

Table III. Chemical Characterization of Batrachotoxin

Reaction	Product	R_f^a	Ehrlich reaction
1. Hydrogenation 5% Pd/C, HOAc, H ₂ , 1 hr.	Minor product C ₂₄ H ₃₃ NO ₄ (dihydrobatrachotoxin)	0.69 0.40	
2. Methyl iodide in acetonitrile, 2 days ^b	Methiodide	0.03	+ (blue)
3. NH ₂ OH, NaOH; semicarbazide, NaOH; 2,4-dinitrophenylhydrazine	No reaction		
4. 2,4-Dinitrophenylhydrazine, H ⁺	2,4-Dinitrophenyl- hydrazone	0.95	
5. LiAlH ₄ , THF, reflux, 15 min.	Reduction product	0.78	
6. NaBH ₄ , MeOH, 15 min. 60°	Reduction product	0.70	+ (blue)
7. Activated MnO ₂	Oxidation product	0.95 0.63	
8. Acetic anhydride, pyridine, 16 hr.	2 acetyl derivatives	0.70, 0.80	+ (blue);
9. Autoxidation	Several products	0.95	
10. MeOH, H ⁺	Several products	0.2; 0.4; 0.6; 0.7	
11. NH ₂ OCH ₃ , pyridine, 16 hr.	O-Methyloxime	0.85	+ (blue)

^a Thin-layer chromatography; silica gel, chloroform-methanol (6:1). ^b Excess methyl iodide (60° 2 hr.) yields an Ehrlich-negative compound (*cf.* the methylation of the carbinolamine cycloneosamandione, C. Schöpf and O. W. Müller, *Ann.*, **633**, 127 (1960)).

reduction with LiAlH₄, oxidation with activated manganese dioxide, acetylation, and treatment with acid (Table III) resulted in loss of the Ehrlich chromogen, while formation of the methiodide and mild sodium borohydride reduction preserved it.⁵ Autoxidation, a serious problem during isolation of batrachotoxin, led to products which were Ehrlich-negative.

(5) The quaternary methiodide on transformation to a pyrrolium salt would be expected to undergo facile demethylation (R. L. Hinman and J. Lang, *J. Org. Chem.*, **29**, 1449 (1964)) to an Ehrlich-positive N-methylpyrrole.

J. W. Daly, B. Witkop

National Institute of Arthritis and Metabolic Diseases
National Institutes of Health, Bethesda, Maryland 20014

P. Bommer, K. Biemann

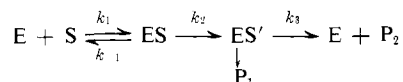
Department of Chemistry
Massachusetts Institute of Technology, Cambridge, Massachusetts

Received October 31, 1964

An Electrophilic Mechanism in the Chymotrypsin-Catalyzed Hydrolysis of Anilide Substrates¹

Sir:

Investigation of the electronic effects of substituents in substrate molecules on their reactivity is a valuable method of elucidating the mechanism of an enzyme catalysis. By using substituted monobenzoylchymotrypsins, Caplow and Jencks² were able to study the mechanism of the deacylation (k_3) of the acyl-enzyme,



ES', in the chymotrypsin catalysis. Bender and Nakamura³ investigated the electronic effects on the acylation step (k_2) using nonspecific ester substrates. Sager and Parks⁴ have studied such effects on the second-order rate constant, k_2/K_m , with substituted anilides of N-benzoyl-L-tyrosine.

(1) This research was supported by United States Public Health Service Grant GM-04725.

(2) M. Caplow and W. P. Jencks, *Biochemistry*, **1**, 883 (1962).

(3) M. L. Bender and K. Nakamura, *J. Am. Chem. Soc.*, **84**, 2557 (1962).

(4) W. F. Sager and P. C. Parks, *ibid.*, **85**, 2678 (1963).

In the chymotrypsin-catalyzed hydrolysis of an amide⁵ and a *p*-nitroanilide,⁶ the acylation step has been shown to be rate limiting. Therefore, a structural modification in the leaving group of the anilide may produce an observable change in the steady-state rate of the hydrolysis of the substrate. This is an advantageous situation for the study of the mechanism of the acylation step, which could not be obtained with a specific ester substrate, because with the latter rate limitation occurs at least in part at the deacylation step, and modification in the leaving group is expected to have less effect on the steady-state rate of the hydrolysis. However, this advantage can be exploited only if k_2 and K_m are determined separately, instead of k_2/K_m . In the present study reasonably good solubility in 5% (v/v.) dimethylformamide-water of the *meta*- and *para*-substituted anilides of N-acetyl-L-tyrosine enabled us to determine these constants (Table I).

Table I. α -Chymotrypsin-Catalyzed Hydrolyses of the Substituted Anilides of Acetyl-L-tyrosine^a

Substituent on aniline	$k_2 \times 10^2$, sec. ⁻¹	$K_m \times 10^4$, M
<i>m</i> -Cl	1.1 ± 0.01	8.2 ± 0.53
<i>p</i> -Cl	1.4 ± 0.05	6.7 ± 0.10
<i>m</i> -CH ₃ O	4.7 ± 0.03	60 ± 1.3
<i>p</i> -CH ₃	8.7 ± 0.1	130 ± 20
<i>p</i> -CH ₃ O	21 ± 0.5	120 ± 5.0

^a Determined by a pH-stat at pH 8.0, 25° in 5% (v/v.) dimethylformamide. The values are averages of duplicate to quadruplicate determinations.

The ρ - σ plot for the first-order rate constant of the acylation, k_2 , determined in 0.1 M KCl containing 5% (v/v.) dimethylformamide at pH 8.0, 25°, gave a ρ -value of -2.0 (Figure 1). K_m also shows a tendency to decrease as σ is increased, but the plots of $\log K_m$ vs. σ are more scattered. Consequently the ρ - σ plot for k_2/K_m cannot be represented by a straight line.

(5) B. Zerner and M. L. Bender, *ibid.*, **85**, 356 (1963).

(6) T. Inagami and J. M. Sturtevant, *Biochem. Biophys. Res. Commun.*, **14**, 69 (1964).

Table II. Substrates. Substituted Anilides of N-Acetyl-L-tyrosine

Substituent	Formula	Compn., % ($\frac{\text{calcd.}}{\text{found}}$)			M.p., °C.
		C	H	N	
<i>m</i> -Cl	C ₁₇ H ₁₇ N ₂ O ₃ Cl	61.34	5.15	8.42	192–193
		61.19	5.34	8.57	
<i>p</i> -Cl	C ₁₇ H ₁₇ N ₂ O ₃ Cl	61.34	5.15	8.42	224–225
		61.14	5.18	7.97	
<i>m</i> -CH ₃ O	C ₁₈ H ₂₀ N ₂ O ₄ · 0.5H ₂ O	64.10	6.24	8.31	161–162
		64.77	6.20	8.27	
<i>p</i> -CH ₃	C ₁₈ H ₂₀ N ₂ O ₃	69.21	6.45	8.97	204–205
		69.06	6.60	9.24	
<i>p</i> -CH ₃ O	C ₁₈ H ₂₀ N ₂ O ₃	65.84	6.14	8.56	220–221
		65.58	6.68	8.21	

The k_2 value for N-acetyl-L-tyrosine *m*-chloroanilide is constant within experimental error between pH 6.2 and 8.0. It decreases at more alkaline values of pH, its profile being represented by a pK' value of 9.7. On the acidic side, there is a suggestion of a decrease in k_2 at pH 6.0. However, the increase in K_m and the limited solubility of the substrate made it impossible to show unequivocally the decrease of k_2 at lower values of pH. On the other hand, the pH profile of the ratio k_2/K_m is a bell-shaped curve with $pK' = 6.8$ for the acidic side and 9.3 for the basic side. These values are in fair agreement with the values obtained by Sager and Parks⁷ for the second-order rate constant of the hydrolysis of the substituted anilides of N-benzoyl-L-tyrosine. The pH profile of k_2/K_m may be considered to represent the prototropic behavior of unbound enzyme and substrate, while the pH dependence of k_2 reflects the ionization of the initial enzyme-substrate complex.⁸ As the substrate has no ionizable group over the pH range studied, the binding of the substrate, N-acetyl-L-tyrosine *m*-chloroanilide, seems to have a remarkable effect on the ionization of the catalytically important group of the enzyme. This effect has not been observed in other specific substrates with a smaller ester or amide structure⁸ and seems to be due to a bulky *m*-chloroanilide structure.

In D₂O, the plateau value of k_2 for the *m*-chloroanilide, as determined at pD 8.2,⁹ is less than that in H₂O by a factor of 3.4. This kinetic isotopic effect and the large negative ρ -value for the rate constant of the acylation suggest that a proton transfer from an acidic group of the enzyme to the anilide substrate occurs in the transition state of the acylation step. Whether this electrophilic nature of the catalysis is a general characteristic of the acylation step specific to the anilide substrates is a problem yet to be elucidated. However, the present data clearly show that an acidic group in the active site of chymotrypsin plays a part in the enzymic catalysis. The fact that k_2 varies depending on the structure of the leaving group allows us to generalize the previous conclusion⁶ that the acylation step is rate limiting in the hydrolysis of N-acetyl-L-tyrosine anilides.

The substrates used and listed in Table II were synthesized by the azide method. N-Acetyl-L-tyrosine hydrazide was converted to the azide by reaction with

(7) W. F. Sager and P. C. Parks, *Proc. Natl. Acad. Sci. U. S.*, **52**, 408 (1964).

(8) M. L. Bender, G. E. Clement, F. J. Kezdy, and H. d'A. Heck, *J. Am. Chem. Soc.*, **86**, 1680 (1964).

(9) pD was defined by assuming that the relationship, pD = meter pH + 0.4 found by K. Glason and F. A. Long, *J. Phys. Chem.*, **64**, 188 (1960), holds for a 5% dimethylformamide-deuterium oxide mixture.

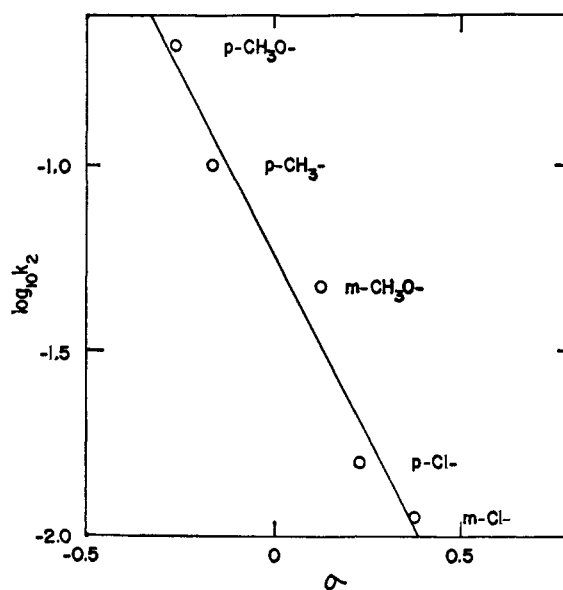


Figure 1. ρ - σ plot of the first-order rate constants of the chymotrypsin-catalyzed hydrolyses of *meta*- and *para*-substituted anilides of N-acetyl-L-tyrosine in 5% (v./v.) dimethylformamide-water at 25°, pH 8.0.

nitrous acid. The azide was then treated with an appropriately substituted aniline in ethyl acetate.

The k_2 and K_m values were read from Eadie plots of the initial rates (8% or less completion of the reaction) of hydrolysis determined by means of a pH-stat. Each reaction solution contained 0.1 M KCl and 5% (v./v.) dimethylformamide. Enzyme concentrations ranged between 3 and 4×10^{-5} M and the substrate concentration, well in excess of that of the enzyme, was varied approximately 10-fold.

Acknowledgment. The authors wish to express their gratitude to Professor J. M. Sturtevant for his valuable advice. We are also indebted to helpful discussions with Professors M. L. Bender and W. P. Jencks.

(10) Contribution Number 1763.

Tadashi Inagami, Sheldon S. York
Departments of Molecular Biology and Biophysics and Chemistry¹⁰
Yale University, New Haven, Connecticut

Abraham Patchornik
Department of Biophysics
Weizmann Institute of Science, Rehovoth, Israel
Received September 15, 1964

The Structure of a Pentaoxyphosphorane by X-Ray Analysis¹

Sir:

Triisopropyl phosphite and phenanthrenequinone form a crystalline 1:1 adduct (C₁₄H₈O₂)P(OC₃H₇)₃ which is representative of a new type of phosphorus compound.² This type of compound is characterized by a large positive chemical shift in the P³¹ n.m.r.

(1) This work was performed in part under the auspices of the U. S. Atomic Energy Commission, the National Cancer Institute of the Public Health Service (Grant No. CA-04769-05), and the National Science Foundation (GP-3341).

(2) F. Ramirez, S. B. Bhatia, R. B. Mitra, Z. Hamlet, and N. B. Desai, *J. Am. Chem. Soc.*, **86**, 4394 (1964), which contains complete documentation.