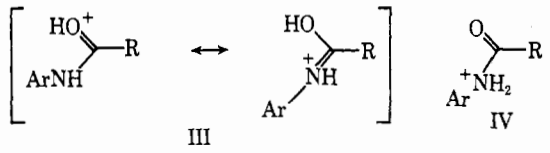
$$k = \frac{k_f}{k_b} = \frac{(K_a)_{serine}}{(K_a)_{anilide}} \quad (3)$$

the *p*-aceto substituent (Figures 2 and 3) observed in this investigation would indicate that the aryl oxy group in the *specific* substrates takes up a different orientation on chymotrypsin from that of acetate esters.

The difference in ρ values between specific ester substrates and acetate esters may be due to the different orientations of the ester carbonyl brought about by the different locations (on the enzyme) of the aryloxy leaving groups which allows an electrophilic interaction at the ester carbonyl oxygen of the specific substrate which is impossible for the bound acetate ester.

It has been suggested that electrophiles assist the acylation of chymotrypsin by anilides (see Wang, 1968, for a review); acylation by substituted anilides (k_2) obeys a Hammett relationship and depends on a negative ρ value consistent with acid catalysis. Electrophilic assistance is not necessary in the case of phenyl ester substrates because aryloxy anions are usually weak bases, and the departure of the leaving group involves the transfer of a proton from the general acid to the departing group and for anilides the protonation is far advanced but in aryl esters would be very little advanced in the transition state for the departure of the leaving group. It follows from the fact that deacylation involves a proton transfer from nucleophile to the general base (imidazole) that in the k^2 step (the microscopic reverse of deacylation) the proton transfer to the leaving group is from the imidazolium ion (from His-57). Scheme I (similar to one proposed by Caplow, 1969) incorporates all the presently available structural-kinetic data and includes electrophilic assistance for the formation of the tetrahedral adduct.

Wang and Parker's (1967) mechanism deserves comment as it would predict the observed structural-kinetic relationships. Wang proposes that anilide hydrolysis (essentially Scheme II) proceeds *via* a zwitterionic structure II from the enzyme–substrate complex I. The equilibrium constant between I and II is given by eq 3. Reasonable estimates are made by Wang for k_b (10^{14} sec^{-1}) and $(K_a)_{serine}$ (10^{-13}) but $(K_a)_{anilide}$ is taken as $10^{0.5}$ from measurements of the basicity of anilides in strong acids. Using this data k_f was calculated to be greater than 1 sec^{-1}; since this value is larger than the largest value for k_2 for acylation of chymotrypsin (0.21 sec^{-1} ~ Inagami *et al.*, 1965) the mechanism was considered consistent with the observed kinetics. Wang's value for $(K_a)_{anilide}$, however, is derived from the model where the protonation giving the pK_a of -0.5 arises from O protonation as in III whereas the desired model involves N protonation as in IV. Anilides



III IV

and amides are well known to protonate on the carbonyl oxygen (Arnett, 1963; Katritzky and Jones, 1961; Edward *et al.*, 1960) and N protonation would require the destruction of about 20 kcal/mole of resonance energy (Pauling, 1960). In addition, the species III is stabilized by resonance whereas IV is not. In order that k_2 should be sustained by Wang's mechanism $k_t \geq k_2$. In the case of acylation by *N*-acetyl-L-tyrosine *p*-anisidide (Inagami *et al.*, 1965; Parker and Wang, 1968) $k_2 = 0.21$ sec^{-1} thus $k_t = 10^{14} \times 10^{-13}/(K_a)_{\text{anilide}} \geq 0.21$ sec^{-1} hence $(K_a)_{\text{anilide}} \leq 0.5$. This maximum value is much less than would be expected for N protonation where the conjugate acid is 20 kcal/mole more energetic by virtue of resonance energy loss. On these grounds Scheme II cannot sustain the observed acylation rate constants for anilide substrates. Wang's mechanism does predict the correct order of magnitudes for k_2; this, however, follows from the fact that similar substituent effects should be observed in N protonation of the departing aniline from the tetrahedral intermediate. A similar set of arguments eliminates a mechanism involving protonation of the ether oxygen in ester substrates prior to tetrahedral intermediate formation.

Bender and Heck (1967) observed that deacylation of cinnamoyl- and *p*-nitrobenzoylchymotrypsin did not involve concurrent carbonyl oxygen exchange. This result could be interpreted as evidence for the nonequivalence of the two exchangeable oxygens (of a tetrahedral intermediate) induced by the asymmetric enzyme surface. The hypothesis of electrophilic participation at the carbonyl oxygen supports this explanation because the electrophile would "protect" that oxygen in the tetrahedral intermediate arising from the carbonyl oxygen. A study of the constituents of the active site[5] indicates that if serine-195 were involved in acylation and if imidazole (His-57) acted as a general base none of the available amino acid side chains could act as an electrophile. Steitz *et al.* (1969) suggest from their X-ray studies that an oxygen of the carboxyl group of the *N*-formyl-L-tryptophanate inhibitor is hydrogen bonded to serine-195 (we assume at the β-hydroxyl group) and to the NH group of the peptide backbone of glycine-193 and serine-195; the other oxygen is hydrogen bonded to the imidazole of histidine-57. Possibly the hydrogen bonding to the peptide backbone of serine-195 and glycine-193 exists in ester and amide enzyme–substrate complexes and provides the electrophilic assistance discussed earlier. The electrophilic interaction is probably identified with the ρ_3–R_3 binding postulated by Hein and Niemann (1962).

[5] A model of the active-site region of chymotrypsin was built using Kendrew Models (Cambridge Repetition Engineers, Cambridge, U. K.), and coordinates from Birktoft *et al.* (1969).

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Partial Purification and Properties of Cytidine Deaminase from Baker's Yeast*

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ABSTRACT: Enzymological studies on partially purified preparations of baker's yeast cytidine deaminase are described. The enzyme is inhibited both by cytidine 5′-monophosphate (CMP) and substrate excess. Plots of kinetic data for CMP take the form of sigmoidal inhibition curves. Substrate saturation curves show first-order kinetics at low cytidine concentrations, suggesting the absence of interaction between catalytic sites; however, substrate inhibition seems to involve cooperative binding of cytidine at inhibitory sites. Cooperativity is also observed between CMP and cytidine inhibitory sites. Heating the enzyme preparation above 50° abolishes both CMP and substrate excess inhibition, while catalytic activity is left unchanged even upon heating at 60°. The data obtained are interpreted as indicating the allosteric nature of inhibition by both CMP and cytidine; as a mere hypothesis, the same kind of inhibitory sites could be thought to be implied in the binding of both compounds, thus providing a satisfactory explanation for the kinetic data reported.

Cytidine deaminase (cytosine nucleoside aminohydrolase, EC 3.5.4.5) was originally found by Schmidt (1932) in dog liver extracts and has since been identified in a variety of biological sources (Conway and Cooke, 1939; Greenstein *et al.*, 1947; Creasey, 1963; Tomchick *et al.*, 1968; Wang *et al.*, 1950; Wang, 1955; Wisdom and Orsi, 1967; Achar *et al.*, 1966). The enzyme has been partially purified from mouse kidney (Creasey, 1963; Tomchick *et al.*, 1968), sheep liver (Wisdom and Orsi, 1967), and green-gram seedlings (Achar *et al.*, 1966). An enzyme activity in yeast, catalyzing the deamination of cytidine, was originally found in crude extracts by Wang *et al.* (1950) and appears to be distinct from cytosine deaminase. The properties of the enzyme, however, have never been studied in purified systems. The present paper reports some kinetic properties of yeast cytidine deaminase. The data show that the enzyme is allosterically inhibited both by substrate excess and by CMP.[1] Cytosine deaminase activity, in accordance with the results obtained by Lochmann (1965), was also found in yeast extracts; in the course of purification, however, the ratio of the two enzymatic activities changes from step to step; after gel filtration on G-100 Sephadex two distinct peaks are obtained for cytidine and cytosine deaminase activities.

Experimental Section

Materials. Nucleosides and nucleotides were obtained either from Sigma Chemical Co. or from Boehringer und Soehne. Whale skeletal myoglobin was obtained from Seravac Laboratories. Pancreatic ribonuclease was obtained from Sigma Chemical Co. Adenosine deaminase from calf intestinal mucosa was obtained from Boehringer und Soehne. Tris (Sigma) was used as a buffer. Other chemicals were of reagent grade or of the highest quality available.

Assay Procedure. A spectrophotometric assay, based on the differential absorption of cytosine and uracil and their nucleosides, was used. The assay was conducted in microcuvets with 1-cm light path and was monitored at 286 mμ with a Zeiss PQM II absorbance recording spectrophotometer, at room temperature. The assay mixture contained, in a final volume of 1 ml, 0.45 M Tris-HCl buffer (pH 7.2), different amounts of enzyme preparation, and substrate solution; the reaction was started by addition of the enzyme preparation and the decrease in optical density at 286 mμ was recorded against a reference cuvet in which substrate was substituted by water. The molarities of all substrate and inhibitors solutions were measured spectrophotometrically from the extinction coefficients at 260 mμ at pH 7 (Cohn, 1955). Modifications of the standard conditions are described in the presentation of ex-

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[1] Abbreviation used is: *p*-MB, *p*-mercuribenzoate.