Table III. Chemical Characterization of Batrachotoxin

Reaction	Product	R_f[a]	Ehrlich reaction
1. Hydrogenation	Minor product	0.69	
5% Pd/C, HOAc, H_2, 1 hr.	$C_{24}H_{35}NO_4$ (dihydrobatrachotoxin)	0.40	
2. Methyl iodide in acetonitrile, 2 days[b]	Methiodide	0.03	+ (blue)
3. NH_2OH, NaOH; semicarbazide, NaOH; 2,4-dinitrophenylhydrazine	No reaction		
4. 2,4-Dinitrophenylhydrazine, H^+	2,4-Dinitrophenylhydrazone	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_2OCH_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 $LiAlH_4$, 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.

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An Electrophilic Mechanism in the Chymotrypsin-Catalyzed Hydrolysis of Anilide Substrates[1]

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 Jencks[2] were able to study the mechanism of the deacylation (k_3) of the acyl-enzyme,

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2$$
$$\downarrow$$
$$P_1$$

ES′, in the chymotrypsin catalysis. Bender and Nakamura[3] investigated the electronic effects on the acylation step (k_2) using nonspecific ester substrates. Sager and Parks[4] 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 amide[5] 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
m-Cl	1.1 ± 0.01	8.2 ± 0.53
p-Cl	1.4 ± 0.05	6.7 ± 0.10
m-CH_3O	4.7 ± 0.03	60 ± 1.3
p-CH_3	8.7 ± 0.1	130 ± 20
p-CH_3O	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., % (calcd. / found) C	H	N	M.p., °C.
m-Cl	$C_{17}H_{17}N_2O_3Cl$	61.34	5.15	8.42	192–193
		61.19	5.34	8.57	
p-Cl	$C_{17}H_{17}N_2O_3Cl$	61.34	5.15	8.42	224–225
		61.14	5.18	7.97	
m-CH_3O	$C_{18}H_{20}N_2O_4 \cdot 0.5H_2O$	64.10	6.24	8.31	161–162
		64.77	6.20	8.27	
p-CH_3	$C_{18}H_{20}N_2O_3$	69.21	6.45	8.97	204–205
		69.06	6.60	9.24	
p-CH_3O	$C_{18}H_{20}N_2O_3$	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 Parks[7] 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 structure[8] and seems to be due to a bulky *m*-chloroanilide structure.

In D_2O, the plateau value of k_2 for the *m*-chloroanilide, as determined at pD 8.2,[9] is less than that in H_2O 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 conclusion[6] 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 nitrous acid. The azide was then treated with an appropriately substituted aniline in ethyl acetate.

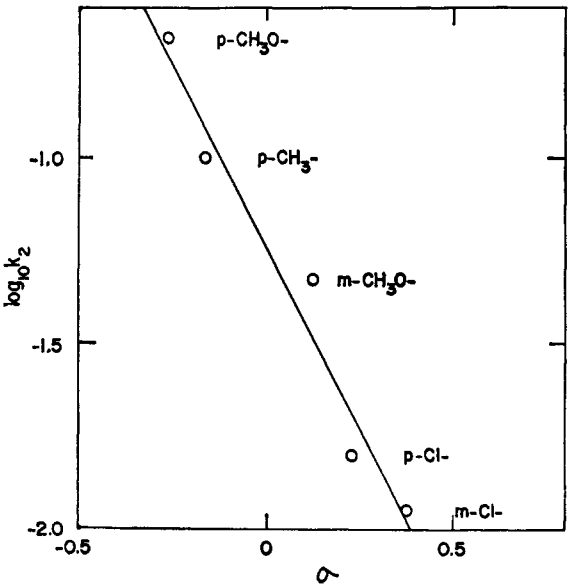

Figure 1. ρ–σ plot of the first-order rate constants of the chymotrypsin-catalyzed hydrolyses of *meta*- and *para*-substituted anilides of N-acetyl-L-tyrosine in 5% (v./v.) dimethylformamide–water at 25°, pH 8.0.

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.

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The Structure of a Pentaoxyphosphorane by X-Ray Analysis[1]

Sir:

Triisopropyl phosphite and phenanthrenequinone form a crystalline *1:1 adduct* $(C_{14}H_6O_2)P(OC_3H_7)_3$ 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 P^{31} n.m.r.

(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.

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(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.