the phototube and by reducing the aperture anterior to the analyzing polaroid. It is probable that in the dispersion curves obtained earlier with the Rudolph instrument the anomaly may also have been caused by stray light, partially polarized by reflection.

Experimental

p-(*p*-Dimethylaminobenzeneazo)-benzoylaminoacetic acid was synthesized according to Karush.[2]

Human serum albumin was obtained from Cutter Laboratories.

(2) F. Karush, *J. Phys. Chem.*, **56**, 70 (1952).

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[CONTRIBUTION NO. 1504 FROM THE STERLING CHEMISTRY LABORATORY, YALE UNIVERSITY]

The Mechanism of Chymotrypsin-catalyzed Reactions. III

BY TREVOR SPENCER AND JULIAN M. STURTEVANT

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Kinetic experiments, employing fast reaction techniques, on the chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate are reported. These experiments demonstrate that the acetylchymotrypsin isolated by Balls and Wood is a true intermediate in the reaction and give further indication that the position of acylation is at a serine hydroxyl rather than at a histidine imidazolyl group. The rate of the final hydrolysis of the acetyl enzyme is controlled by a group at the catalytic site having an apparent pK of 7.4. It is shown on the basis of experiments involving competition between *p*-nitrophenyl acetate and a specific substrate, acetyl-L-tyrosine ethyl ester, that hydrolysis of the latter probably involves the same three-step mechanism as the hydrolysis of *p*-nitrophenyl acetate. This conclusion emphasizes that the specificity of the enzyme can be even more important in later stages of the catalysis than it is in the initial formation of a Michaelis–Menten complex. A new stopped-flow fast reaction apparatus is briefly described.

Introduction

It has been shown in previous publications[1,2] that the kinetics of the chymotrypsin (CT)-catalyzed hydrolysis of *p*-nitrophenyl acetate (NPA) is consistent with a mechanism involving three distinct steps

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (ES')_t \qquad (1)$$

$$(ES') \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (ES'')_t + P' \qquad (2)$$

$$(ES'') \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + P'' \qquad (3)$$

In the first step there is a very rapid formation of a Michaelis–Menten complex, ES′, which in the second step is decomposed to give the product P′ (*p*-nitrophenol (NP) in the case of NPA) and an acylated derivative, ES″, of the enzyme. Finally, the acyl enzyme is hydrolyzed to regenerate the enzyme and form the product P″ (acetate in the case of NPA). The rates of the second and third steps are dependent on the state of ionization of a group in the catalytic site, only the unprotonated form being active. In equations 1 to 3, $(ES')_t$ and $(ES'')_t$ represent the sum of both the ionized and unionized forms of the intermediates, the distribution between these forms following, at least approximately, the equation for a single group ionization

$$K_i' = a_H \frac{[ES']}{[ES'H^+]} \qquad K_i'' = a_H \frac{[ES'']}{[ES''H^+]} \qquad (4)$$

where the quantities in square brackets represent the molar concentrations of the corresponding species and a_H is the hydrogen ion activity (in this paper operationally defined as the quantity measured by a glass electrode).

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

(2) H. Gutfreund and J. M. Sturtevant, *Proc. Nat. Acad. Sci.*, **42**, 719 (1956).

The differential equations of this kinetic scheme are readily solved by the method of Laplace transforms, if attention is confined to the early stages of the reaction so that the substrate concentration can be assumed to remain essentially constant at its initial value, $[S]_0$, and the reversal of the second and third steps can be neglected. It can be shown that the concentration of the product P′ is given by the expression

$$[P'] = k_1k_2'[E]_0[S]_0\left[\frac{nm - k_3'(n+m)}{n^2m^2} + \frac{k_3'}{nm}t + \frac{k_3' - m}{m^2(n-m)}e^{-mt} - \frac{k_3' - n}{n^2(n-m)}e^{-nt}\right] \qquad (5)$$

where t is the time, $k_2' = k_2K_i'/(K_i' + a_H)$ and $k_3' = k_3K_i''/(K_i'' + a_H)$ are apparent first-order rate constants, and m, n are given by

$$2m = k_1A' + k_3' - \sqrt{(k_1A' + k_3')^2 - 4k_1(k_2'[S]_0 + k_3'A')} \qquad (6)$$

$$m + n = k_1A' + k_3' \qquad (7)$$

$$A' = [S]_0 + K_m' \qquad (8)$$

$$K_m' = \frac{k_{-1} + k_2'}{k_1} \qquad (9)$$

The second exponential in equation 5 reflects the build up of the steady-state concentration of the Michaelis–Menten complex ES′, while the first exponential describes the formation of the steady-state concentration of the acyl enzyme, ES″. In the present case, available techniques are inadequate to detect the phase of the reaction involving the second exponential so that we may conclude that $n >> m$. This implies that $k_1A' >> k_2'$ and $k_1A' >> k_3'$. It then follows with adequate accuracy that

$$m = \frac{k_2'[S]_0 + k_3'A'}{A'}; \quad n = k_1A' \qquad (10)$$

Equation 5 becomes, after decay of the second exponential

$$[P'] = \frac{k_2'[E]_0[S]_0}{k_2'[S]_0 + k_3'A'}\left[k_3't + \frac{k_2'[S]_0}{k_2'[S]_0 + k_3'A'}\left(1 - e^{-\frac{k_3'[S]_0 + k_3'A'}{A'}t}\right)\right] \quad (11)$$

Measurements made during the period when the exponential term in equation 11 is comparable in magnitude to unity may be employed in favorable cases to evaluate the quantity $k_2'[S]_0/A' + k_3'$. Measurements made by classical methods usually involve values of t large enough so that the exponential term is negligible compared to unity. The rate of formation of P' is then given by

$$r = \frac{d[P']}{dt} = \frac{k_2'k_3'[E]_0[S]_0}{k_2'[S]_0 + k_3'A'} \quad (12)$$

It is important to note that application of Eadie[3] plots to data giving r as a function of $[S]_0$ can then only lead to the evaluation of the *apparent* kinetic constants

$$K_m'_{(app)} = \frac{k_3'}{k_2' + k_3'}K_m' \quad (13)$$

and

$$\frac{1}{k_3'_{(app)}} = \frac{1}{k_2'} + \frac{1}{k_3'} \quad (14)$$

The approximations involved in arriving at these equations are certainly valid in connection with experiments where *initial* rates are determined with substrate concentrations much larger than enzyme concentrations. In experiments such as those illustrated in Fig. 3, in which the substrate concentrations were only two to five times the enzyme concentrations, the adequacy of equation 11 is indicated by the fact that the expected linear variation of [P'] with time was observed for a considerable period after the decay of the exponential term was complete.

The present communication is concerned primarily with three questions relating to this mechanism: (1) is the acetylchymotrypsin (ACT) isolated by Balls and Wood[4] a true intermediate in the reaction; (2) at which position is the enzyme acylated; and (3) does the same three-step mechanism apply to other substrates of CT?

Materials

Buffer.—In most of the experiments where buffering was necessary, appropriate mixtures of acetic acid and tris-(hydroxymethyl)-aminomethane (THAM) of 0.02–0.04 *M* ionic strength were employed. All solutions were also 0.10 *M* in NaCl. *p*H values were determined with reference to Beckman *p*H 7.0 standard buffer by means of a glass electrode and Beckman Model G *p*H-meter.

Enzyme.—Salt-free crystalline α-chymotrypsin was purchased from Worthington Biochemical Sales, Freehold, N. J. Nitrogen determinations by the micro-Kjeldahl method indicated 14.2% N. Where molar enzyme concentrations are given, they were computed on the assumption that CT was the only nitrogenous material present, taking the enzyme to contain 16.2% N and to have a molecular weight[5] of 23,000.

Acetylchymotrypsin.—ACT was prepared according to the method of Balls and Wood.[4] The product was assayed before and after being held at *p*H 9, using acetyl-L-tyrosine ethyl ester (ATEE) as substrate at *p*H 6 and 25°. A typical preparation was found to contain 9% active CT, and 96.5% of the original activity was recovered after treatment at *p*H 9 for 30 minutes. Some experiments were also performed with a sample of ACT kindly supplied by Dr. George Hess of Cornell University.

Substrates.—ATEE was purchased from Mann Research Laboratories, New York, N. Y., and was used without further purification; m.p. 79.1° cor., lit.[6] 79–80°. NPA was prepared by the acetylation[7a] of NP by acetic anhydride and was purified by recrystallization from aqueous ethanol; m.p. 77.9° cor., lit.[7b] 77.5–78°.

Kinetic Methods

Titration Method.—Hydrolyses of ATEE were followed by the titration method of Schwert, *et al.*,[8] using 1 *M* NaOH delivered from a 0.1-ml. microburet with manual control.

Stopped-flow Apparatus.—Most of the kinetic observations were made with a modification of the spectrophotometric stopped-flow apparatus described by Gibson.[9] A schematic diagram of the apparatus is shown in Fig. 1.

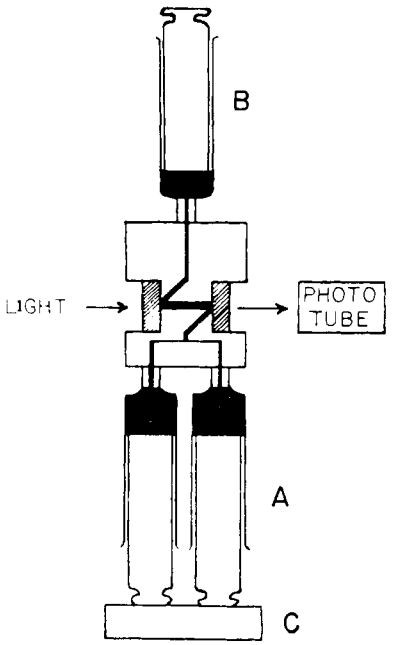

Fig. 1.—Schematic diagram of stopped-flow apparatus. See text for details.

The solutions which are to be mixed are contained in two 2-ml. Luer-Lok glass syringes, A. The syringes are connected by 1 mm. capillary channels in a Lucite block to a "mixing chamber" which in this design is a simple T-joint. A 1 cm. length of 1 mm. channel connects the mixing chamber to the glass-lined observation chamber, which is of 2 mm. diameter with quartz end plates 1.00 cm. apart. From the observation chamber the fluid passes to the stopping syringe, B. The apparatus is kept full of liquid at all times. At the start of a reaction, the two solution syringes are pushed by a common pushing bar, C, to expel about 1/4 ml. from each syringe. This amount of liquid is adequate to flush the old liquid from the observation chamber. Time is counted from the instant the flow of liquid is stopped by the stopping syringe hitting an adjustable stop. Side-arms (not shown) together with appropriate 3-way stopcocks are provided for removing air bubbles trapped when the syringes are screwed onto the plastic block and for expressing old fluid from the stopping syringe. Copper blocks through which water from a thermostat is circulated enclose the plastic block and the solution syringes. Provision is included for inserting a small low-lag thermocouple to a

(3) G. S. Eadie, *J. Biol. Chem.*, **146**, 85 (1942).

(4) A. K. Balls and H. N. Wood, *ibid.*, **219**, 245 (1956).

(5) M. S. N. Rao and G. Kegeles, THIS JOURNAL, **80**, 5724 (1958).

(6) S. Kaufman, H. Neurath and G. W. Schwert, *J. Biol. Chem.*, **177**, 793 (1949).

(7) (a) F. D. Chattaway, *J. Chem. Soc.*, 2495 (1931); (b) M. Bender and B. W. Turnquest, THIS JOURNAL, **79**, 1652 (1957).

(8) G. W. Schwert, H. Neurath, S. Kaufman and J. E. Snoke, *J. Biol. Chem.*, **172**, 221 (1948).

(9) Q. H. Gibson, *J. Physiol.*, **117**, 49P (1952). See also B. Chance, in "Investigations of Rates and Mechanisms of Reactions," edited by S. L. Friess and A. Weissberger, Interscience Publishing Co., New York, N. Y., 1953, p. 690.

position just beyond the observation chamber for checking the adequacy of temperature control.

The rate of flow of fluid during the initiation of the reaction averages 5 ml. sec.$^{-1}$. Thus the average age of the mixed solution which comes to rest in the observation chamber is approximately 0.005 sec. The efficiency of mixing in the present design is not particularly good, so that reactions having half-times below about 0.02 sec. cannot be properly observed. Thus the flow rate is high enough to avoid significant timing errors.

A Unicam[10] SP.500 spectrophotometer is employed as a monochromator, the light source being either a hydrogen lamp and associated power supply[10] or a tungsten lamp and regulated power supply.[11]

The stopped-flow apparatus fits in place of the usual cell holder of the SP.500. The photocell compartment of the SP.500 is replaced by a similar compartment containing a 1P28 photomultiplier and associated voltage divider network for supplying appropriate voltages to the dynodes. The high voltage for the photomultiplier is delivered by a very stable electronic power supply.[12] The load resistor of the 1P28 is the 1 megohm input resistor of a stable linear D-C amplifier[13]; a variable low-pass resistor-capacitor filter is inserted between the phototube and the amplifier so that noise voltages can be reduced when high speeds of response are not necessary. The amplifier drives a direct-inking oscillograph,[14] the combination having a flat response up to 100 cycles per sec., which is adequate for recording any processes for which the present speed of mixing is sufficient. A very useful feature of the amplifier is that the zero can be offset 5 or more chart widths by a very stable and accurately reproducible adjustment, so that high amplifier gain can be employed to give a full scale deflection with as little as 5% change in transmission. The oscillograph is supplied with 6 readily interchanged chart speeds ranging from 2.5 mm. per min. to 25 mm. per sec.

Over-all sensitivity is adequate for observations down to 220 mμ. The stability of the light sources has been found to be excellent, so that experiments extending over many minutes can be performed even though there is no opportunity to check light intensity during an experiment. In fact, the chief source of instrumental instability appears to be phototube fatigue.

The detection and recording system is linear in per cent. transmission, so that conversion to optical density is always necessary. In usual practice, the system is adjusted, by means of the monochromator slit width, to give full scale deflection with water in the observation tube and the amplifier set at a low gain. The amplifier gain and zero offset are then adjusted as necessary to give a satisfactory record of the reaction under study. Optical densities are thus obtained by direct calculation, calibrating solutions of known optical density being unnecessary.

A very convenient feature of the apparatus is that no more than 5 min. are needed to replace the stopped-flow components by the usual SP.500 cell holder, either with the normal photocell detector and circuitry of the SP.500 (which is similar to that of the Beckman DU spectrophotometer) or with the 1P28 detector and associated recording equipment described above.

The Value of pK_i'' for the Hydrolysis of NPA.—In an earlier publication,[1] the variation of the over-all rate of hydrolysis of NPA by CT was investigated by ordinary spectrophotometry over the range of pH 6.45 to 7.75 at 27°. In these measurements the solvent contained 20% (v./v.) isopropyl alcohol and 0.05 M phosphate, and the initial substrate concentration was 2.5×10^{-3} M. These results, when interpreted in terms of a single group ionization, indicated a value of 7.3 for pK_i'' (*cf.* equation 4). However, since interference from non-enzymic hydroxide ion catalyzed hydrolysis prevented observations at pH values above 7.75, the distinction between the pH dependence to be expected from equation 4 and that corresponding to a direct hydroxide ion catalysis of the last step of the enzymic process was not as definite as desirable.

Dixon and Neurath[15] measured the rate of recovery of activity toward ATEE by ACT as a function of pH and found the variation with pH to be in accord with a single group ionization with pK = 7.0. If ACT is identical with ES'' in equation 3, the pK for this process should be expected to be equal to pK_i'' for the NPA hydrolysis.

In view of this discrepancy and the restricted pH range of the earlier data, it seemed important to redetermine pK_i''. The stopped-flow apparatus makes it possible to determine rates with relatively large enzyme concentrations and thus to minimize the importance of the contribution to the rate resulting from non-enzymic catalysis. Experiments were performed at 25° in the pH range 7.0 to 8.8 with the results summarized in Fig. 2. In each experiment, a solution containing CT, NPA and 0.008 M acetic acid was mixed in the stopped-flow machine with an equal volume of THAM–acetate buffer, and the reactions were followed at 400 mμ. The initial "burst" of NP liberation was essentially complete in the low pH solution before mixing. The observed rates, in optical density units per sec., were converted to mole l.$^{-1}$ sec.$^{-1}$ using the measured absorption of NP as a function of pH. The substrate concentration used in these experiments was sufficient nearly to saturate the enzyme, so that it is very unlikely that the results were significantly affected by variation of $K_m'_{(app)}$ within the pH range covered.

The observed rates include the contribution due to hydroxide ion catalysis. As illustrated in Fig. 2, it was found, by successive approximations, that a plot of r_0 *vs.* $r_0 a_H$, where r_0 is the corrected initial enzymic rate, adhered reasonably well to a straight line if the hydroxide ion catalysis was assumed to be governed by the equation

$$r_{OH} = k' \frac{[S]_0}{a_H} \qquad (15)$$

with $k' = 43 \times 10^{-14}$ mole l.$^{-1}$ sec.$^{-1}$. The slope of the line gave a value of 7.41 for pK_i'', in fair agreement with the earlier value obtained in a different solvent.

A series of measurements of the initial rate of the hydroxide ion catalyzed reaction was performed with the results listed in Table I. The mean value of k' is identical with that which had to be assumed to have the enzymic rates fall as well as possible on a single group ionization curve. Holleck and Melkonian[16] studied the hydroxide ion catalyzed reaction in 0.1 M borate buffers by a polarographic method, obtaining a rate constant over twice as large as that reported here. This discrepancy is due at least in part to the fact that they employed 10% ethanol as solvent.

From the enzymic rate extrapolated to zero hydrogen ion activity and the enzyme concentration, we obtain a first-order rate constant of 0.021 sec.$^{-1}$,

(10) Unicam Instruments Ltd., Cambridge, England. The stopped-flow apparatus has been briefly described and illustrated by photographs in Spectrovision, No. 5, p. 5 (1957), published by Unicam.

(11) Nobatron MA6501, Sorensen and Co., Inc., Stamford, Conn.

(12) Model HV-3A, Technical Measurements Corp., New Haven, Conn.

(13) Model BL-550, Brush Electronics Co., Cleveland, Ohio.

(14) Model BL-201, Brush Electronics Co., Cleveland, Ohio.

(15) G. H. Dixon and H. Neurath, *J. Biol. Chem.*, **225**, 1049 (1957).

(16) L. Holleck and G. A. Melkonian, *Z. Elektrochem.*, **58**, 867 (1954).

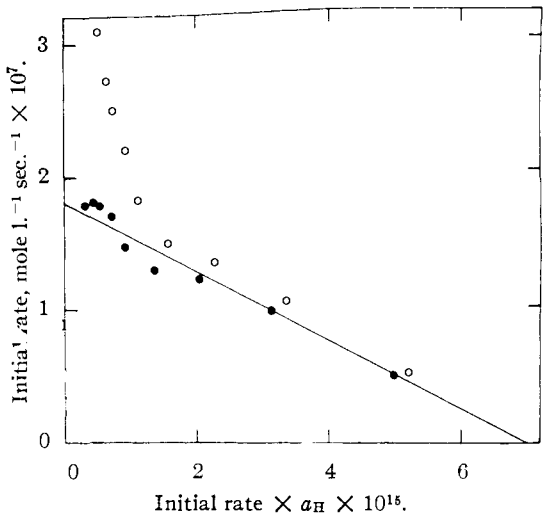

Fig. 2.—The initial rate of the steady-state CT-catalyzed hydrolysis of NPA at 25° in THAM–acetate buffers plotted against the product of the initial rate times the hydrogen ion activity. Total ionic strength 0.12 *M*; $[S]_0 = 5.0 \times 10^{-4}$ *M*; $[E]_0 = 8.8 \times 10^{-6}$ *M*; 2.5% (v./v.) ethanol. Open circles, rates including OH^- ion catalysis; filled circles, rates corrected for OH^- ion catalysis.

in satisfactory agreement with the previously published[1] estimate.

In the experiments of Dixon and Neurath,[15] ATEE was presumably specifically bound during the hydrolysis of the ACT. It is possible that this might be the cause of the lower *pK* observed by these authors.

TABLE I

THE HYDROXIDE ION CATALYZED HYDROLYSIS OF NPA AT 25°

*p*H	Initial rate, mole l.$^{-1}$ sec.$^{-1}$ × 10^7	*k'*, mole l.$^{-1}$ sec.$^{-1}$ × 10^{14}
8.21	0.41	52
8.52	.68	42
8.63	.79	42
8.78	1.13	38
	Mean	43

Solution composition as in Fig. 2 with enzyme omitted.

Experiments with Acetylchymotrypsin

Acetylchymotrypsin as an Intermediate in NPA Hydrolysis.—It has seemed reasonable to assume that the ACT isolated by Balls and Wood[4] is indeed an intermediate in the hydrolysis of NPA. Dixon and Neurath[15] found that ACT is converted to a form of the enzyme active in catalyzing the hydrolysis of ATEE, the conversion following first-order kinetics with a rate constant of 8.8×10^{-3} sec.$^{-1}$ at *p*H 7 and 25°. Using their value for the *pK*, 7.0, of the group controlling the rate of this process, the limiting value of the rate constant at high *p*H is calculated to be 1.8×10^{-2} sec.$^{-1}$, which is fairly close to the limiting rate constant for the hydrolysis of NPA.

The experiments illustrated in Fig. 3 give direct indication that ACT is an intermediate, at least so far as the lyophilized ACT redissolved at low *p*H is concerned. In the experiment represented by curve A, CT at *p*H 4.8 was mixed in the stopped-flow apparatus with a solution of NPA in a buffer such that the final *p*H was 8.2. Rapid acylation of the enzyme with liberation of somewhat less than a mole of NP per mole of CT was followed by a zero-order steady-state liberation of NP and acetate. Curve B corresponds to a similar experiment performed with ACT. A small "burst" of NP liberation was observed because the enzyme was incompletely acylated in the sample[17] of ACT used; as soon as this was completed to give the steady-state concentration of ACT, the reaction proceeded with essentially the same zero-order rate as observed with the free enzyme. Thus, within a time interval no larger than 10 to 20 millisec., the ACT was behaving precisely as expected for the true intermediate contaminated by some unchanged enzyme.

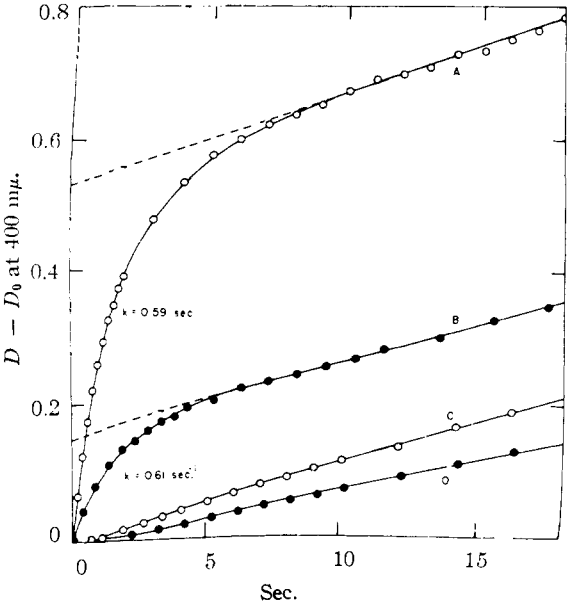

Fig. 3.—Optical density changes at 400 mμ accompanying the hydrolysis of NPA by CT and ACT at 25° in THAM–acetate buffers. Total ionic strength 0.12 *M*; $[E]_0 = 4.4 \times 10^{-5}$ *M*. Curve A, CT, and curve B, ACT, mixed with NPA at *p*H 8.2, $[S]_0 = 2.0 \times 10^{-4}$ *M*, 1.0% (v./v.) ethanol; the rate constants refer to the approach to the steady state with respect to ACT. Curve C, CT plus NPA at *p*H 4.8 mixed with buffer to give *p*H 8.2, $[S]_0 = 2.5 \times 10^{-4}$ *M*, 2.5% (v./v.) ethanol. Curve D, ACT plus NPA at *p*H 4.8 mixed with buffer to give *p*H 8.2, $[S]_0 = 1.0 \times 10^{-4}$ *M*, 0.5% (v./v.) ethanol.

Curves C and D were obtained in similar experiments in which CT (curve C) or ACT (curve D) and substrate were present in the same solution at *p*H 4.8 which was then mixed in the stopped-flow machine with an equal volume of buffer to give a final *p*H of 8.2. Under these circumstances, the acylation took place before the *p*H was raised, so that no rapid liberation of NP was observed. In each case the initial rate of NP liberation was actually a little less than the steady-state value, presumably because the steady-state degree of

(17) The incomplete acylation was probably in part due to the fact that the sample was two months old. It has been observed (H. Neurath, private communication) that lyophilized ACT undergoes slow hydrolysis during storage.

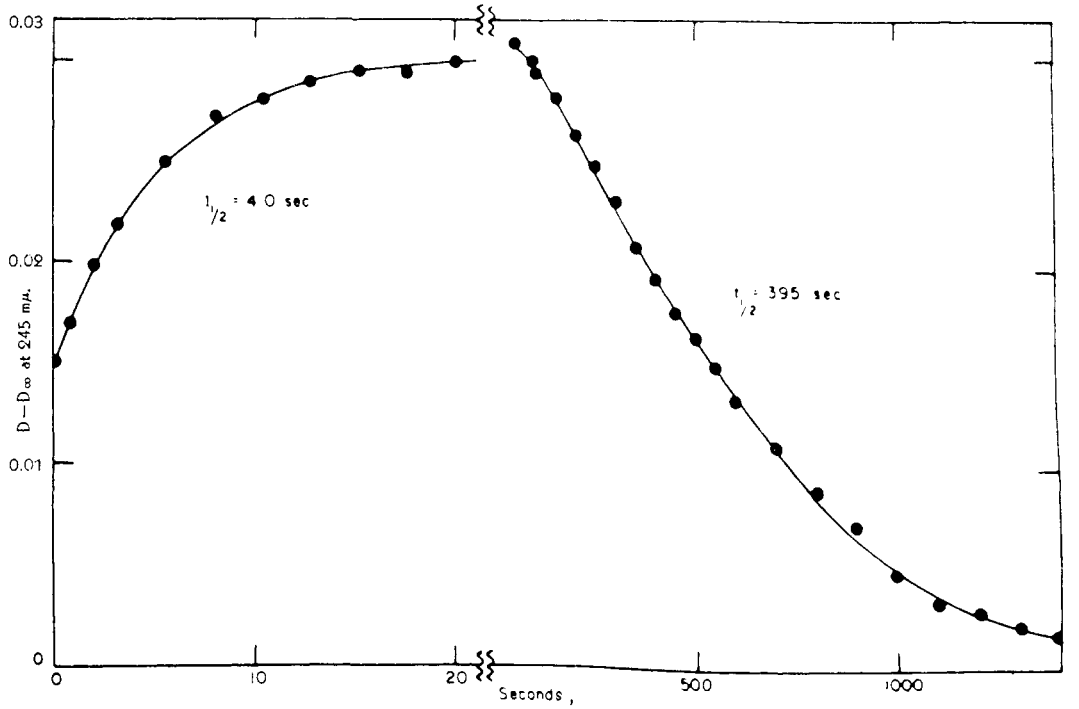

Fig. 4.—Optical density changes at 245 mμ following raising the *p*H of an ACT solution from 4.8 to 8.2 at 25°, THAM–acetate buffer, total ionic strength 0.14 *M*, 4.4 × 10^{-5} *M* ACT.

acylation is somewhat larger at low than at high *p*H.

The Position of Acylation of the Enzyme.—It is now generally agreed that both the imidazolyl group of a histidine residue and the hydroxyl group of a serine residue are involved in the active site of CT. There has, however, been considerable disagreement as to which of these groups is acylated in the most stable intermediate ES'' formed during the hydrolysis of NPA. In a recent publication, Dixon and Neurath[18] reported that a slow decrease in absorption at 245 mμ occurs when the *p*H of a solution of ACT is raised from 4 to 8 or 9. Since it is known that acetylimidazole absorbs at this wave length, they postulated that the observed decrease in absorption is due to the hydrolytic removal of the acetyl group from the imidazolyl group at the active site of the enzyme and that the decrease in absorption is preceded by a very rapid increase as the acetyl group shifts from a serine residue to the histidine residue. Their proposed mechanism would thus place the acyl group on imidazolyl for most of the time during which it is bound to the enzyme in the actual hydrolytic process at high *p*H.

There are several reasons for believing that in fact the acetyl group is bound most of the time at a serine residue during hydrolysis of NPA. The experiments summarized in Fig. 3 show that if there is an acyl shift from oxygen to nitrogen as postulated by Dixon and Neurath, it must be extremely rapid. Furthermore, it has been shown,[2] by experiments in NP buffer, that when CT is acetylated by NPA at a *p*H of 6.6, hydrogen ions are taken up by the enzyme from the buffer. At this *p*H, the imidazolyl group at the active site should be partially protonated, and since the *pK* of acetylimidazolyl should be much lower than that of imidazolyl, we would expect protons to be *liberated* by the enzyme if the imidazolyl group became appreciably acetylated. Although there is some doubt as to the actual value of pK_i', there is no doubt that it is considerably smaller than pK_i''. This fact is not only consistent with the observed uptake of protons during acetylation but is in itself clear indication that during the final rate-controlling hydrolysis the imidazolyl group is free and in equilibrium with respect to proton binding.

There remains the problem of accounting for the spectral changes at 245 mμ observed by Dixon and Neurath.[18] We have investigated the behavior of ACT in the stopped-flow apparatus with results similar to those of Dixon and Neurath, as shown in Fig. 4. Immediately after raising the *p*H from 4.8 to 8.2, there is a rapid increase in absorption, followed by a slow decrease which proceeds according to approximately first-order kinetics with a rate constant of 1.7 × 10^{-3} $sec.^{-1}$. The decrease in absorption was approximately $^3/_4$ as large as observed by Dixon and Neurath.

It is immediately evident that the process shown in Fig. 4, which took place at 25°, cannot be directly associated with the rate-controlling step in the hydrolysis of NPA since it proceeded at a rate approximately *one fifteenth* as large as the rate of the final step in the hydrolysis of NPA. It is interesting that the half-time of the decay of absorption at 245 mμ at 25° shown in Fig. 4 is 395 sec., as compared with the value of 415 sec. found by Dixon and Neurath at 10°. In several experiments at 15° we have also observed half-times of about 400 sec. It thus appears that this reaction,

(18) G. H. Dixon and H. Neurath, THIS JOURNAL, **79**, 4558 (1957).

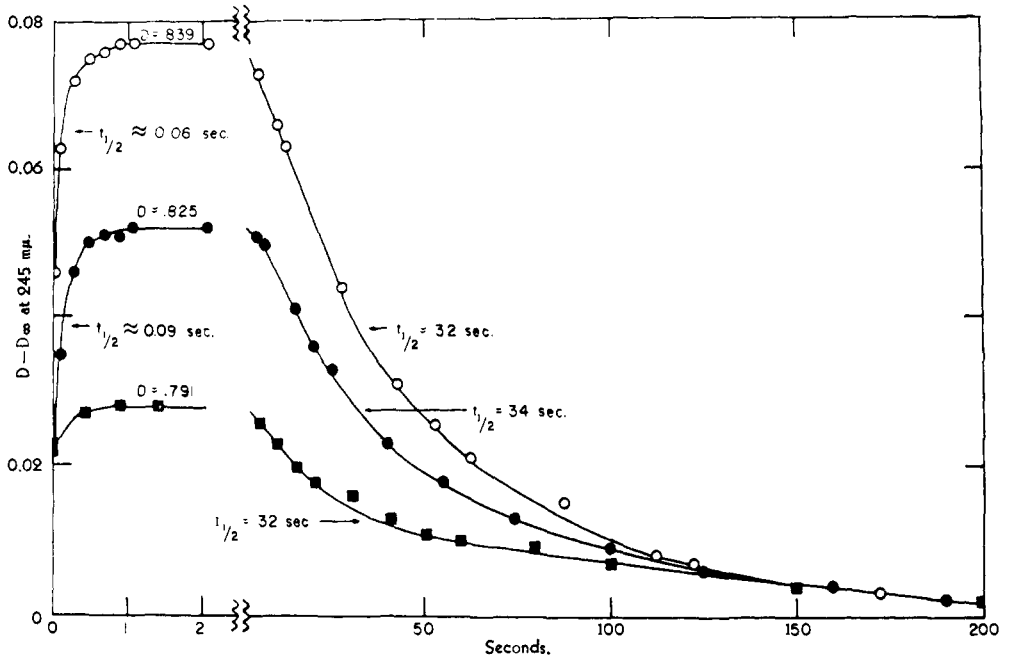

Fig. 5.—Optical density changes at 245 mμ following raising the *p*H of a CT solution from 4.8 to 8.2 at 25°. THAM–HCl buffer, total ionic strength 0.13 *M*, 4.5 × 10^{-6} *M* CT. Age of CT solution at start of experiment: open circles, 0.3 hr.; filled circles, 6.1 hr.; filled squares, 26.6 hr. The maximum optical density reached is indicated for each solution.

whatever it may be, has a rate essentially independent of temperature. Quite coincidentally, the rate of the conversion of ACT to active CT was found by Dixon and Neurath[13] at 10° and *p*H 7 to have a rate almost identical with the rate of decay of absorption at 245 mμ at 10° and *p*H 9.

The significance of the changes in absorption at 245 mμ, so far as the hydrolysis of NPA is concerned, is further made doubtful by the experiments shown in Fig. 5. A solution of *chymotrypsin* at *p*H 4.8 was raised after various time intervals to *p*H 8.2 in the stopped-flow apparatus, using a THAM–HCl buffer containing no acetate. Again, rapid increases in absorption at 245 mμ followed by slow decreases were observed, though the rates of both processes were considerably faster than in the case of ACT. The magnitudes of the changes decreased markedly with increasing age of the low *p*H solution. (It is not known whether a similar effect of age is observable with ACT.)

A solution of CT which had been aged overnight at *p*H 4 and which was shown to give a small change in absorption at 245 mμ when its *p*H was raised to 8.2 was held at *p*H 8.2 for 30 min. and its *p*H was then again dropped to 4.5. When the *p*H of this solution was returned to 8.2 in the stopped-flow apparatus, no significant changes in absorption at 245 mμ were detected. This experiment indicates that the process observed at 245 mμ is not reversed by lowering the *p*H.

It appears that some change in the enzyme is caused by the process of lyophilization; this change is reversed when the enzyme is redissolved, the rate of reversal increasing with *p*H. The present experiments give no basis for an understanding of the changes involved or for the large differences in rates observed with CT and ACT.

Inhibition Experiments

There is at present no direct experimental evidence that the kinetic scheme embodied in equations 1–3 is applicable to the CT-catalyzed hydrolysis of specific substrates such as ATEE. Even if acylated enzyme were formed in considerable amount, the reaction rates in this case are so large as to preclude the possibility of detecting the acylation reaction. If the method of "initial acceleration" devised by Gutfreund[19] is extended to the 3-step mechanism, it can be shown that the intercept τ of the steady-state hydrolysis curve with the time axis must be less than $1/k_3$, under circumstances such that the approximations employed in the treatment of equation 6 are valid. In the case of ATEE, k_3 = 160 sec.$^{-1}$ (see below), so that $\tau < 0.006$ sec. With a substrate such as acetyl-L-tyrosinamide, which is hydrolyzed approximately 1000 times slower than ATEE, rate-limitation undoubtedly occurs at the acylation step, so that the steady-state concentration of acylated enzyme is extremely small.

If the hydrolyses of NPA and ATEE utilize the same active site on the enzyme, it should be possible to observe mutual inhibitory effects of these two substrates. We have accordingly investigated[20] the influence of NPA on the hydrolysis of ATEE and of ATEE on the hydrolysis of NPA.

Inhibition of ATEE Hydrolysis by NPA.—The rate of hydrolysis of ATEE by CT at *p*H 7.0 and 25° was determined in the presence of various

(19) H. Gutfreund, *Disc. Faraday Soc.*, **20**, 167 (1955).

(20) The inhibition experiments reported here were undertaken as a result of a conversation with Professor Carl Niemann of the California Institute of Technology. We acknowledge our indebtedness to Professor Niemann for directing our attention to the problem of inhibition of NPA hydrolysis.

concentrations of NPA. The solutions contained 0.005 M THAM and 0.10 M NaCl. Two series of measurements were made, one at an initial substrate concentration of 7.15×10^{-4} M and one at 180×10^{-4} M. Sufficiently low enzyme concentrations were employed so that reliable initial rates could be obtained in the manually operated pH-state.[8] Hydrolysis of the inhibitor, including the amount necessary to acetylate the enzyme, was negligible during the interval required to evaluate the initial rate of hydrolysis. The data are summarized in the form of rate *vs.* rate × [NPA] plots in Fig. 6, where the rates are expressed in arbitrary units.

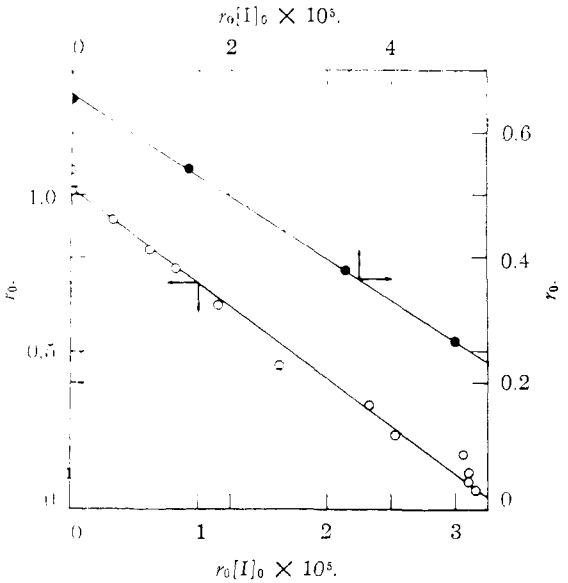

Fig. 6.—Inhibition by NPA of the CT-catalyzed hydrolysis of ATEE. Initial rates (arbitrary units) at pH 7.0, 25°, plotted against the product of initial rate (same arbitrary units) times molarity of NPA. Total ionic strength 0.10 M; 10% (v./v.) ethanol. Open circles, 1.80×10^{-2} M ATEE; closed circles, 7.15×10^{-4} M ATEE. Different enzyme concentrations were used in the two sets of experiments.

It can be shown that for a case of competitive[21] inhibition, the initial rate for a system following simple Michaelis–Menten kinetics should be given by the expression

$$r_0 = r_{max} \frac{[S]_0}{K_m + [S]_0} - \frac{K_m}{K_m + [S]_0} \frac{1}{K_I} r_0[I] \quad (16)$$

where r_{max} is the initial rate in the absence of inhibitor and under conditions of enzyme saturation, K_I is the inhibitor constant, and [I] is the inhibitor concentration (strictly the concentration of free inhibitor, which is practically the same as the total concentration in the present experiments). For non-competitive[21] inhibition, the corresponding equation is

$$r_0 = r_{max} \frac{[S]_0}{K_m + [S]_0} - \frac{1}{K_I} r_0 [I] \quad (17)$$

In either case, a plot of r_0 *vs.* r_0[I] should be a straight line; the slope should be a function of $[S]_0$ in the competitive case but not in the non-competitive case. If a competitive inhibitor is itself a substrate, the inhibitor constant is identical with the Michaelis–Menten constant.[22]

It is evident that the two lines in Fig. 6 have markedly different slopes, so that the inhibition cannot be purely non-competitive. If we assume competitive inhibition, we calculate from the slopes that $K_m = 5.1 \times 10^{-3}$ M and $K_I = 2.7 \times 10^{-5}$ M. Since this value for K_m is considerably different from those previously reported,[23,24] we have redetermined this quantity under the conditions of the inhibition experiments. The results are shown in Fig. 7 in the form of an Eadie[3] plot.

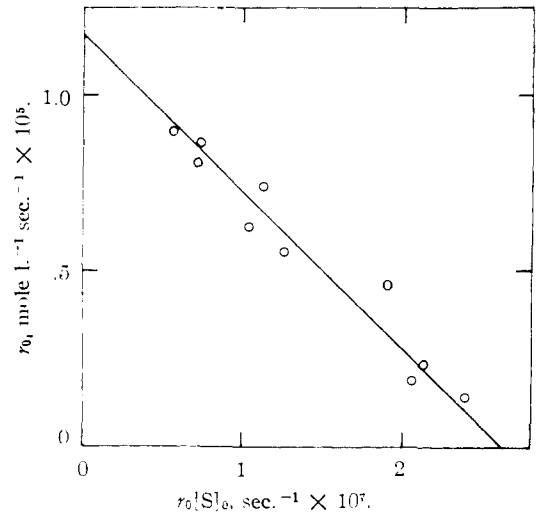

Fig. 7.—The CT-catalyzed hydrolysis of ATEE. Initial rates at pH 7.0, 25°, as a function of substrate concentration, plotted in the form of an Eadie[3] plot. Total ionic strength 0.10 M; 10% (v./v.) ethanol; $[E]_0 = 1.25 \times 10^{-7}$ M.

The slope of this plot gives $K_m = 5.0 \times 10^{-3}$ M, in good agreement with the value obtained from the inhibition experiments. This value is intermediate between the values given by earlier workers,[23,24] the differences probably being attributable to differences in the media used. The agreement between the K_m evaluated from the inhibition experiments and that determined directly substantiates the conclusion that the inhibition is competitive in kinetic form.

From the extrapolated rate in Fig. 7, we find that $k_3'_{(app)} = 94$ sec.$^{-1}$ at pH 7.0. If we assume the same pH dependence as observed by Dixon and Neurath[15] in the case of δ-chymotrypsin, we obtain $k_{3(app)} = 160$ sec.$^{-1}$. Previously reported values are 190 sec.$^{-1}$ [23] and 162 sec.$^{-1}$.[24]

In conformity with the notation used earlier in this paper, the inhibition constant determined here should properly be represented by $k_{II(app)}$, since it describes the effective inhibition by NPA *after* the steady-state concentration of ACT has been established. It has a very small value (*i.e.*, NPA is a very effective inhibitor), and it thus appears that the strong inhibition produced by NPA actually results from its successful competition with ATEE

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

(22) L. L. Ingraham, THIS JOURNAL, **79**, 666 (1957).

(23) G. W. Schwert and S. Kaufman, *J. Biol. Chem.*, **180**, 517 (1949).

(24) L. W. Cunningham, Jr., *ibid.*, **207**, 443 (1954).

for the acylation site of the enzyme. It is most unlikely that the presence of so small a group as the acetyl group on the acylation site could interfere in any way with the hydrolysis of ATEE unless the same acylation site is required by both substrates.

The true K_m for NPA is rather large, having a value of approximately 5×10^{-3} M. It should therefore be difficult to observe any inhibition of the hydrolysis of ATEE resulting from competition for the initial binding site of the enzyme. Experiments performed in the stopped-flow machine, using brom thymol blue to detect the acid liberated by the hydrolysis of ATEE, confirmed this prediction.

The Inhibition of NPA Hydrolysis by ATEE.—Since ATEE is much more rapidly hydrolyzed by CT than is NPA, it is impossible to observe the effect of ATEE on the steady-state hydrolysis of NPA, that is, on the last step of the process. It is, however, possible in the stopped-flow apparatus to investigate the effect of ATEE on the rate of the acylation by NPA. Experiments were performed at ATEE concentrations up to 1.5×10^{-2} M in the presence of 8.8×10^{-6} M CT and 2.5×10^{-4} M NPA, at pH 7.8 and 25°. At the lowest inhibitor concentration employed, 2.5×10^{-3} M, hydrolysis of 20% of the inhibitor would be expected to take place in the first second, if we neglect the inhibitory effect of NPA. By using a high amplifier gain and a chart speed of 25 mm. sec.$^{-1}$, it was possible to obtain a reliable value for the initial rate of liberation of NPA during the first few tenths of a second after initiation of the reaction. The results of these experiments are shown in Fig. 8. The value of K_I obtained from the slope of the line is 3.7×10^{-3} M on the assumption of competitive inhibition. If the inhibition is non-competitive this figure is raised to 3.9×10^{-3} M. The value of K_I in either case is close to the value of $K_{m(app)}$ for ATEE. Since the true K_m cannot be smaller than $K_{m(app)}$, as shown by equation 13, it appears probable that ATEE interferes with the interaction of NPA with the enzyme by competing for the initial adsorption site. If the ATEE interfered by competing for the acylation site, the steady-state concentration of acetyltyrosyl enzyme would have to be large, and this would in turn imply that $k_2 > k_3$ for ATEE and that K_m for ATEE is considerably larger than 5×10^{-3} M. It seems unlikely that the true binding of a good substrate would be much weaker than the apparent binding of ATEE. If this argument is accepted, it would follow that k_3 is at least somewhat larger than k_2 for ATEE.

In any case, it can be stated that the inhibitory action of ATEE is exerted at either the adsorption site or the acylation site of the enzyme, or perhaps at both sites. This result and that of the preceding section give definite support to the assumption that the hydrolysis of ATEE also proceeds by the 3-step mechanism.

Discussion

The partial mechanism for chymotrypsin catalyses presented schematically in Fig. 9 is in accord with the salient facts at present available. This

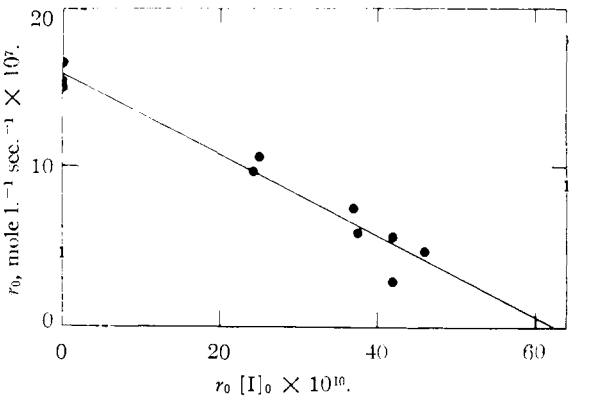

Fig. 8.—Inhibition by ATEE of the NPA acetylation of CT. Initial rates at pH 7 8, 25°, plotted against the product of initial rate times molarity of ATEE. Total ionic strength 0.14 M; 12.5% (v./v.) ethanol; $[E]_0 = 8.8 \times 10^{-6}$ M.

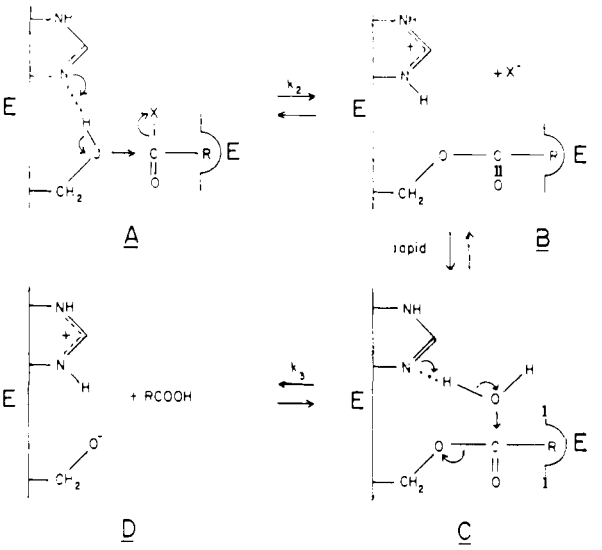

Fig. 9.—Proposed partial mechanism for CT-catalyzed reactions.

mechanism is similar to that proposed by Cunningham[25] in that the position of acylation is at a serine hydroxyl group and that the serine hydroxyl is hydrogen-bonded to imidazolyl. The Michaelis–Menten complex, A, is converted to the acylated enzyme, B and C, by a step involving acceptance of a proton by imidazolyl and nucleophilic attack on carbonyl by the oxygen of the serine hydroxyl. In the final hydrolytic step, imidazolyl again accepts a proton, this time from a water molecule the oxygen of which is involved in nucleophilic attack of the carbonyl.

The hydrogen bond in A is considered to be the cause of the change of the apparent pK of the imidazolyl group resulting from acetylation by NPA. When this hydrogen bond is broken by acetylation, the imidazolyl becomes available for (weaker) hydrogen bonding with a water molecule.

As is to be expected, there are difficulties inherent in this mechanism, which actually are encountered in one form or another by the other

(25) L. W. Cunningham, *Science*, **125**, 1145 (1957). See also F. H. Westheimer, *Proc. Nat. Acad. Sci.*, **43**, 969 (1957).

mechanisms so far proposed. For example, why does imidazolyl in the k_2 step serve to transfer a proton from serine hydroxyl to H_2O, while in the final step it serves to transfer a proton from H_2O to serine hydroxyl? One would suppose that H_2O molecules are readily available at all times, and that the intermediate acylation of serine would be unnecessary.[26] It is possible that there is some significant entropic gain for the final step resulting from the additional point of attachment of the substrate to the enzyme furnished by serine hydroxyl.

A more specific problem arises from some of the very large differences in rates observed in CT reactions. Table II summarizes values for various kinetic constants; some of those listed are approximate values only, but this is of no concern in the present discussion. Since it is not certain at which step the rate of hydrolysis of ATEE is limited, comparison of the rate constants for this reaction with those for the NPA hydrolysis can only be expressed in the form of inequalities

$$\frac{k_2(\text{ATEE})}{k_2(\text{NPA})} \geq 50; \quad \frac{k_3(\text{acetyltyrosyl})}{k_3(\text{acetyl})} \geq 6000 \quad (18)$$

In each case the inequality becomes an equality if the hydrolysis of ATEE is rate-limited at the corresponding step.

An important general conclusion is obvious from a comparison of these two substrates: the specificity of an enzyme may be *primarily* exerted at a stage in its catalysis subsequent to the initial binding of substrate. The true K_m for ATEE is at least as large as that for NPA; the fact that it is a much better substrate arises from the much greater ease of either the formation or the hydrolysis of acetyltyrosylchymotrypsin as compared with acetylchymotrypsin.

If we assume that $k_3 > k_2$ for ATEE hydrolysis (see the preceding section), we must conclude that the rate of hydrolysis of acetyltyrosylchymotrypsin exceeds that of ACT by a factor of more than 6000, which corresponds to a difference in free energies of activation of more than 5260 cal. per mole. It seems unlikely that very much of this difference can be attributed to a difference in heats of activation, in view of the fact that neither hippurylchymotrypsin nor hydrocinnamylchymotrypsin appears to be hydrolyzed much faster than ACT (see Table II), and we must therefore suppose that there is a significant difference in entropies of activation. This suggests that in acetyltyrosylchymotrypsin the benzene ring and the acetamino group are bound at the specificity site of the enzyme in such a way as to produce the proper orientation of the carbonyl group for the hydrolytic attack, whereas in ACT configurational entropy is lost in forming the transition state for the last step. If we adopt the approximate procedure suggested by Laskowski and Scheraga,[27] we can estimate in the case of ACT this disadvantage might contribute as much as 3500 cal. per mole to the free energy of activation.

(26) This difficulty has been pointed out by Dr. Jui Wang in discussions with the authors.

TABLE II

SOME KINETIC CONSTANTS FOR CHYMOTRYPSIN-CATALYZED REACTIONS AT 25°

Substrate	k_2, sec.$^{-1}$	pK_p	k_3, sec.$^{-1}$	pK_p	K_m, M
p-Nitrophenyl acetate	3	6.6	0.025	7.4	0.005
		$k_{3(app)}$, sec.$^{-1}$		$pK_{i(app)}$	$K_{m(app)}$
Acetyl-L-tyrosine ethyl ester		160		6.8[a]	0.005
Methyl hippurate[b]		0.23		..	.0038
Methyl hydrocinnamate[c]		0.028		..	.004

[a] G. H. Dixon and H. Neurath, ref. 15. [b] At *p*H 7.9, 0.15 *M* NaCl. R. B. Martin and C. Niemann, THIS JOURNAL, **79**, 4814 (1957). [c] In 20% (w./w.) methanol. K. J. Laidler and M. L. Barnard, *Trans. Faraday Soc.*, **52**, 497 (1956).

Very recently Rydon[28] has suggested that in place of serine at the active site there is a Δ^2-oxazoline group formed by a ring closure involving the serine hydroxyl and the adjacent peptide bond to an aspartyl residue. This hypothesis appears to be consistent with the information available concerning the acylation step and perhaps to have some advantages over the mechanism represented in Fig. 9. However, Rydon further suggests that the hydrolysis of the acyl enzyme is aided by the adjacent carboxylate of the aspartyl residue and that the stability of ACT at low *p*H is due to the fact that the aspartyl carboxylate is protonated. This view seems untenable in view of the fact that the hydrolysis of ACT is shown by our experiments to be controlled by a group having a pK' of approximately 7.4. It thus appears that an additional group, presumably an imidazolyl, is present at the catalytic site and must be in the unprotonated form for the final hydrolytic step to take place.

Acknowledgments. The authors wish to acknowledge the help given by Dr. H. Gutfreund in several stimulating discussions and the technical assistance of Mr. A. E. Gregory. This research was aided by grants from the National Science Foundation and the United States Public Health Service.

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(27) M. Laskowski, Jr., and H. A. Scheraga, THIS JOURNAL, **76**, 6305 (1954).

(28) H. N. Rydon, *Nature*, **182**, 978 (1958).