formed (resulting in an apparent increase in absorbance at all wave lengths down to 245 mμ) which then slowly redissolved (resulting in an apparent decrease in absorbance with a half-life of approximately five minutes). However, at no time did any preparation of *trans*-cinnamoyl-α-chymotrypsin undergo these precipitation and redissolution phenomena.[63] Thus there appears to be only one experimentally verifiable type of acyl-chymotrypsin intermediate, which is the subject of the present paper.

Both the spectral data, which are somewhat ambiguous, and the kinetic data in 7.74 *M* urea, which are relatively unambiguous, indicate that the acyl group of the acyl-enzyme is attached to an oxygen atom of the enzyme in the form of an ester linkage. This evidence supports completely the suggestions made from isolation experiments that the acyl group is attached to the oxygen atom of a serine moiety of the enzyme.[16] The occurrence of this acyl-enzyme intermediate in the α-chymotrypsin-catalyzed hydrolyses of many labile esters has now been demonstrated without doubt. The occurrence of a similar intermediate in the α-chymotrypsin-catalyzed hydrolysis of a non-labile ester and in a trypsin-catalyzed hydrolysis will be reported in subsequent papers in this series.

(63) It has been reported that acetyl-chymotrypsin loses its acetate residue (as measured by ^{14}C activity) at a much slower rate than the rate of appearance of enzymic activity. This observation by T. Viswanatha and W. B. Lawson, *Arch. Biochem. Biophys.*, **93**, 128 (1961), implies that some acylation at other than the active site occurs, an observation confirmed in this Laboratory. However, this complication apparently does not apply to the more specific cinnamoyl systems.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, NORTHWESTERN UNIVERSITY, EVANSTON, ILL.]

The Formation of an Acyl-enzyme Intermediate in the α-Chymotrypsin-catalyzed Hydrolyses of Non-labile *trans*-Cinnamic Acid Esters[1-3]

BY MYRON L. BENDER[4] AND BURT ZERNER

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The α-chymotrypsin-catalyzed hydrolyses of methyl and benzyl *trans*-cinnamates have been investigated under pseudo-first-order conditions in which the enzyme concentration is much greater than the substrate concentration. Under these conditions, the acylation and deacylation reactions can be described as two consecutive first-order reactions. In each enzymatic process, unequivocal evidence has been obtained for the involvement of *trans*-cinnamoyl-α-chymotrypsin as an intermediate in the reaction, both from rate measurements and absorption spectral characteristics. A quantitative fit of the absorbance *vs.* time curve for the α-chymotrypsin-catalyzed hydrolysis of methyl cinnamate was obtained using the measured molar absorptivities of reactant, intermediate and product, together with the independently determined (apparent) rate constant of acylation and the rate constant of deacylation. In addition, zero-order kinetics of the methyl cinnamate system enabled the calculation of the Michaelis constant for acylation and the (true) acylation rate constant. The ratio of these constants is consistent with that obtained directly from the first-order kinetics. It is concluded that the formation of an acyl-enzyme intermediate is not an artifact of α-chymotrypsin catalyses resulting from the lability of the ester function, but is part of the general mechanism of such catalyses.

Introduction

The hydrolysis of labile acyl derivatives (*e.g.*, nitrophenyl esters) is catalyzed by α-chymotrypsin in a three-step process: (i) adsorption of the substrate on the enzyme; (ii) acylation of the enzyme with the release of the phenol; and (iii) deacylation of the acyl-enzyme giving the carboxylic acid product and regenerating the enzyme.[5] In other papers of this series[6,7] the evidence supporting the involvement of the acyl-enzyme intermediate, *trans*-cinnamoyl-α-chymotrypsin, in the enzyme-catalyzed hydrolyses of five labile *trans*-cinnamic acid derivatives (*o*-, *m*-, *p*-nitrophenyl cinnamates, *p*-cresyl cinnamate and N-cinnamoylimidazole) has been thoroughly documented.

However, it has been maintained on the basis of kinetic arguments that the α-chymotrypsin-catalyzed hydrolysis of methyl hippurate[8] does not proceed through a hippuryl-enzyme intermediate and (by implication) that the α-chymotrypsin-catalyzed hydrolyses of other non-labile substrates also do not involve acyl-enzyme intermediates.[9] With a view to establishing the generality of acyl-enzyme formation in the α-chymotrypsin-catalyzed hydrolysis of carboxylic acid derivatives, we therefore investigated the enzyme-catalyzed hydrolysis of two non-labile (alkyl) esters of *trans*-cinnamic acid, methyl and benzyl cinnamate. An account of this work is given in the present paper.

Experimental

Materials.—α-Chymotrypsin (3 × crystd., salt-free) was obtained from Worthington Biochemical Corporation and was used without further purification. Stock solutions ($1.5 - 3 \times 10^{-3}$ *M*) of the enzyme were prepared in appropriate buffers. All enzyme solutions were centrifuged to remove traces of lint and (at the higher concentrations) insoluble protein. The normality of the enzyme solutions was determined by titration[10] at the beginning and end of

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

(2) Paper XI in the series, The Mechanism of Action of Proteolytic Enzymes; previous paper, M. L. Bender, G. R. Schonbaum and B. Zerner, *J. Am. Chem. Soc.*, **84**, 2540 (1962).

(3) Some of the results of this paper have been presented previously: M. L. Bender and B. Zerner, *ibid.*, **83**, 2391 (1961).

(4) Alfred P. Sloan Foundation Research Fellow.

(5) For a detailed list of references, see ref. 6, 7.

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

(7) M. L. Bender, G. R. Schonbaum and B. Zerner, *ibid.*, **84**, 2562 (1962).

(8) S. A. Bernhard, W. C. Coles and J. F. Nowell, *ibid.*, **82**, 3043 (1960).

(9) M. L. Bender and W. A. Glasson, *ibid.*, **82**, 3336 (1960), also obtained kinetic results in the enzyme-catalyzed methanolysis of N-acetyl-L-phenylalanine methyl ester which do not appear to be readily reconciled with the formation of an acyl-enzyme intermediate in the reaction.

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

each series of runs. Methyl cinnamate (Eastman Kodak Co., white label) was purified by distillation at ~1 mm. and partial fractional crystallization; m.p. 33.5–34.5° (lit.[11] m.p. 34.7°). The purity of this compound was checked by spectrophotometric measurements on cinnamate ion liberated in alkaline hydrolysis and was found to be not less than 99.3%. Benzyl cinnamate was prepared by the reaction of *trans*-cinnamoyl chloride with benzyl alcohol in pyridine solution. The product was recrystallized to constant melting point from 95% ethanol; m.p. 34.5° (lit.[12] m.p. 39°). *p*-Nitrophenyl cinnamate[6] was recrystallized three times from chloroform–hexane; m.p. 146.5–147.5° (lit.[13] m.p. 146°). Stock solutions of the substrates were prepared in acetonitrile (Eastman Kodak Co. spectro grade). Carbonate-free sodium hydroxide solutions were prepared from saturated solutions of reagent grade sodium hydroxide and were standardized against N.B.S. primary standard potassium hydrogen phthalate. Phosphate buffers (0.1 *M*) and tris-(hydroxymethyl)-aminomethane (Tris) buffers were prepared as previously described.[6] Owing to the buffering action of the enzyme at the concentrations used in these experiments, the *p*H was adjusted as necessary by the addition of carbonate-free sodium hydroxide using a Radiometer TTTl titrator.

pH **Measurement.**—The *p*H of the solutions was determined immediately after reaction directly in the spectrophotometer cuvette using a Radiometer *p*H meter, model 4C, equipped with a G 222B (semi-micro) glass electrode. The meter was standardized as recommended by Bates, *et al.*[14] However, accurate *p*H measurement in these highly concentrated enzyme solutions is difficult and the values reported here may be in error by ±0.02 *p*H unit.

Kinetic Measurements. Methyl Cinnamate. Pseudo-first-order Kinetics.—As shown in the Appendix, given suitable values of k_2', k_3', K_m, $[E]_0$ and $[S]_0$, the enzyme-catalyzed hydrolysis of methyl cinnamate (assuming the formation of the acyl-enzyme intermediate, *trans*-cinnamoyl-α-chymotrypsin) may be reduced to two pseudo-first-order consecutive reactions. The reason for choosing such conditions is that in this way one is able to "see" significant concentrations of the acyl-enzyme in the spectrophotometer, and hence quantitatively relate the observed absorbance *vs.* time curve to the measured constants, k_{obs} and k_3' (see Appendix). A series of runs at varying *p*H was carried out at high enzyme concentrations ($1.3 - 1.7 \times 10^{-3}$ *M*) with the concentration of methyl cinnamate maintained at 1.20×10^{-4} *M*. Parallel to these runs, the deacylation of *trans*-cinnamoyl-α-chymotrypsin (generated from the N-*trans*-cinnamoylimidazole) was investigated using the same stock enzyme solution, thus establishing independently the rate constant, k_3', of eq. 5. A Cary model 11 recording spectrophotometer was used throughout this work and all reactions were followed at 310 mμ (*vide infra*). A typical pair of experiments is described: 3.00 ml. of a standardized enzyme solution was equilibrated at 25.0 ± 0.1° in the sample compartment of the spectrophotometer; 100 μl. of an acetonitrile stock solution of methyl cinnamate was added on the tip of a flat-ended stirring rod and recording was commenced after approximately 20 seconds. The absorbance *vs.* time curve shows the growth and decay of a maximum, followed after the expected time (governed by k_{obs} and k_3' and the molar absorptivities[15] of all species present) by a true exponential decay. A typical experimental trace is shown in Fig. 1. Conventional "infinity" plots of log $(A_t - A_\infty)$ *vs.* time yield the rate-constant, k_{obs}, which in this system is always less than the deacylation rate constant and is therefore directly related to the acylation reaction (see Appendix). A reaction, identical in form, was then carried out using N-*trans*-cinnamoylimidazole as substrate. In this case, of course, one observes only the deacylation of the acyl-enzyme, *trans*-cinnamoyl-α-chymotrypsin, since acylation of the enzyme is virtually instantaneous.

In addition to the runs at high enzyme concentration and varying *p*H, a series of runs was done at constant *p*H (7.80) and substrate concentration, with varying enzyme concentration. These runs were performed in exactly the same manner as the above runs, and similar behavior was observed, although the maximum absorbance change was of course diminished at the lower enzyme concentrations.

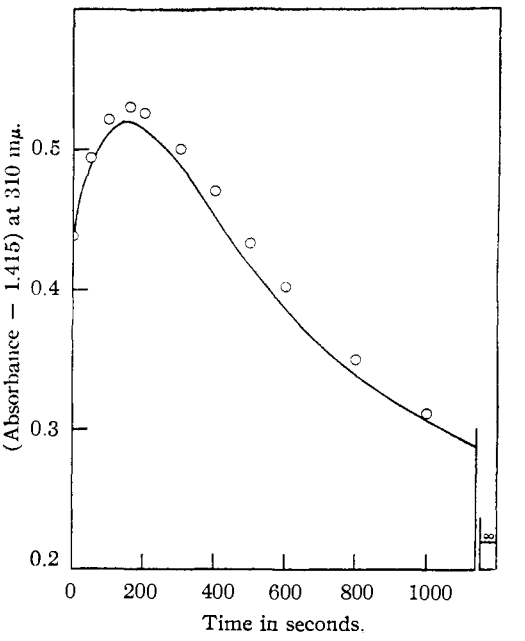

Fig. 1.—The α-chymotrypsin-catalyzed hydrolysis of methyl cinnamate: solid curve, experimental trace of run 7; open circles, points calculated using the rate constants obtained in runs 7, 8 (Table I) and the molar absorptivities of the species.

Methyl Cinnamate. Zero-order Kinetics.—These runs were performed on a Cary model 14PM spectrophotometer at wave lengths between 310 and 320 mμ. The enzyme concentration was maintained constant at 4.80×10^{-5} *M* and the substrate concentration was varied from 1.94×10^{-3} to 2.76×10^{-4} *M*. A typical experiment is described: 3.00 ml. of 0.1 *M* phosphate buffer (*p*H 7.90) was equilibrated at 25.0 ± 0.1° in the cuvette in the sample compartment of the spectrophotometer; 100 μl. of an acetonitrile stock solution of methyl cinnamate was added and recording commenced. The hydroxide-ion catalyzed hydrolysis of the ester is completely negligible at this *p*H, so that the molar absorptivity of the ester at the particular wave length may be checked in each experiment. Excellent agreement was found with values independently determined volumetrically. After about 1 minute, 50 μl. of the stock enzyme solution was added and recording was commenced after 20 seconds. Again one sees the development of a (weak) maximum, followed by true zero-order kinetics. The absorbance data are readily converted to rate data by combination with the difference in molar absorptivities of the ester and cinnamate ion at the particular wave lengths.

Benzyl Cinnamate. Pseudo-first-order Kinetics.—These runs were performed under very similar conditions to the methyl cinnamate runs and similar behavior was noted. Here, however, the rate constant obtained from the first-order portion of the absorbance *vs.* time curve agreed with the independently determined deacylation rate constant, k_3'. Detailed analysis of this system, however, was precluded in the present experiments because of the low solubility of the substrate. Indeed, the successful observation of the reaction in the present experiments is made possible by the rapid consumption of the substrate present in a supersaturated solution. In the runs at lowest enzyme concentration, precipitation did occur and lasted for as long as 2–3 minutes; the initial part of the absorbance *vs.* time curve was altered, but the rate constants obtained after the precipitate had been consumed were in good agreement with the independently determined deacylation constant, k_3'.

Molar Absorptivities at 310 mμ.—The wave length 310 mμ was chosen as the wave length for following the reactions,

(11) J. Kendall and J. E. Booge, *J. Am. Chem. Soc.*, **38**, 1712 (1916).

(12) "Dictionary of Organic Compounds," I. M. Heilbron, ed., Oxford University Press, New York, N. Y., 1934, Vol. I, p. 241.

(13) R. Anschütz, *Ber.*, **60**, 1322 (1927).

(14) R. G. Bates, G. D. Pinching and E. R. Smith, *J. Research Natl. Bur. Standards*, **45**, 418 (1950).

(15) H. K. Hughes, *et al.*, *Anal. Chem.*, **24**, 1349 (1952).

TABLE I

THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF METHYL CINNAMATE. PSEUDO-FIRST-ORDER KINETICS[a]

Run	pH[b]	$[E]_0/[S]_0$[c]	$k_{obs} \times 10^3$, sec.$^{-1}$	$k_3' \times 10^3$, sec.$^{-1}$	ΔA_{max} (calcd.)[d]	ΔA_{max} (exptl.)	t_{max} (calcd.),[e] sec.	t_{max} (exptl.), sec.
1,2	6.02	13.6	0.32	0.67	0.599	0.604	1470	1430 ± 100
3,4	6.51	14.4	0.72	2.10	.569	.583	532	510 ± 90
5,6	6.89	12.6	1.19	4.15	.553	.548	285	280 ± 30
7,8	7.23	11.3	1.73	8.02	.530	.520	158	146 ± 20
9,10	7.49	11.9	2.05	10.2	.522	.511	120	125 ± 5
11,12	7.89	12.6	2.78	12.2	.506	.505	94	91 ± 15

[a] 25.0°, 3.2% CH_3CN; each line shows a pair of runs. [b] 0.1 *M* phosphate buffers. [c] Substrate, methyl cinnamate; $[S]_0 = 1.20 \times 10^{-4}$ *M* throughout. [d] Substrate, N-*trans*-cinnamoylimidazole; $[S]_0 = 8.65 \times 10^{-5}$ *M* throughout. [e] ΔA_{max} represents the absorbance change at maximum absorbance; t_{max} is the time of maximum absorbance.

since the molar absorptivities[15] at this wave length are suitable for the observation of a maximum in the absorbance *vs.* time curve; *i.e.*, $\epsilon_{AE} > \epsilon_S > \epsilon_{Cin^-}$.[16] The (difference) molar absorptivity of the acyl-enzyme *vs.* enzyme at 310 mμ has been previously determined[6,10] and shown to be essentially *p*H-independent in the *p*H range 5–7. The value $\Delta\epsilon_{AE} = 10.96 \times 10^3$ at 310 mμ has been used throughout the calculations. The molar absorptivity of methyl cinnamate at 310 mμ, measured in the absence of enzyme, is 3.27×10^3. However, it has been shown that the small volume of acetonitrile added has a significant effect on the molar absorptivity of the enzyme and therefore the value required for the calculations is the (apparent) molar absorptivity determined directly in the highly concentrated solutions. Seven determinations yielded the value $\epsilon^{310}_{MeCinnamate} = 3.62 (\pm 0.1) \times 10^3$.[17] The molar absorptivity of cinnamate ion in water solution at 310 mμ is 207 ± 2. However, at high concentrations of enzyme, the (apparent) molar absorptivity of cinnamate ion obtained under the conditions of the reaction is considerably increased. Therefore, the value used in the calculations is that obtained under the particular experimental conditions. These values ranged from 1.3 to 1.8 × 10^3. The calculations are not sensitive to this value.

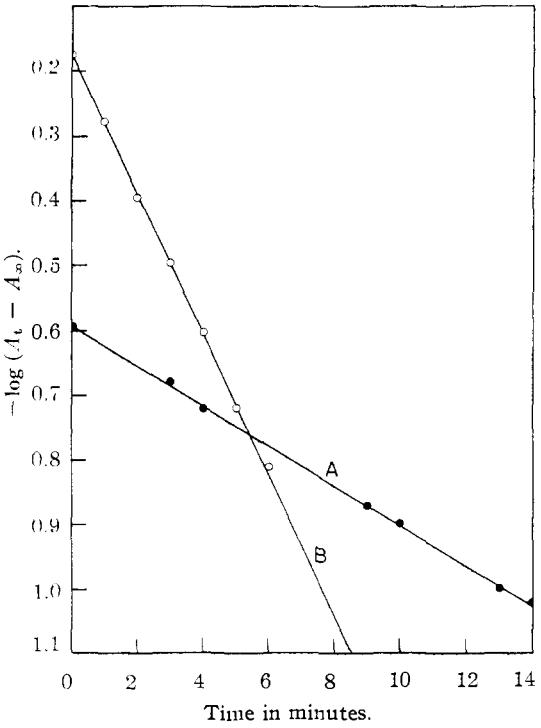

Fig. 2.—A, acylation of α-chymotrypsin with methyl cinnamate. B, deacylation of *trans*-cinnamoyl-α-chymotrypsin. Data of runs 5, 6 (Table I).

Alkaline Hydrolysis.—The rates of alkaline hydrolysis of methyl cinnamate, benzyl cinnamate and *p*-nitrophenyl cinnamate were determined under pseudo-first-order conditions in dilute standard carbonate-free sodium hydroxide solutions spectrophotometrically at 295, 295 and 400 mμ, respectively. In all cases a 0–0.1 absorbance wire (Cary 14 PM) was used and in addition 10-cm. cells were used for the benzyl ester. The concentration of substrates was $\sim 10^{-5}$ *M* for methyl cinnamate, 4×10^{-6} *M* for *p*-nitrophenyl cinnamate and 7×10^{-7} to 3×10^{-6} *M* for the benzyl ester. Pseudo-first-order constants were obtained from "infinity" plots and the second-order constants, k_{OH}, were obtained by dividing by the base concentration. Two independently standardized base solutions were used at various dilutions and each constant is the mean of at least four runs.

Results and Discussion

The results of experiments at a fixed concentration of methyl cinnamate, high concentrations of the enzyme and varying *p*H are presented in Table I. Figure 1 shows a typical experimental absorbance *vs.* time curve, together with calculated points obtained from the measured rate constants (k_{obs} and k_3') and the molar absorptivities of methyl cinnamate, *trans*-cinnamoyl-α-chymotrypsin and cinnamate ion. Typical kinetic plots used to obtain k_{obs} and k_3' are shown in Fig. 2. In addition, Table I lists the experimental and calculated values of $\Delta A_{max} = A_{max} - A_0$, the maximum absorbance change and t_{max}, the time of maximum absorbance. Qualitatively, the presence of a maximum in the absorbance *vs.* time curve shows the formation of a highly absorbing intermediate. Further, the excellent fit of the calculated curve to the experimental curve (Fig. 1) and the excellent agreement between the calculated and experiment values of ΔA_{max} and t_{max} throughout the series of runs in Table I establishes beyond doubt that the intermediate formed in the enzyme-catalyzed hydrolysis of methyl cinnamate is *trans*-cinnamoyl-α-chymotrypsin. While this system is convenient for the display of the intermediate, as will be subsequently clear, it is not useful for determining the ratio of the constants k_2' and K_m (eq. 5).

The results of the experiments at constant *p*H and constant methyl cinnamate concentration but with varying enzyme concentration are presented in Table II and graphically in Fig. 3, in which k_{obs} is plotted against the initial enzyme concentration $[E]_0$. Since true first-order kinetics are still observed in this system, even at the lowest enzyme concentration, k_{obs} is given by the equation

$$k_{obs} = k_2' [E]_0/K_m$$

(see eq. 11 and case III, Appendix). It will be seen from Fig. 3, that at low enzyme concentrations

(16) AE = acyl-enzyme, S = substrate, Cin^- = cinnamate ion.

(17) In a preliminary communication of this work,[3] the value 3.27×10^3 was used.

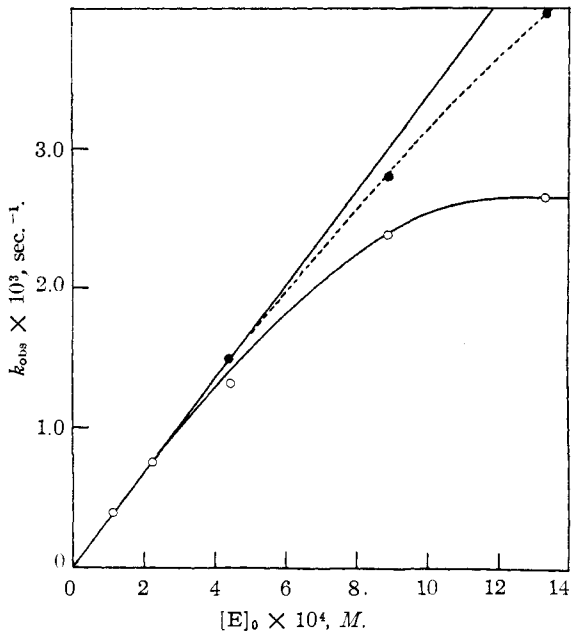

Fig. 3.—Effect of enzyme concentration on k_{obs} in the α-chymotrypsin-catalyzed hydrolysis of methyl cinnamate; data of Table II: open circles, solid curve, experimental points; full circles, broken curve, points calculated using eq. 10.

k_{obs} is strictly linear in $[E]_0$, as required by the above equation, but approaches a maximum value at high enzyme concentrations. The linear portion of the curve gives the values $k_2'/K_m = 3.36\ M^{-1}$ sec.$^{-1}$ at pH 7.80. This procedure, however, does not allow the separation of the constants k_2' and K_m.

TABLE II

THE EFFECT OF ENZYME CONCENTRATION ON k_{obs} IN THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF METHYL CINNAMATE[a,b]

$[E]_0 \times 10^4$, M	$k_{obs} \times 10^3$, sec.$^{-1}$
1.11	0.377
2.23	0.742
4.45	1.31
8.90	2.39
13.3	2.66

[a] 25.0°, 3.2% CH_3CN, pH 7.80 ± 0.02. [b] $[S]_0 = 1.20 \times 10^{-4}\ M$ throughout.

Since it has been shown that eq. 5 does indeed describe the system methyl cinnamate-α-chymotrypsin–water, zero-order kinetics should allow the determination of a $K_{m(app)}$ and k_{cat}' given by eq. 1 and 2.[18]

$$K_{m(app)} = k_3'K_m/(k_2' + k_3') \quad (1)$$

$$1/k_{cat}' = 1/k_2' + 1/k_3' \quad (2)$$

Then, since k_3' is known independently from the deacylation of *trans*-cinnamoyl-α-chymotrypsin, both K_m and k_2' may be evaluated as well as the ratio k_2'/K_m. A Lineweaver–Burk[19] plot of the data obtained under conditions of zero-order kinetics is shown in Fig. 4. Least-squares calculation of the data gives $K_{m(app)} = 2.05 \times 10^{-3}\ M$ and $k_{cat}' = 7.3 \times 10^{-3}$ sec.$^{-1}$, at pH 7.90. Since the (pH-independent) deacylation constant (k_3) of *trans* - cinnamoyl - α - chymotrypsin has been previously determined[7] as 12.5 ± 0.5 × 10^{-3} sec.$^{-1}$, one can correct this value to pH 7.90, using $pK_{deacylation} = 7.15$.[7] This gives the value of k_3' at pH 7.90 as 10.6×10^{-3} sec.$^{-1}$, and hence $k_2' = 23.9 \pm 2.4 \times 10^{-3}$ sec.$^{-1}$, $K_m = 6.7 \pm 0.6 \times 10^{-3}\ M$ and $k_2'/K_m = 3.57\ M^{-1}$ sec.$^{-1}$. If k_2'/K_m is corrected to pH 7.80, one can compare the value so obtained ($3.44\ M^{-1}$ sec.$^{-1}$) with that determined under first-order conditions at pH 7.80, namely, $3.36\ M^{-1}$ sec.$^{-1}$. The agreement is good, perhaps fortuitously good, considering the many separate experiments involved in this comparison. Be this as it may, there is here an independent check on the validity of the first-order kinetic approach to this enzymatic system.

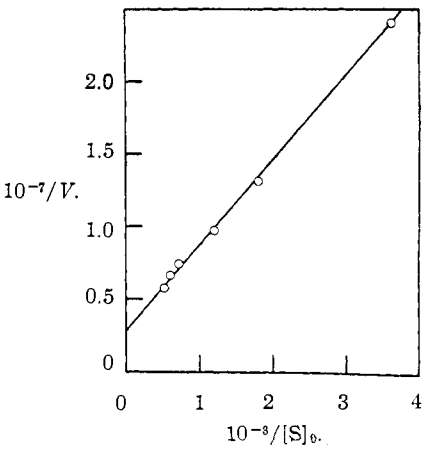

Fig. 4.—Lineweaver–Burk plot of the α-chymotrypsin-catalyzed hydrolysis of methyl cinnamate; units of V, M sec.$^{-1}$; units of $[S]_0$, M; $[E]_0 = 4.80 \times 10^{-5}\ M$; 0.1 M phosphate buffer, pH 7.90, 25.0 ± 0.1°.

Since the value of k_2' and K_m are now known, using eq. 10, it is possible to calculate the k_{obs} *vs.* $[E]_0$ curve and this is shown as a dashed line in Fig. 3. The measured rate constant falls off much faster than predicted by eq. 10. This may reasonably be ascribed to inhibition by enzyme, although insufficient data has been obtained to allow an assessment of this possibility. Experimentally, however, the rate does become virtually independent of enzyme concentration at high values of $[E]_0$.[20] An estimate of the $pK_{acylation}$ can therefore be obtained directly from k_{obs} and pH. The data show a fit to a $pK_{acylation} = 7.1$ In these same highly concentrated enzyme solutions, $pK_{deacylation} = 7.3$. The precision of these measurements, however, is not as good as that obtained at lower enzyme concentrations, where $pK_{deacylation} = 7.15$. Certainly within experimental error, there is good agreement. Further, the data *indicate* that the same ionization is controlling both reactions.

Gutfreund and Hammond[21a] have compared the individual kinetic constants obtained in the α-chymotrypsin-catalyzed hydrolysis of three N-

(18) H. Gutfreund and J. M. Sturtevant, *Biochem. J.*, **63**, 656 (1956); H. Gutfreund and J. M. Sturtevant, *Proc. Natl. Acad. Sci., U. S.*, **42**, 719 (1956).

(19) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).

(20) Measurements made at lower pH values show the same effect.

(21) (a) H. Gutfreund and B. R. Hammond, *Biochem. J.*, **73**, 526 (1959); (b) N-benzyloxycarbonyl-L-tyrosine p-nitrophenyl ester.

benzoyl-L-tyrosine derivatives—an amide, an ethyl ester and a *p*-nitrophenyl ester.[21b] This comparison has been criticized,[22] since it assumes the formation of the acyl-enzyme, N-benzoyl-L-tyrosyl-α-chymotrypsin, in the hydrolysis of the amide and ethyl ester. It would be of interest, therefore, to make such a comparison in the case of a labile and non-labile ester, both of which are *known* to hydrolyze *via* an acyl-enzyme intermediate. The two compounds selected are methyl cinnamate and *p*-nitrophenyl cinnamate. Attempts to obtain k_2' and K_m for a nitrophenyl cinnamate in a high water-content solvent (not more than 10% v./v. organic solvent) in the stopped-flow apparatus[23] have been uniformly unsuccessful owing to the sparing solubility of these esters. However, since k_2'/K_m has been successfully measured[6] for *p*-nitrophenyl cinnamate by a different experimental approach, an intelligent guess at K_m allows the comparison made in Table III. Of the 12 constants shown, only *two* are estimated values, and even if the estimate of K_m were in error by an order of magnitude, it would not affect any of the conclusions.

TABLE III

KINETIC CONSTANTS FOR THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF TWO *trans*-CINNAMIC ACID ESTERS

	Methyl ester[a]	*p*-Nitrophenyl ester
K_m, M	$6.7 \pm 0.6 \times 10^{-3}$	$3\text{–}5 \times 10^{-3}$[c]
k_2'/K_m, M^{-1} sec.$^{-1}$	3.57	$1.13 \pm 0.04 \times 10^{4}$[b,6]
k_2', sec.$^{-1}$	$23.9 \pm 2.4 \times 10^{-3}$	33.9–56.5[d]
k_3', sec.$^{-1}$	10.6×10^{-3}	11.1×10^{-3}[f]
k_{cat}', sec.$^{-1}$	7.3×10^{-3}	11.1×10^{-3}[f]
k_{OH}, M^{-1} sec.$^{-1}$	$6.04 \pm 0.10 \times 10^{-2}$[e]	2.85 ± 0.05[e]

[a] 25.0°, 3.2% CH_3CN, 0.1 M phosphate, *p*H 7.90. [b] 25.0°, 11.4% CH_3CN, 0.05 M Tris, *p*H 8.34. [c] Estimated from $K_m = 1.6 \times 10^{-3}$ M for *p*-nitrophenyl trimethylacetate[24] and the effect of organic solvent on K_m.[6,7,25] [d] Estimated value, dependent on K_m. [e] 25.0°, 0.3% CH_3CN; k_{OH} for benzyl cinnamate $= 6.78 \pm 0.10 \times 10^{-2}$ M^{-1} sec.$^{-1}$. [f] 25.0°, 10% CH_3CN, 0.05 M Tris, *p*H 8.34.

The rates of alkaline hydrolysis for methyl and *p*-nitrophenyl cinnamates show the expected ratio of about 50. However, when one compares k_{cat}' for the two esters, the difference is small, the more so when one takes account of the slightly different *p*H's at which the experiments were performed. An exactly similar effect is noted with the tyrosine derivatives. The most striking result of the comparison, however, is the tremendous difference between the values of k_2' for the two esters, the ratio favoring the *p*-nitrophenyl ester being of the order 10^3. Gutfreund and Hammond[21] had been able to set a lower limit of k_2 for N-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester as 10^3 sec.$^{-1}$. The present work indicates strongly that it may be very much larger than this, and further indicates the complete reasonableness of their rationalization of their data. On these grounds, the possibility of observing the acylation step with a good substrate (*e.g.*, one of the reactivity of N-acetyl-L-tyrosine ethyl ester), even if the intermediate had suitable spectral characteristics, would appear to be very remote.[26]

The data obtained for the α-chymotrypsin-catalyzed hydrolysis of benzyl cinnamate are presented in Table IV. It is clear that under the conditions of the reactions, $k_{obs} = k_3'$. The deacylation step must therefore be rate controlling. This does not allow a conclusion on the relative magnitudes of k_2' and k_3' for this ester, however, since k_{obs} is a complex constant. It is only due to the favorable K_m in the methyl cinnamate system that the pseudo-first-order acylation reaction is rate controlling for as complete analysis has shown, $k_2' > k_3'$. Table IV also clearly shows the "solvent" effect of the enzyme at high concentrations, exactly similar to the effects in the methyl cinnamate system.

TABLE IV

THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF BENZYL CINNAMATE[a]

$[E]_0 \times 10^4$, M	$[S]_0 \times 10^5$,[b] M	$k_{obs} \times 10^3$,[c] sec.$^{-1}$	$k_3' \times 10^3$,[d] sec.$^{-1}$
8.68	7.12	5.3_1	5.24
8.65	3.62	5.2_1	5.52[e]
5.67	7.12	5.0_0	5.0_3
2.84	7.12	4.7_4, 4.50[f]	4.7_3

[a] 25.0°, 3.2% CH_3CN, 0.1 M phosphate buffer, *p*H 7.00 ± 0.02. [b] S = benzyl cinnamate. [c] Substrate, benzyl cinnamate. [d] Substrate, N-*trans*-cinnamoylimidazole, 7.68×10^{-5} M. [e] [N-*trans*-cinnamoylimidazole] = 3.90×10^{-5} M. [f] Precipitate of approximately 2–3 minutes duration formed in these runs.

It has now been adequately demonstrated that the α-chymotrypsin-catalyzed hydrolyses of two non-labile substrates, methyl and benzyl cinnamate, proceed through the acyl-enzyme intermediate, *trans*-cinnamoyl-α-chymotrypsin.[27] Thus, it is established beyond doubt that the same general mechanism is involved in the α-chymotrypsin-catalyzed hydrolysis of both labile and non-labile substrates; the formation of the acyl-enzyme intermediate is therefore not an artifact resulting from the lability of the ester function, but is part of the general mechanism of α-chymotrypsin catalyses.

Appendix.—Consider the application of spectrophotometry to the system of consecutive first-order reactions represented by eq. 3

$$A \xrightarrow{k'} B \xrightarrow{k''} C \qquad (3)$$

where $[B]_0 = [C]_0 = 0$. Let the molar absorptivities[15] (at a particular wave length) of A, B, and C be ϵ_A, ϵ_B and ϵ_C, and set the condition that $\epsilon_A > \epsilon_B > \epsilon_C$. Then, in its most general form, the absorbance *vs.* time curve will exhibit a maximum followed after a suitable time (governed by k'/k'' and ϵ_A, ϵ_B, ϵ_C) by a true exponential decay. Using the general solutions[28] for $[A]_t$, $[B]_t$ and $[C]_t$

(22) S. A. Bernhard, in discussion of M. L. Bender, G. R. Schonbaum and G. Hamilton, *J. Polymer Sci.*, XLIX, 75 (1961).

(23) M. L. Bender and B. Zerner, unpublished observations.

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

(25) T. H. Applewhite, R. B. Martin and C. Niemann, *ibid.*, **80**, 1457 (1958).

(26) On the basis of other evidence, however, which will form the substance of a future publication, it might be anticipated that k_{cat} for the ethyl and *p*-nitrophenyl esters of N-acetyl-L-tyrosine are the same. This work and its implications for the generality of acyl-enzyme formation in the action of α-chymotrypsin is being pursued in these laboratories at the present time.

(27) Preliminary experiments with β-phenylethyl and cyclohexyl cinnamate again show the presence of the intermediate.

(28) A. A. Frost and R. G. Pearson, "Kinetics and Mechanism," John Wiley and Sons, Inc., New York, N. Y., Sec. Ed., 1961, p. 166 *et seq.*

from eq. 3, it follows directly that the first-order portion of the decay curve will yield k' or k'', depending on whether $k' < k''$ or $k' > k''$. While this is mathematically sound, if $k' \simeq k''$, the exponential decay may occur so late in the reaction as to preclude the collection of meaningful data.

The time (t_{max}) and magnitude of maximum absorbance are experimentally useful quantities. It can be shown that

$$t_{max} = \ln \{(\epsilon_B - \epsilon_C)/[(\epsilon_A - \epsilon_C) + k'/k''(\epsilon_B - \epsilon_A)]\}/(k'' - k') \quad (4)$$

The species concentrations may be calculated at t_{max} giving, after combination with the molar absorptivities,[15] the required maximum absorbance.

The extension of the above system to the α-chymotrypsin-catalyzed hydrolysis of methyl cinnamate, assuming the formation of the acyl-enzyme intermediate, *trans*-cinnamoyl-α-chymotrypsin, requires only that acylation of the enzyme occurs under pseudo-first-order conditions. Consider the scheme

$$E + S \underset{}{\overset{K_m}{\rightleftharpoons}} ES \xrightarrow{k_2'} ES' \xrightarrow{k_3'} E + P_2 \quad (5)$$
$$\qquad\qquad\qquad\qquad\quad + P_1$$

where k_2' and k_3' are the (pH — dependent) acylation and deacylation constants.[18]

Case I: If $k_3' >> k_2'$, eq. 5 reduces to

$$E + S \underset{}{\overset{K_m}{\rightleftharpoons}} ES \xrightarrow{k_2'} E + P_1 + P_2 \quad (6)$$

If $[E]_0 >> [ES]$

$$\text{rate} = dP_1/dt = dP_2/dt = k_2'([S]_0 - [P_1])/(1 + K_m/[E]_0) \quad (7)$$
$$= k_{obs}([S]_0 - [P_1])$$

where

$$k_{obs} = k_2'/(1 + K_m[E]_0) \quad (8)$$

Equation 7 requires that the rate be pseudo first-order in substrate concentration, with a first-order constant, k_{obs}, given by eq. 8.

If $K_m >> [E]_0$, from eq. 8

$$k_{obs} = k_2'[E]_0/K_m \quad (9)$$

Case II: If $k_3' << k_2'$, eq. 3 reduces to

$$E + S \underset{}{\overset{K_m}{\rightleftharpoons}} ES \xrightarrow{k_2'} ES' + P_1$$

If $[E]_0 >> [S]_0$,

$$\text{rate} = dP_1/dt = k_2'([S]_0 - [P_1])/(1 + K_m/[E]_0) \quad (10)$$

which is the same as eq. 7. However, here the condition ($[E]_0 >> [S]_0$) for obtaining pseudo first-order kinetics is more stringent than in case I ($[E]_0 >> [ES]$), since the enzyme is not regenerated as the reaction progresses.

Again, if $K_m >> [E]_0$, from eq. 10

$$k_{obs} = k_2'[E]_0/K_m \quad (11)$$

Case III: If k_2' is of the same order of magnitude as k_3' in eq. 5, and if $[E]_0$ is not *very much* greater than $[S]_0$, by the time the first-order decay is observed, [E] will be considerably greater than [S], for the enzyme is fed back into the system in the k_3' step, while the substrate is continuously consumed (compare case II). In so far as the first-order decay is concerned, the initial substrate concentration is ($[S]_0 - [ES] - [P_1]$) at the time at which the decay curve becomes truly exponential.

From the foregoing, given suitable values of K_m, $[E]_0$ and $[S]_0$, it follows that regardless of the ratio k_2'/k_3', it is possible (theoretically) to establish conditions under which the acylation reaction will be pseudo first-order. The rate constant obtained from the first-order part of the absorbance *vs.* time curve will measure, therefore, the smaller of the values, $k_2'/(1 + K_m/[E]_0)$ or k_3'. For the methyl cinnamate system, k_{obs} is always less than the independently measured deacylation constant, k_3'. Since it has been shown that k_3' is a rate-controlling constant in this hydrolysis it therefore follows that k_{obs} is related to the acylation reaction, *i.e.*, $k_{obs} = k_2'/(1 + K_m/[E]_0)$. However, for benzyl cinnamate, $k_{obs} = k_3'$ and, therefore, the pseudo-first-order acylation constant in this system must be greater than k_3'.

From eq. 7 and 10, if $[E]_0 >> K_m$

$$dP_1/dt = k_2'([S]_0 - [P_1]) \quad (12)$$

i.e., the rate should become independent of enzyme concentration at sufficiently high concentrations of enzyme, but remain first order in substrate. Under these conditions, one may say that the substrate is "saturated with enzyme."[29] It should be noted that the observed first-order constant, k_{obs}, becomes in this event equal to the acylation constant, k_2'. This, of course, would require that $k_3' > k_2'$ for successful observation. It is thus precluded for the methyl cinnamate system (where $k_2' > k_3'$), apart from any practical difficulties of the prohibitively high enzyme concentrations required.

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(29) *Cf.* O. H. Straus and A. Goldstein, *J. Gen. Physiol.*, **26**, 559 (1943); A. Goldstein, *ibid.*, **27**, 529 (1944).