[CONTRIBUTION FROM THE DEPARTMENTS OF CHEMISTRY, ILLINOIS INSTITUTE OF TECHNOLOGY, CHICAGO 16, ILL., AND NORTHWESTERN UNIVERSITY, EVANSTON, ILL.]

# The Effect of Structure on the Rates of Some α-Chymotrypsin-catalyzed Reactions[1,2]

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The relative rates of acylation of α-chymotrypsin by series of substituted phenyl acetate, phenyl cinnamates and phenyl trimethylacetates have been determined. In all cases electron-withdrawing substituents facilitate the acylation reaction. The Hammett ρ-constant of the second-order acylation constants of a series of phenyl acetates ($k_{obs} = k'_{acylation}/K_m$) is 1.8, while the ρ-constant of the first-order acylation for a series of phenyl trimethylacetates ($k'_{acylation}$) is 1.4. These ρ-constants are similar to those found in (non-enzymatic) nucleophilic reactions of phenyl acetates and quite different from those found in electrophilic reactions. The ρ-constants show greater similarity to those involving nucleophilic attack than the one known reaction involving general basic catalysis.

## Introduction

The correlation of structure with reactivity has been a powerful tool in the elucidation of the mechanism of organic reactions. The success of this approach has been due to the separation of electronic and steric effects upon reaction rate and their separate correlation with particular reaction mechanisms. Attempts to apply structure–reactivity correlations to enzymatic systems have been plagued by the difficulties inherent in systems in which steric effects, defined as specificity behavior, and electronic effects cannot be separated in any straightforward fashion. However, a number of limited correlations have been found between the structure of a substrate and its relative reactivity in a given enzymatic system and it is the purpose of the present paper to explore such correlations with the enzyme α-chymotrypsin.

The most striking effects of structure on reactivity are those effects normally called specificity. The many studies of the specificity of substrates toward chymotrypsin have been elegantly summarized.[4,5] However, these studies do not concern us now for they have been oriented to discover what non-electronic structural features will lead to a facile reaction with the enzyme. Enormous differences in reactivity have been found and many features of topographical interest[6] have been found. It is presumed, although not proved, that most of these effects of structure on reactivity must be explained in entropic terms. The approach of the present research is to *minimize* differences in classical specificity and to introduce structural changes into the substrate that will result only in electronic effects on reactivity. Hopefully, then mechanistic conclusions can be drawn from these electronic effects on reactivity.

The point of greatest specificity of chymotrypsin substrates is in the carboxylic acid portion of the molecule and the point of least specificity is in the leaving group portion of the molecule. This has been shown by an analysis of a large number of reactions of proteins and synthetic substrates.[4] Therefore the approach taken here is to investigate variations in the leaving group, keeping the carboxylic acid moiety fixed. For this reason the rates of hydrolysis of several series of substituted phenyl esters by chymotrypsin have been determined.

The hydrolysis of phenyl esters by other hydrolytic enzymes has been studied.[7,8] In hydrolysis reactions catalyzed by hydroxide ion, the relative rates can of course be correlated by means of a Hammett ρ–σ relationship, explained on the basis that electron withdrawal from the site of reaction increases the ease of nucleophilic attack. In studies with eel esterase, cobra venom cholinesterase and serum cholinesterase, a linear Hammett relationship is not observed.[8] Both electron-withdrawing and electron-donating substituents in the *para* position of the phenyl ester decrease the rate of reaction. These results have been interpreted to indicate that the relative rates of reaction are not determined solely by nucleophilic attack but by some combination of nucleophilic and electrophilic catalysis which leads to the concave Hammett relationship observed in these cases. This analysis is clouded by the fact that it is not clear what steps the rate constants signify, for the stepwise kinetic analysis, worked out in detail for chymotrypsin, has not been applied to these systems.

The hydrolysis of substituted phenyl acetates by wheat germ lipase has also been investigated.[9] *p*-Substituents caused only small effects on the rate constants of these enzymatic reactions, the *p*-nitro substituent producing less than a doubling of the rate constant (compared to the unsubstituted compound). The effect of substituents on the hydrolysis of phenyl sulfates[10] by an arylsulfatase and on the hydrolysis of phenyl β-D-glucosides by emulsin[11] have also been studied. In both these instances the rate constant of the catalytic step was significantly faster for the *p*-nitro compound than for the unsubstituted compound (by a factor of 13 to 17). These results were interpreted in terms of domination of the catalytic step by nucleophilic attack. The rate of

(1) This research was supported by grants from the National Institutes of Health.

(2) Paper XV in the series, The Mechanism of Action of Proteolytic Enzymes; previous paper, M. L. Bender and G. A. Hamilton, *J. Am. Chem. Soc.*, **84**, 2570 (1962).

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(4) H. Neurath and G. W. Schwert, *Chem. Revs.*, **46**, 69 (1950).

(5) H. Neurath and B. S. Hartley, *J. Cell. Comp. Physiol.*, **54**, Supp. 1, 179 (1959).

(6) M. L. Bender, G. R. Schonbaum and B. Zerner, *J. Am. Chem. Soc.*, **84**, 2540 (1962).

(7) F. Bergmann, S. Rimon and R. Segal, *Biochem. J.*, **68**, 493 (1958).

(8) L. A. Mounter, *Biochim. et Biophys. Acta*, **27**, 219 (1958); L. A. Mounter and V. P. Whittaker, *Biochem. J.*, **54**, 551 (1953).

(9) O. Gawron, C. J. Grelecki and M. Duggan, *Arch. Biochim. Biophys.*, **44**, 455 (1953).

(10) K. S. Dodgson, B. Spencer and K. Williams, *Biochem. J.*, **64**, 216 (1956).

(11) R. L. Nath and H. N. Rydon, *ibid.*, **57**, 1 (1954).

hydrolysis of diethyl phenyl phosphates catalyzed by erythrocyte cholinesterase is extremely sensitive to *p*-substituents, the *p*-nitro compound reacting 180,000 times as fast as the unsubstituted ester.[12]

Substituent effects involving enzymatic reactions of phenyl esters may be compared with substituent effects in non-enzymatic reactions of phenyl esters. These effects have been summarized by Gaetjens and Morawetz.[13] In all nucleophilic reactions of phenyl esters, electron-withdrawing substituents facilitate and electron-donating substituents decelerate the reaction as predicted by simple theory. Intermolecular reactions of phenyl esters and nucleophiles in general are less sensitive to substituent effects than intramolecular reactions. But in all instances the qualitative effect of substituents in non-enzymatic nucleophilic reactions and in the enzymatic reactions enumerated above parallel one another, implying that the latter reactions are nucleophilic in character.

The above investigations have involved only over-all enzymatic processes. It has been shown that chymotrypsin involves a two-step catalytic reaction, an acylation of the enzyme followed by a deacylation of the acyl-enzyme.[6] Therefore in order to determine rigorously the effect of structure on reactivity with chymotrypsin it is necessary to look at each step individually. In this paper, the effect of structure on the acylation of chymotrypsin is presented.

## Experimental

**Materials. Phenyl Trimethylacetates.**—*p*-Nitrophenyl trimethylacetate was synthesized by adding *p*-nitrophenol (12 g.) to 30 g. of anhydrous pyridine; then trimethylacetyl chloride (10 g.), prepared from trimethylacetic acid (Eastman Kodak. Company) and freshly-distilled thionyl chloride, was added. After the initial reaction had subsided, the mixture was warmed for 10 minutes, and then poured with vigorous stirring into ice-water. The resulting precipitate was washed with 5% sodium carbonate solution and recrystallized from 95% alcohol.

*p*-Acetylphenyl trimethylacetate was synthesized: *p*-hydroxyacetophenone (0.1 mole) was dissolved in 100 ml. of dry pyridine; trimethylacetyl chloride (0.1 mole) was added. The reaction mixture was warmed for 15 minutes at 50°, and then poured into ice-water. The solid residue was extracted with ether; the ether solution was washed with water, 5% sodium hydroxide, 0.1 *N* hydrochloric acid and water, and dried over sodium sulfate. The product was distilled *in vacuo*, b.p. 123–124° (4 mm.), and recrystallized from 40% methanol–water.

The synthesis of *p*-aldehydophenyl trimethyl acetate was carried out as above except that the reaction mixture was flushed with nitrogen to avoid oxidation of the aldehyde group.

**Phenyl Acetates.**—These esters were prepared either from acetic anhydride or from acetyl chloride and the appropriate phenol, using pyridine as the solvent. A typical synthesis follows, that of *o*-nitrophenyl acetate. *o*-Nitrophenol (14 g., 0.1 mole), acetic anhydride (15 g., 0.125 mole) and a trace of pyridine were dissolved in 25 ml. of dry benzene. The reaction mixture was refluxed for 1 hour. After neutralization with saturated sodium bicarbonate solution, the benzene solution was washed with water, 5% sodium hydroxide solution, 0.1 *N* hydrochloric acid and water. The benzene solution was then dried and evaporated to dryness. The residue was recrystallized from ligroin.

The syntheses of *m*-aldehydophenyl acetate and *p*-aldehydophenyl acetate were carried out under a stream of nitrogen gas and the products after fractional distillation were stored under nitrogen.

**Phenyl Cinnamates.**—The preparations of *o*-nitro-, *m*-nitro- and *p*-nitrophenyl cinnamates have been described previously.[6]

The physical constants of all ester substrates are listed in Table I.

TABLE I

PHYSICAL CONSTANTS OF ESTER SUBSTRATES

| Ester | M.p., °C. | M.p. °C. | (lit.), Ref. |
|---|---|---|---|
| Trimethylacetate | | | |
| *p*-Nitrophenyl | 94–95 | 94–95 | *c* |
| *p*-Acetylphenyl | 54 | *a* | |
| *p*-Aldehydophenyl | 120 (4 mm.)[d,e] | *b* | |
| Acetate | | | |
| *o*-Nitrophenyl | 38 | 40–41 | *f* |
| *m*-Nitrophenyl | 54 | 55–56 | *h* |
| *p*-Nitrophenyl | 77.5–78 | 79–80 | *h* |
| *m*-Acetylphenyl | 42–43 | 44–44.5 | *i* |
| *m*-Aldehydophenyl | 110 (5 mm.)[d,g] | 264–265 (760)[d] | *j* |
| *p*-Acetylphenyl | 50–52 | 54 | *k* |
| *p*-Aldehydophenyl | 100 (5 mm.)[d,e] | 264–265 (760)[d] | *l* |
| Cinnamate | | | |
| *o*-Nitrophenyl | 81–82 | 84.5 | *m* |
| *m*-Nitrophenyl | 112–113 | *n* | |
| *p*-Nitrophenyl | 143 | 146 | *m* |

[a] Calcd. for $C_{13}H_{16}O_3$: C, 70.88; H, 7.32. Found: C, 71.02; H, 7.34. [b] Calcd. for $C_{12}H_{14}O_3$: C, 69.88; H, 6.84. Found: C, 69.79; H, 7.11. [c] C. E. McDonald and A. K. Balls, *J. Biol. Chem.*, **227**, 727 (1957). [d] Boiling point. [e] $n^{20}D$ 1.5098. [f] F. D. Chattaway, *J. Chem. Soc.*, 2495 (1931). [g] $n^{25}D$ 1.5337. [h] F. Arnall, *J. Chem. Soc.*, **125**, 814 (1924). [i] E. Bamberger, *Ber.*, **48**, 1355 (1915). [j] F. Tiemann and R. Ludwig, *Ber.*, **15**, 2047 (1882). [k] F. M. Irvine and R. Robinson, *J. Chem. Soc.*, 2091 (1927). [l] W. Richter, *Ber.*, **34**, 4293 (1901); P. E. Papadakis, *J. Am. Chem. Soc.*, **67**, 1799 (1945). [m] R. Anschütz, *Ber.*, 60, 1322 (1927). [n] Calcd. for $C_{15}H_{11}O_4N$: C, 66.91; H, 4.12; N, 5.20. Found: C, 67.04; H, 4.22; N, 5.24. [o] $n^{25}D$ 1.5402.

**Kinetics of Enzymatic reactions.**—The solvent, buffers, enzyme and added salts have been described previously.[6]

Substrate stock solutions were prepared with acetonitrile as solvent. The concentrations of these solutions were based on the weight of the pure substrate. Enzyme stock solutions were prepared by dissolving three-times recrystallized α-chymotrypsin (Worthington Biochemical Corp.) in water. The concentrations of the enzyme solutions were determined by titration with *N-trans*-cinnamoylimidazole as described previously.[14] Appropriate microliter quantities of the enzyme and substrate stock solutions were added to 3 ml. of the appropriate buffer solution in the quartz cuvette. The transmittance or absorbance of the solution was recorded as a function of time with a Beckman DK-2 or Cary model 14 recording spectrophotometer at a wave length suitable for the particular phenol that was formed in the reaction. The cell compartment of the spectrophotometer was thermostated. In those reactions involving high [substrate]/[enzyme] ratios, the concentration of enzyme was limited to the low level of $\sim 0.5 \times 10^{-5} M$ so that the necessary excess substrate would be soluble in 1.6% acetonitrile–water. This situation necessitated the use of the transmission scale of the Beckman DK-2 in order to observe the total absorbance change of 0.09–0.10 absorbance unit.

The rate constants for the acylation reactions with trimethylacetate esters in which substrate concentration was much greater than enzyme concentration were calculated using first-order kinetic plots. The deacylation of trimethylacetyl-α-chymotrypsin is known to be exceedingly slow ($1.3 \times 10^{-4}$ sec.$^{-1}$ at pH 8.5).[15]. However, corrections were made for both the turnover of the acyl-enzyme and the spontaneous hydrolysis of the substrate following the procedure of Gutfreund and Sturtevant.[15,16].

(12) W. N. Aldridge and A. N. Davidson, *Biochem. J.*, **51**, 62 (1952).

(13) E. Gaetjens and H. Morawetz, *J. Am. Chem. Soc.*, **82**, 5328 (1960).

(14) G. R. Schonbaum, B. Zerner and M. L. Bender, *J. Biol. Chem.*, **236**, 2930 (1961).

(15) M. L. Bender and G. A. Hamilton, *J. Am. Chem. Soc.*, **84**, 2570 (1962).

(16) H. Gutfreund and J. M. Sturtevant, *Biochem. J.*, **63**, 656 (1956).

The rate constants for those reactions in which the enzyme concentration was equal to or greater than the substrate concentration were calculated using a usual form of the second-order rate equation.[17] In this instance the deacylation of the acyl-enzyme and the spontaneous hydrolysis of the substrate can reasonably be assumed to be negligibly slow with respect to the acylation reaction since there is no excess substrate; this assumption is borne out by the fact that the second-order plots were invariably linear to greater than two half-lives.

Figure 1 of ref. 15 and Fig. 1 of ref. 17 are similar to the two kinds of kinetics that were observed here, a first-order acylation of α-chymotrypsin by a substrate where the substrate to enzyme ratio is large and the second-order acylation of α-chymotrypsin by a substrate where the substrate to enzyme ratio is 1:1.

## Results

**Phenyl Acetates.**—The second-order rate constants for the reactions of seven substituted phenyl acetates with α-chymotrypsin have been determined. The reaction experimentally observed is the acylation of chymotrypsin by these esters, denoted by

$$\begin{array}{ccccc} E + S & \overset{K_m}{\rightleftharpoons} & ES & \xrightarrow{k_2} & ES' + P_1 \\ \downarrow\uparrow K_1 & & & & \\ EH^+ & & & & \end{array} \qquad (1)$$

where ES is the enzyme-substrate complex, ES′ is the acyl-enzyme and $P_1$ is the phenol. The assumption that the observed kinetics are concerned only with the acylation reaction is based on the following considerations: (1) the rate constant of the acylation of chymotrypsin by a typical member of this series, *p*-nitrophenyl acetate, ($5.6 \times 10^2$ $M^{-1}$ sec.$^{-1}$; see Table II) is much larger than the rate constant for deacylation of acetyl-α-chymotrypsin ($12 \times 10^{-3}$ sec.$^{-1}$)[16,18]; (2) the reactions were carried out at equal substrate and enzyme concentrations so that no turnover of the enzyme is anticipated; and (3) strict second-order kinetics were observed to over 70% of each reaction. Under these conditions one can reasonably interpret the observed second-order rate constant as a function of the acylation rate constant ($k_2$) and the assumed pre-equilibrium constant between enzyme and substrate ($K_m$), as well as the prototropic equilibrium of the enzyme ($K_1$) of eq. 1.

$$k_{obs} = k_1/K_m(1 + [H^+]/K_1) \qquad (2)$$

Since these reactions were carried out at constant *p*H, the *relative* values of the second-order rate constant are a function of $k_2$ and $K_m$. The results of these kinetic investigations are presented in Table II.

The results of the acylation of α-chymotrypsin may be analyzed in terms of a Hammett $\rho$–$\sigma$ plot. Such an analysis results in Fig. 1 in which log $k_{obs}$ is plotted *versus* $\sigma^-$ (the special $\sigma$ defined originally by Hammett as pertaining to reactions of phenols and anilines). The relative rate constants of *m*-nitrophenyl acetate and *p*-nitrophenyl acetate (the latter rate constant is about 25 times the former) indicate immediately that a Hammett plot utilizing the normal $\sigma$-constants (defined in terms of the $pK_a$'s of substituted benzoic acids) will not suffice for this analysis. This conclusion is further indicated by the exceptionally high reactivity of the *p*-aldehydo- and *p*-acetylphenyl acetates with respect to the corresponding *meta* compounds.

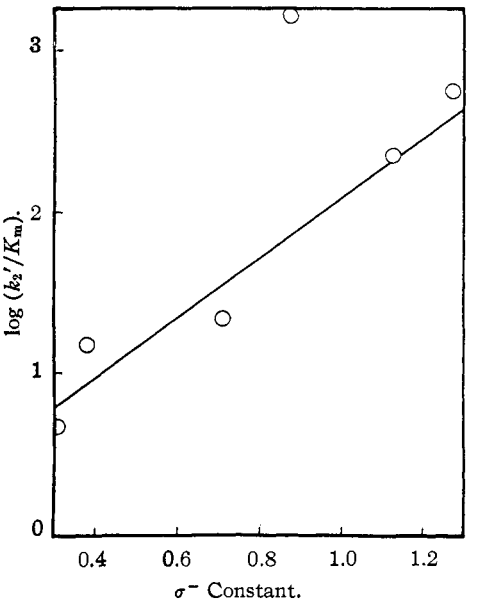


Fig. 1.—Hammett plot of the acylation of α-chymotrypsin by a series of substituted phenyl acetates (rate constant $k_2'/K_m$ has units of $M^{-1}$ sec.$^{-1}$).

TABLE II

THE ACYLATION OF α-CHYMOTRYPSIN BY A SERIES OF SUBSTITUTED PHENYL ACETATES[a]

| Phenyl acetate | $[S]_0 = [E]_0 \times 10^5$, $M$ | $k_2'/K_m$, $M^{-1}$ sec.$^{-1}$[d] | $pK_a$ of phenol[b] | Hammett $\sigma^-$[f] |
|---|---|---|---|---|
| *p*-Nitro | 4.13 | $5.63 \times 10^2$ | 7.14 | 1.27 |
| *p*-Aldehydo | 3.83 | $2.27 \times 10^2$ | 7.66[e] | 1.126 |
| *m*-Nitro | 4.13 | 21.3 | 8.35 | 0.71 |
| *m*-Aldehydo | 3.83 | 15.8 | 9.02[c] | .381 |
| *m*-Acetyl | 3.83 | 4.6 | 9.19 | .306 |
| *p*-Acetyl | 3.83 | $1.63 \times 10^2$ | 8.05 | .874 |
| *o*-Nitro | 4.13 | $1.54 \times 10^2$ | 7.21 | |

[a] In 10% acetonitrile–water, 0.1 $M$ (total) phosphate buffer, *p*H 7.94, 25.1 ± 0.1°. [b] In water; H. C. Brown, D. H. McDaniel and O. Häfliger in "Determination of Organic Structures by Physical Methods," E. A. Braude and F. C. Nachod, eds., Academic Press, Inc., New York, N. Y., 1955, p. 589. [c] R. A. Robinson and A. K. Kiang, *Trans. Faraday Soc.*, 52, 327 (1956). [d] A second-order rate constant may be obtained from the data in ref. 18 by using the equation $k_{obs} = k_2'/K_m$. This leads to a value of $8 \times 10^2$ $M^{-1}$ sec.$^{-1}$ which is in good agreement with the value in the table above considering that the two determinations were carried out under somewhat different solvent and buffer conditions. [e] 7.52 in 8% dioxane–water at 38°; O. Gawron, M. Duggan and C. J. Grelecki *Anal. Chem.*, 24, 969 (1952). [f] There is an excellent fit between $\sigma^-$ (determined from reaction rates: L. P. Hammett, "Physical Organic Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1940, p. 184) and the $pK_a$'s of the phenols (equilibrium constants: F. G. Bordwell and G. D. Cooper, *J. Am. Chem. Soc.*, 74, 1059 (1952)).

It is seen in Fig. 1 that of the seven rate constants determined, five conform to a reasonably good linear relationship. The sixth, that of *o*-nitrophenyl acetate, is not expected to fit such a linear

(17) M. L. Bender, G. R. Schonbaum and B. Zerner, *J. Am. Chem. Soc.*, **84**, 2562 (1962).

(18) H. Neurath and B. S. Hartley, *J. Cell. Comp. Physiol.*, **54**, Supp. 1, 184 (1959).

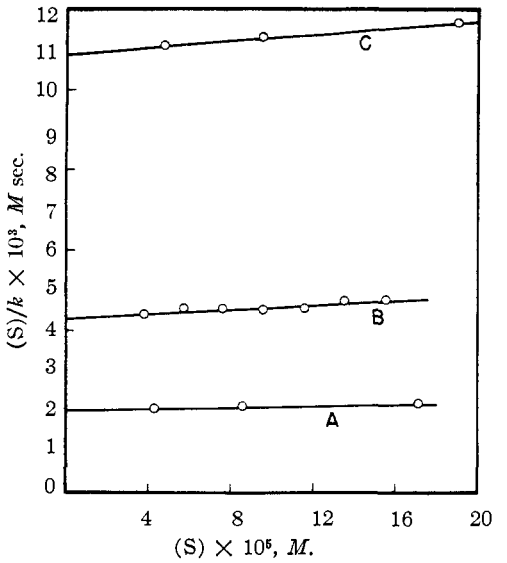


Fig. 2.—Dixon-Webb plots of the α-chymotrypsin-catalyzed hydrolysis, *p*H 8.2, 0.01 *M* Tris buffer, ionic strength 0.06, temperature 25.6 ± 0.1°, of: A, *p*-acetylphenyl trimethylacetate; B, *p*-nitrophenyl trimethylacetate; C, *p*-aldehydophenyl trimethylacetate.

relationship because of steric effects usually observed with *o*-substituents, and is not included in the plot. The seventh compound, *p*-acetylphenyl acetate, exhibits a rate constant which is by far the fastest of the group and is the only member of the group which inexplicably fails to obey a linear relationship between log $k_{obs}$ and $pK_a$. One might postulate that a specific interaction of the carbonyl group with the enzyme accounts for its exceptional reactivity. But such a premise predicts that the *p*-aldehydo compound, which contains an even more reactive carbonyl group in essentially the same position as the *p*-acetyl compound, would also behave abnormally and it does not. Therefore one is left with no ready explanation of the behavior of *p*-acetylphenyl acetate.

It should be pointed out that the correlation being discussed is one which involves a complex constant $k_{obs} = k_2'/K_m$. Therefore the variations observed with structural changes could conceivably arise from either $k_2'$ or $K_m$, or from a combination of both constants. It will first be assumed, however, that $K_m$ is independent of structural changes in the *p*-position of the leaving group and that the variations in $k_{obs}$ reflect variations in $k_2'$. If this is done, then one can say that the variation in rate constants for the acylation of α-chymotrypsin with substituted phenyl acetates parallels the effect of structure on reactivity in a number of nucleophilic reactions of phenyl acetates.

**Phenyl Cinnamates.**—If indeed $K_m$ is independent of structural change in the leaving group, the effect of structure on reactivity noted above in the acetate series should also be observable with another group of phenyl esters. For this purpose the rate constants of the acylation of chymotrypsin with two *p*-substituted phenyl cinnamates were determined. The results of these studies[6] are shown in Table III. It is seen that the *p*-nitro/*m*-nitro ratio is qualitatively the same in both families of reactions (the ratio is 10.5 in the cinnamate acylations and 26 in the acetate acylations). The independence of the substituent effect of the acyl group of the esters indicates that the substituent effect is a real one presumably associated with some electronic effects on the reaction.

TABLE III

THE ACYLATION OF CHYMOTRYPSIN BY SOME SUBSTITUTED PHENYL CINNAMATES[a]

| Phenyl cinnamate | $[S]_0 \times 10^6$, *M* | $[E]_0/[S]_0$ | $k_2'/K_m \times 10^{-4}$, $M^{-1}$ sec.$^{-1}$ | $pK_a$ of phenol[b] |
|---|---|---|---|---|
| *p*-Nitro | $4.8 \times 10^{-6}$ | 1.7 –4.25 | 11.3 ± 0.4 | 7.14 |
| *m*-Nitro | $4.7 \times 10^{-5}$ | 1.02–1.69 | 1.08 ± 0.08 | 8.35 |

[a] In 10% acetonitrile-water, 0.05 *M* (total) Tris buffer, *p*H 8.35, 25.0 ± 0.1°. [b] See footnote *b*, Table II.

**Phenyl Trimethylacetates.**—The second-order acylation constants for the phenyl acetates and phenyl cinnamates give a consistent picture of the effect of substituents on the acylation of α-chymotrypsin. However, these data are equivocal since the second-order constant is a complex constant. What is really desired is the effect of substituents on the acylation rate constant, $k_2'$. This result can only be accomplished by applying a Michaelis–Menten treatment to the *acylation* reaction so that the $K_m$ and $k_2'$ can be separated from one another. Such an approach has been successfully carried out by Gutfreund and Sturtevant for *p*-nitrophenyl acetate[17] and 2,4-dinitrophenyl acetate[19] using a stopped-flow device in order to follow these extremely rapid (pre-steady state) reactions. We have applied a similar approach to follow the kinetics of the acylation of α-chymotrypsin with three substituted phenyl trimethylacetates. Since the acylations with these sterically hindered esters are considerably slower than the corresponding acetates (by one to two orders of magnitude) these reactions have the advantage that they may be observed using conventional apparatus.

Plots of the acylations of α-chymotrypsin with *p*-nitrophenyl, *p*-acetylphenyl and *p*-aldehydophenyl trimethylacetate are shown in Fig. 2[20] and the derived data for these reactions are shown in Table IV.

TABLE IV

THE ACYLATION OF α-CHYMOTRYPSIN WITH SOME SUBSTITUTED PHENYL TRIMETHYLACETATES[a]

| Phenyl trimethylacetate | $k_2' \times 10$, sec.$^{-1}$ | $K_m \times 10^3$, *M* | Sigma of leaving group[c] $\sigma$ | $\sigma^-$ |
|---|---|---|---|---|
| *p*-Nitro[b] | 3.7 | 1.6 | 0.778 | 1.27 |
| *p*-Acetyl | 9.8 | 2.0 | .516 | 0.874 |
| *p*-Aldehydo | 2.3 | 2.5 | .216 | 1.126 |

[a] In 1.6% acetonitrile–water, 0.01 *M* (total) Tris buffer, ionic strength 0.06, *p*H 8.2, 25.6 ± 0.1°. [b] Ref. 15. [c] H. H. Jaffé, *Chem. Revs.*, **53**, 191 (1953).

The data in Table IV indicate that the assumption that $K_m$ is independent of structure in these substituted phenyl esters is incorrect; both $K_m$ and $k_2'$ are affected by the *p*-substituent. It will be recalled that the second-order rate constant of

(19) H. Gutfreund and J. M. Sturtevant, *Proc. Natl. Acad. Sci., U. S.*, **42**, 719 (1956).

(20) M. Dixon and E. C. Webb, "Enzymes," Academic Press, Inc., New York, N. Y., 1958, p. 21.

the acylation of *p*-acetylphenyl acetate was abnormally high as seen in Fig. 1. It was thought at first that this abnormal behavior might be a function of an abnormal $K_m$. However, the $K_m$ for the corresponding compound in Table IV is not abnormal. Rather, the rate constant $k_2'$ is abnormally high.[21] No reasonable explanation for this result is forthcoming at the present time. The three reactions shown in Table IV are the only ones involving substituted phenyl esters that are accessible to experimental measurement (the only reactions in which the absorbance of the leaving group is spectrophotometrically observable under the conditions of this reaction). With the omission of the abnormal *p*-acetylphenyl trimethylacetate, one is, therefore, forced to make an analysis of the substituent effects on the acylation of α-chymotrypsin with only two reactions. This is a reasonable procedure since the *p*-nitrophenyl and *p*-aldehydophenyl esters appear to be part of a large group of esters exerting "normal" substituent effects in their second-order acylations (see Fig. 1).

In Fig. 3 are shown Hammett plots of the Michaelis constant $K_m$ and the acylation rate constant $k_2'$. For the former plot normal Hammett σ-constants have been used since the phenoxide ion is not liberated in the adsorption step. For the latter plot $\sigma^-$-constants have been used for the reasons given earlier. All three esters were used in the plot of log $K_m$ *vs.* σ resulting in a linear relationship with slope (ρ) equal to −0.33. As noted above only two esters were used in the plot of log $k_2'$ *vs.* $\sigma^-$ defining a line of slope (ρ) equal to 1.43.

## Discussion

Now that substituent effects on one step of an enzymatic reaction, the acylation of α-chymotrypsin, have been isolated, it is of interest to relate them to known substituent effects.

Qualitatively electron-withdrawing substituents facilitate the acylation of α-chymotrypsin. This behavior is noted in both the second-order and the first-order rate constants for acylation. A quantitative comparison of the substituent effect in the chymotrypsin acylation may be made with other reactions involving substituted phenyl esters, as shown in Table V.

From a consideration of the Hammett ρ constants, the acylation of α-chymotrypsin is not at all similar to hydronium ion catalysis (reaction 2) where electronic effects in the pre-equilibrium and rate-determining steps cancel, leading to little effect of substituents over-all. On the other hand, the acylation of α-chymotrypsin appears to resemble a number of reactions in Table V which involve nucleophilic attack at the carbonyl carbon atom of the ester. The acylation of α-chymotrypsin thus resembles a nucleophilic reaction of the ester more than an electrophilic reaction. If indeed both an electrophile and a nucleophile are involved in the acylation reaction, the dominating influence as judged by substituent effects is nucleophilic attack.

(21) *p*-Acetylphenyl sulfate showed abnormally high reactivity ($V_{max}$) in its hydrolysis by an aryl sulfatase; K. S. Dodgson, B. Spencer and K. Williams, *Biochem. J.*, **64**, 216 (1956).

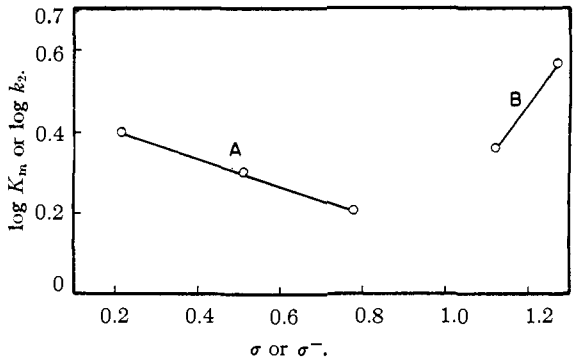


Fig. 3.—Hammett plot of the acylation of α-chymotrypsin with phenyl trimethylacetates: $\rho_{K_m} = -0.33$ (A); $\rho_{k_2} = 1.43$ (B).

From a consideration of the Hammett substituent constants, it appears that the acylations of α-chymotrypsin do not resemble intermolecular nucleophilic reactions (reactions 1, 3, 4, 5), but rather resemble intramolecular nucleophilic reactions (reactions 6 and 7). The analogy between an intramolecular organic reaction and an intracomplex enzymatic process has been noted many times before[26]; this observation may add substance to this analogy.

TABLE V

SUBSTITUENT EFFECTS IN REACTIONS OF PHENYL ESTERS

| | Reaction | Hammett ρ | Hammett substituent constant used in correlation | Ref. |
|---|---|---|---|---|
| 1 | Hydrol. of phenyl acetates, $OH^-$ catalysis | 1.0–1.1 | σ | 22 |
| 2 | Hydrol. of phenyl acetates, $H_3O^+$ catalysis | −0.22 | σ | 22 |
| 3 | Hydrol. of phenyl acetates, imidazole catalysis | >2[b] | σ | 23 |
| 4 | Ammonol. of phenyl acetates | >2[b] | σ | 24 |
| 5 | Ammonia-catalyzed ammonol. of phenyl acetates | 0.56 | σ | 24 |
| 6 | Hydrol. of phenyl acid glutarates | 2.0 | $\sigma^-$ | 13 |
| 7 | Hydrol. of γ-(4-imidazolyl)-butyric acid phenyl esters | 1.2 | $\sigma^-$ | 25 |
| 8 | Acyln. of chymotrypsin with phenyl acetates ($k_{obs} = k_2'/K_m$) | 1.8 | $\sigma^-$ | [a] |
| 9 | Acyln. of chymotrypsin with phenyl trimethylacetates ($k_2'$) | 1.4 | $\sigma^-$ | [a] |

[a] This investigation. [b] The correlation was recalculated from the data in the reference using σ instead of $\sigma^-$, giving a better fit.

One of the unanswered questions about the α-chymotrypsin catalysis is whether the catalysis occurs through the action of a general base or nucleophile on the enzyme surface. On the basis of the *p*H dependence it would appear that one or the other of these two possibilities must occur.[17] The indication from Table V on the basis of the Hammett ρ constants is that the acylation of α-chymotrypsin does not resemble the lone instance of general basic catalysis (reaction 5) but

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

(23) T. C. Bruice and G. L. Schmir, *J. Am. Chem. Soc.*, **79**, 1663 (1957).

(24) T. C. Bruice and M. F. Mayahi, *ibid.*, **82**, 3067 (1960).

(25) T. C. Bruice and J. M. Sturtevant, *ibid.*, **81**, 2860 (1959).

(26) M. L. Bender, *Chem. Revs.*, **60**, 53 (1960).

rather resembles the reactions involving nucleophilic catalysis (reactions 1, 3, 6, 7). This would appear to be a rather tenuous comparison since there appears to be a considerable difference in Hammett $\rho$ constants even between oxyanions (reaction 1) and neutral nitrogen nucleophiles (reactions 3 and 4). However, this is the only conclusion that may be drawn from the above data, tentative as it may be.

It is seen that there is a small difference in Hammett $\rho$ constants between the correlations involving first- and second-order acylations of chymotrypsin. This difference of course results from the fact that there is a small but real substituent effect on the adsorption constant, $K_m$. Since the substituent effect on $K_m$ is not large, it appears justified to discuss both the first-order and second-order acylations in the same context.

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# The Mechanism of $\alpha$-Chymotrypsin-catalyzed Hydrolyses[1-3]

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Experimental evidence bearing on the mechanism of action of $\alpha$-chymotrypsin has been summarized from previous papers in this series and the literature. Mechanistic proposals for $\alpha$-chymotrypsin action have been developed on the basis of the following evidence: (1) the occurrence of a two-step catalytic mechanism in which the intermediate compound, the acyl-enzyme, is an alkyl ester; (2) the equivalence of the two catalytic steps of acylation and deacylation through experimental observations, and symmetry and microscopic reversibility arguments; (3) the nucleophilic character of each catalytic step; (4) the effect of *p*H on the rates of the individual steps, indicating the participation of a base or nucleophile in the catalytic process; (5) spectrophotometric and kinetic experiments indicating no bulidup of an intermediate containing the acyl group bound to imidazole in deacylation; and (6) the effect of deuterium oxide on the rates of the individual steps, indicating the occurrence of a rate-determining proton transfer. These mechanisms are given in eq. 7, 8 and 9.

A class of proteolytic enzymes may be defined as those enzymes which react uniquely with a labile phosphate derivative to produce an inactive phosphorylated enzyme in which the phosphorus atom resides on the hydroxyl group of a serine moiety of the enzyme. This class of enzymes has been called the "serine proteinases."[5] $\alpha$-Chymotrypsin is the member of this class of enzymes most amenable to experimental investigation from the point of view of availability, purity, background of information and ease of experimentation. For these reasons, most of the mechanistic experiments described in previous papers were carried out with $\alpha$-chymotrypsin. It is reasonable to believe that the discussion presented here for $\alpha$-chymotrypsin will be pertinent as well for other members of the serine proteinase family such as trypsin, plasmin, thrombin and elastase.

Although a considerable amount of information concerning the mechanism of $\alpha$-chymotrypsin-catalyzed hydrolyses is now available, the three-dimensional structure of the enzyme (protein) is not available. Even the sequential analysis of the structure of chymotrypsin has not as yet been completed. Therefore, any conclusions in this paper must be tempered by the realization that the all-important structural information is yet to come.

From a mechanistic viewpoint ignorance of the protein structure reduces to ignorance of the structure of the one active site in the molecule. The active site is probably small compared to the entire enzyme, since most of the substrates of $\alpha$-chymotrypsin, even specific substrates, are quite small molecules compared to $\alpha$-chymotrypsin itself, which has a molecular weight of 24,800.[6] Furthermore, several hydrolytic enzymes have been cleaved of appreciable fractions of their total bulk with little or no loss of catalytic activity.[7] The active site is pictured as a *region* among several chains which contribute nucleophiles, electrophiles and specificity loci to the totality called the active site. This paper will attempt to specify the catalysis carried out by this site.

**Specificity and Binding.**—Before proceeding to a consideration of the catalytic features of the $\alpha$-chymotrypsin mechanism, it is of interest to consider the specificity exhibited by various kinds of substrates, including those used in the preceding investigations. It is well known that many poor substrates bind as well as good substrates. For example the association constant for the binding of the two members of an enantiomorphic pair with an enzyme will often be approximately the same, but only one will react subsequently to give products. This phenomenon may be easily explained by saying that the reactive substrate leads to a three-dimensional configuration on binding in which the susceptible linkage is positioned correctly with respect to the catalytic entities on the enzyme surface whereas the unreactive linkage, while bound, is incorrectly positioned. This explanation is based on the implicit assumptions that the binding of the substrate occurs to a relatively rigid enzyme surface and further that the binding is performed by groups on the enzyme

(1) This research was supported by grants from the National Institutes of Health.

(2) Paper XVI in the series, The Mechanism of Action of Proteolytic Enzymes; previous paper, M. L. Bender and K. Nakamura, *J. Am. Chem. Soc.*, **84**, 2577 (1962).

(3) Some of this material was presented in preliminary form: M. L. Bender, G. R. Schonbaum, G. A. Hamilton and B. Zerner, *ibid.*, **83**, 1255 (1961).

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(5) B. S. Hartley, *Ann. Revs. Biochem.*, **29**, 45 (1960).

(6) P. E. Wilcox, J. Kraut, R. D. Wade and H. Neurath, *Biochim. et Biophys. Acta*, **24**, 72 (1957); P. E. Wilcox, E. Cohen and W. Tan, *J. Biol. Chem.*, **228**, 999 (1956).

(7) R. L. Hill and E. L. Smith, *Biochim. et Biophys. Acta*, **19**, 376 (1956); R. L. Hill and E. L. Smith, *J. Biol. Chem.*, **235**, 2332 (1960); G. E. Perlmann, *Nature*, **173**, 406 (1954).