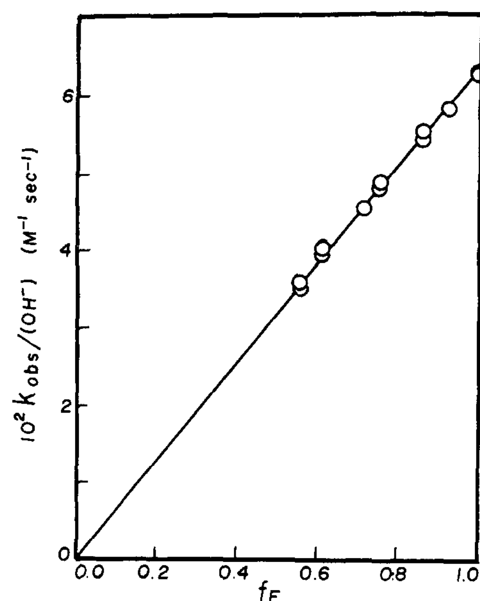
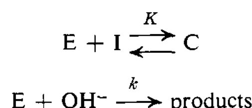


Figure 1. Plot of the equation $k_{obsd}/(OH^-) = kf_E$ for the imidazole-inhibited hydrolysis of methyl cinnamate.

were (in sec.$^{-1}$), respectively, 0.00112, 0.00105, 0.00098, 0.00086, and 0.00082.

The inhibitory effect of the imidazole, though small, is real. The decrease in rate of hydrolysis of methyl cinnamate caused by imidazole is more pronounced than would be expected from a nonspecific solvent effect. (Addition of 0.4 *M* acetonitrile changes the rate constant from 0.00112 to 0.00108 sec.$^{-1}$.) A very pertinent comparison to be made is between these data and similar studies carried out with ethyl acetate[3]; in the latter case a slight acceleration of the rate was observed. An interpretation of the results reported here, which is consistent with all available information, postulates an equilibrium interaction of the ester (E) and the imidazole (I) to form a 1:1 complex (C) that is relatively unreactive toward hydroxide ion.[4]



$$E + I \overset{K}{\rightleftharpoons} C$$

$$E + OH^- \xrightarrow{k} \text{products}$$

With this reaction scheme, the velocity may be written $v = k(OH^-)f_E(E_t)$, where (E_t) is the sum of concentrations of free and complexed ester and f_E is the fraction of ester in the free form. The experimental rate equation is $v = k_{obsd}(E_t)$. Combining these relations gives $k_{obsd}/(OH^-) = kf_E$. A plot of $k_{obsd}/(OH^-)$ *vs.* f_E should give a straight line passing through the origin; the slope should equal k, which of course can be independently determined.

The calculation of f_E requires knowledge of K, the complex formation constant. This was obtained at 25.0° and at several pH's using the solubility techniques.[5] As expected, evidence of interaction was obtained, and K was estimated to be 1.0 ± 0.1. K was independent of pH in the range 7.2–8.3. The plot of $k_{obsd}/(OH^-)$ against f_E is shown in Figure 1 (which includes more data than are reported above). Evidently the postulated scheme is consistent with the data. This interpretation of the results follows closely the analysis of Higuchi and his co-workers in their studies of the inhibition of hydrolysis of local anesthetic esters by xanthine derivatives.[6,7]

This communication is, we believe, the first report of an inhibitory effect of imidazole in a nonenzymatic homogeneous system. The results may be of significant aid in suggesting mechanisms for the specificity behavior of enzymes and of drug–receptor interactions. It is particularly interesting that a functional entity (*i.e.*, imidazole) that has been widely implicated as a catalytic function in proteolytic enzymes may also act to prevent attack on a substrate by a nucleophilic reagent (hydroxide ion in the present case). This observation leads to the idea that enzyme specificity could, in part, be a result of a functional unit in the active site interacting to a greater or lesser extent with the substrate, thus (in one conceivable mode) preventing attack by a catalytic function in the active site; a range of reactivities would be possible, with the rate of reaction dependent upon the extent of complexation between the substrate and the interacting function of the active site. Other mechanisms could be postulated. We are presently extending this study to consider the effects that the structures of the substrate, inhibitor, and attacking agent have on the inhibition phenomenon.

Acknowledgments. This research was supported by PHS Research Grant GM 11136 from the National Institutes of Health, Public Health Service. We wish to thank Dr. M. L. Bender, in whose laboratory K. A. C. carried out some preliminary experiments on this problem.

(4) This mechanism ignores some possible reactions, such as imidazole-catalyzed hydroxide ion attack of the ester, but the data may be accounted for with the postulated scheme.

(5) T. Higuchi and K. A. Connors, *Advan. Anal. Chem. Instrumentation*, in press.

(6) T. Higuchi and L. Lachman, *J. Am. Pharm. Assoc.*, **44**, 521 (1955); L. Lachman, L. J. Ravin, and T. Higuchi, *ibid.*, **45**, 290 (1956).

(7) An alternate interpretation may be given in terms of the effect of imidazole on the activity coefficients of the reactants. In this interpretation f_E becomes an activity coefficient, and the solubility study provides an estimate of this quantity. In a sense this hypothesis is equivalent to the complex formation hypothesis, with the latter interpretation furnishing a mechanism for the activity coefficient effect. It is interesting that T. C. Bruice and R. N. Topping, *J. Am. Chem. Soc.*, **85**, 1488 (1963), have demonstrated complex formation between imidazole species and α-aminophenylacetic acid.

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Batrachotoxin. The Active Principle of the Colombian Arrow Poison Frog, *Phyllobates bicolor*

Sir:

The venom obtained by Märki and Witkop[1] from the skin of the Colombian arrow poison frog, *Phyllobates bicolor*, is *the most active venom so far known* (Table I). Our recent expedition (December 1963–January 1964) to the Choco rain forest of Western Colombia[2] netted 2400 frogs whose skin extracts yielded a total of 30 mg. of the crystalline major active principle which we name *batrachotoxin*.

(1) F. Märki and B. Witkop, *Experientia*, **19**, 239 (1963).

(2) We are greatly indebted to Mrs. Martè Latham, who organized and guided this difficult and dangerous venture. A colorful report on this expedition is scheduled to appear in the National Geographic Magazine.

Table I

	Batrachotoxin, $C_{24}H_{33}NO_4$	Batrachotoxinin A, $C_{24}H_{35}NO_5$	Batrachotoxinin B, $C_{24}H_{35}NO_5$	Batrachotoxinin C, $C_{24}H_{35}NO_5$	Conclusions
Ultraviolet spectrum	End absorption	End absorption	End absorption	End absorption	No conjugated system
O.R.D (CH_3OH, *c* 0.23, 24°)	$[\alpha]_{589}$ +5–10°; $[\alpha]_{300}$ −260°				
Infrared spectrum, cm.$^{-1}$	1695, 1250	1645	1610 (broad)	Not tested	Carbonyl, vinyl ether
N.m.r. spectrum (reference, TMS δ = 0.0, solvent $CDCl_3$)	0.85	0.85	0.85	0.9	$\geqslant$C–CH_3
	1.48 (doublet)	1.42	1.38		>CH–CH_3
	2.25, 2.2; 2.25; 2.45				Allylic methyl or methylene
	2.35	2.30 2.66	2.34 2.70	2.30	>N—CH_3[a]
	3.40 (2H)	3.42 (~2H)	3.45 (~4H)	3.48	Exchangeable H's (D_2O)
	5.6–6.3 (~3H)	5.5–6.3 (~4H)	5.4–6.3 (~3H)	5.4–6	Olefinic and –CH<(O–)(N–)
	8.74 broad (<0.4H)				Aldehyde ?
R_f values[b]	0.60	0.54	0.19	0.08	
Color reactions					
(1) iodoplatinate	+	+	+	+	
(2) Ehrlich reaction	Red	None	None	None	Batrachotoxin has potential pyrrole system
(3) dimethylamino-cinnamaldehyde	Blue	None	None	None	
LD_{50}[c]	1.15–2.7 µg./kg. (mice)	1/10 of batrachotoxin	Very low	Not tested	LD_{50} varies with history and batch of batrachotoxin sample
ED_{50}[c,d]	0.37 µg./kg. (mice)	Not tested	Not tested	Not tested	

[a] Peak shifted downfield in batrachotoxinin A on acidification and in batrachotoxin methiodide. [b] Thin-layer chromatography; silica gel, chloroform–methanol (6:1). [c] We are indebted to Dr. Louis S. Harris, Sterling-Winthrop Research Institute, for these determinations. [d] Minimum effective dose causing mice to fall off inclined screen.

The exact molecular weight of batrachotoxin was determined by high-resolution mass spectrometry[3] to be 399.2413, which corresponds to the empirical formula $C_{24}H_{33}NO_4$ (mol. wt. calcd. 399.2409).

In addition *three related basic congeners* were isolated by preparative thin-layer chromatography in quantities of 15, 6, and 1 mg., respectively. Analyzed by mass spectrometry all three congeners gave the same empirical formula, $C_{24}H_{35}NO_5$. The physical and biological properties of these venoms are summarized in Table I.

The neutral lipophilic fraction did not contain compounds related to batrachotoxin, but, according to the n.m.r. profiles, long straight-chain aliphatic compounds.

The high resolution mass spectrum of batrachotoxin, in the form of an element map,[4] indicates a contiguous skeleton of at least 17 carbon atoms (*e.g.*, $C_{17}H_{15}^+$ and $C_{17}H_{17}^+$ ions), suggestive of a steroid-type ring system. Other significant features are fragments due to loss of CH_3, CHO, C_4H_9NO, and $C_6H_{11}NO$ from the molecular ion ($C_{24}H_{33}NO_4$). This would indicate the presence of one (or more) methyl groups, a (potential) aldehyde group, and the fact that the nitrogen atom is easily lost together with one oxygen and four to six carbon atoms. These conclusions are corroborated by the presence of intense peaks due to the ions C_4H_8NO, $C_4H_{10}NO$, and $C_6H_{10}NO$. The appearance of an abundant ion $C_7H_8NO_2$ requires that a second oxygen atom is nearby, within a total of seven carbon atoms of the nitrogen atom. Based on these findings, and conclusions (Table II), a steroidal structure with one nitrogen and two oxygen atoms attached to ring D (or A) is our present working hypothesis.

(3) All mass spectrometric data were obtained with a double focusing mass spectrometer (CEC 21-110) with a photoplate for ion detection.

(4) K. Biemann, P. Bommer, and D. Desiderio, *Tetrahedron Letters*, **26**, 1725 (1964).

Table II. Evaluation of Mass Spectrogram Obtained from a Sample of 50 µg. of Batrachotoxin

Results	Conclusions
Mol. wt. 399.2413	$C_{24}H_{33}NO_4$ 9 rings or double bonds
CH 17/17	Carbon skeleton C_{17} (potentially) highly unsaturated OH groups in ring system
No intense C_4H_7NO	Not ring A of Samandarine
No intense $C_6H_{12}NO$	Not ring F of Tomatidine
$C_4H_{10}NO$ $C_7H_{6-8}NO$ $C_7H_8NO_2$	NO within 4 C NO_2 within 7 C

Chemical Properties of Batrachotoxin. As previously described,[1] batrachotoxin contains no acidic group, but is a weakly basic amine (p*K* = 7–8), presumably a carbinolamine ether.[1] That the nitrogen is involved in a rather unique chemical environment is borne out by color reactions, such as the immediate strong red color with Ehrlich reagent and a strong blue color with *p*-dimethylaminocinnamaldehyde, suggestive of a potential pyrrole grouping. The ultraviolet spectrum, the ease of catalytic reduction of the chromogenic group, and the methylation to an Ehrlich-positive quaternary salt militate against the presence of an actual pyrrole. Most reactions, such as hydrogenation to a dihydro derivative, formation of a 2,4-dinitrophenylhydrazone,

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 $C_{24}H_{35}NO_4$ (dihydrobatrachotoxin)	0.69 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-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_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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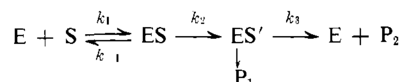
An Electrophilic Mechanism in the Chymotrypsin-Catalyzed Hydrolysis of Anilide Substrates[1]

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,



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

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).