The Kinetics of the α-Chymotrypsin-Catalyzed Hydrolysis of p-Nitrophenyl Acetate*

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Received May 14, 1962

The kinetics of the hydrolysis of p-nitrophenyl acetate by α-chymotrypsin have been investigated by a number of different methods. The kinetics of acylation were determined under conditions in which (1) enzyme concentration is much less than substrate concentration, (2) enzyme concentration is much greater than substrate concentration, and (3) enzyme and substrate concentrations are equal to one another. The steady state kinetics were determined under conditions in which (4) the enzyme concentration is much less than substrate concentration. A saturation of the substrate by the enzyme is observed under condition (2), the opposite of the normal enzymatic conditions. Values of k_2/K_m determined by the four methods outlined above are consistent with one another. The ionization constants of the enzyme, enzyme-substrate complex, and acyl-enzyme intermediate have been determined from the dependence of the kinetics on pH. Two extraneous reactions have been observed, one in the steady state at high substrate concentrations and high pH, and one in the presteady state at low pH and high enzyme concentrations. The former reaction rationalizes a number of discrepant values of the turnover in the literature.

Since the discovery by Hartley and Kilby (1952) that chymotrypsin catalyzes the hydrolysis of p-nitrophenyl acetate, a large research effort has been devoted to the kinetics of this reaction (reviewed by Neurath and Hartley, 1959) because of the feasibility of following each step of this reaction by convenient spectrophotometric methods. When chymotrypsin is treated with p-nitrophenyl acetate at low pH it is possible to isolate an acetyl-α-chymotrypsin (Balls and Aldrich, 1955). This derivative is inactive as an enzyme, but the acetyl group can be readily removed with nucleophiles at pH's around 8 (Balls and McDonald, 1956). The hydrolysis of N-acetyl-L-tyrosine ethyl ester, a "specific substrate" for chymotrypsin, is inhibited by p-nitrophenyl acetate (Spencer and Sturtevant, 1959), indicating that the same part of the enzyme is involved in both processes.

In the hydrolysis of p-nitrophenyl acetate (S) by chymotrypsin (E), an initial rapid liberation of approximately one mole of p-nitrophenol (P_1) per mole of chymotrypsin was observed, followed by a slow (zero-order) release of p-nitrophenol and acetate ion (P_2) (Hartley and Kilby, 1952, 1954). This behavior was interpreted in terms of a catalytic sequence involving two distinct steps in addition to the primary adsorption, a total of three steps.

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

* This investigation was supported by a grant from the National Institutes of Health. This is paper XVII in the series, The Mechanism of Action of Proteolytic Enzymes; previous paper, Bender (1962).

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The rate of formation of the acyl-enzyme (ES') was determined by stopped-flow techniques which indicated that the kinetics of acylation are consistent with the above scheme (Gutfreund and Sturtevant, 1956a,b).

The kinetic parameters of the over-all (turnover) reaction, however, are not well established and the rate constants recorded in the literature differ widely from one report to another. Moreover the apparent Michaelis constant ($K_m$app) of the turnover reaction has never been measured and therefore the proposed kinetic scheme is lacking one essential piece of information. An additional point of confusion is the report (Hartley and Kilby, 1954) that even diisopropylphosphoryl-chymotrypsin, which is reputedly completely inactive enzymatically, catalyzes the hydrolysis of p-nitrophenyl acetate at a measurable rate. No further investigation of this reaction has been carried out, in spite of the fact that this reaction could interfere with the normal enzymatic hydrolysis and could modify the observed turnover rate by a large amount. Finally, the pH dependence of the various kinetic parameters has not been measured in a comprehensive fashion nor are the results consistent (Neurath and Hartley, 1959).

The aim of the present work is to determine under one set of experimental conditions (which include the precise determination of the absolute enzyme concentration) all kinetic parameters of the hydrolysis of p-nitrophenyl acetate by α-chymotrypsin, in order to show that the proposed kinetic scheme is valid within quite large concentration and pH limits and further to show that several different kinetic approaches are available for this reaction, all of which give concordant results.

The kinetic scheme for the *p*-nitrophenyl acetate–α-chymotrypsin reaction developed by Spencer and Sturtevant (1959) includes the prototropic ionization of the enzyme-substrate complex (ES) and the prototropic ionization of the acyl-enzyme intermediate (ES') but lacks one essential feature, the ionization of the free enzyme ($E + H \rightleftharpoons EH$). The latter ionization leads to the possibility that both forms of the enzyme are able to form a complex with the substrate, a possibility which is supported, among other evidence, by studies on inhibitors which usually are better bound to the enzyme as the *p*H diminishes (Doherty and Vaslow, 1952; Vaslow and Doherty, 1953; Foster and Niemann, 1955). Moreover, previous experimental work does not allow one to rule out the possibility of noncompetitive inhibition by hydrogen ion in the acylation step (k_2'). Therefore a more complete kinetic scheme can be written as follows:

$$\begin{array}{ccccccc} & & \overset{K_a}{\rightleftharpoons} & & \overset{k_2'}{\longrightarrow} & & \overset{k_3'}{\longrightarrow} \\ EH & + S & HES & & EHS' & & EH \\ K_1\downarrow\uparrow & & K_2\downarrow\uparrow & & K_3\downarrow\uparrow & +P_1 & \downarrow\uparrow + P_2 \\ H & + E + S & \underset{}{\rightleftharpoons} & ES & \longrightarrow & ES' & \longrightarrow E \\ & & \underset{K_b}{} & & \overset{k_2}{} & & \overset{k_3}{} \end{array} \quad (1)$$

On the basis of a large amount of kinetic evidence indicating that deacylation always obeys a single *p*H dependence, we can *a priori* rule out k_3' (Bender *et al.*, 1962). For the particular case of *p*-nitrophenyl acetate other experimental results will strongly suggest that $k_2' = 0$, or at least that $k_2 \gg k_2'$, but this is far from the general rule for all chymotrypsin substrates; for example, the kinetics of hydrolysis of *N*-trans-cinnamoylimidazole by α-chymotrypsin can best be explained by assuming that k_2' is of the same order of magnitude as k_2 (Bender *et al.*, 1962). We shall therefore derive the mathematical expression for the above general case.

The proposed reaction scheme can lead in several ways to the determination of the individual constants. The first, most general way would be to assume that the rate constants of all steps would be of the same order of magnitude. The authors did not attempt to solve this knotty problem. The second, more restricted method is the mixed equilibrium-steady state method used by Gutfreund and Sturtevant (1956a,b) and Spencer and Sturtevant (1959) in which one postulates that prototropic reactions are equilibria from the kinetic point of view, and then solves the rate equation by assuming a steady state for (ES) and (ESH). A third method, apparently more restricted but more elegant, is the use of the hypothesis that both prototropic reactions and complex formation are equilibria, *i.e.*, much faster than the rate-determining steps. It can easily be shown that the last two methods give exactly the same equation, except that the significance of the rate constants is different in the two treatments. A sufficient argument for stating that the prototropic reactions and complex formation are not rate-controlling under our experimental conditions is the failure to observe any initial acceleration or any other deviation from strictly first-order kinetics in the very early phases of *p*-nitrophenol release in the acylation step. Therefore from the *kinetic point of view*, our observations indicate that K_1, K_a, and K_b are pure equilibria. For these reasons we shall use the equilibrium hypothesis throughout.

Defining the complex constants in the following way:

$$K_m = \frac{K_a H + K_b K_2}{K_2 + H} = \frac{K_a(K_1 + H)}{K_2 + H}$$

$$k_2^{tot} = \frac{k_2' H/K_2 + k_2}{1 + H/K_2}$$

$$k_3^{tot} = \frac{k_3}{1 + H/K_3}$$

$$a = \frac{k_2^{tot} E_0}{1 + K_m/S_0}$$

$$b = \frac{(k_2^{tot} + k_3^{tot})S_0 + k_3^{tot} K_m}{K_m + S_0}$$

we obtain

$$dP_1/dt = \frac{k_2^{tot} E_0}{1 + K_m/S_0} - \frac{k_2^{tot} a/b(1 - e^{-bt})}{1 + K_m/S_0} \quad (2)$$

or the integrated form, with the initial condition $P_1 = 0$ at $t = 0$

$$P_1 = \frac{k_2^{tot} E_0 - k_2^{tot} a/b}{1 + K_m/S_0} t + \frac{k_2^{tot} a}{b^2(1 + K_m/S_0)}(1 - e^{-bt}) \quad (3)$$

These equations enable us to determine the different parameters in several ways.

(a) *Measurement of the Presteady State.*—At this stage of the reaction the exponential term e^{-bt} is almost exclusively rate-controlling, and experimentally (by using calculation methods which eliminate the nonexponential terms) b can be determined. One calculation method is the use of a modified Guggenheim plot (Gutfreund and Sturtevant, 1956). Equation (2) offers another method: one can measure dP_1/dt from the experimental P *vs.* t. When the steady state is reached,

$$(dP_1/dt)\ \text{steady state} = \frac{k_2^{tot} E_0}{1 + K_m/S_0} - \frac{k_2^{tot} a/b}{1 + K_m/S_0}$$

and therefore

$$(dP_1/dt) - (dP_1/dt)\ \text{steady state} = V_t - V_\infty = \frac{k_2^{tot} a/b}{1 + K_m/S_0} e^{-bt} \quad (4)$$

Plotting log $(V_t - V_\infty)$ *versus* t, one obtains a straight line with slope $-b$. This method is less cumbersome than use of the Guggenheim plot and has about the same accuracy. Another convenient graphical method is to extrapolate the steady-

state zero-order P *vs.* t curve to the presteady state and calculate simple first-order kinetics with the values of differences between the extrapolated line and the experimental curve.

In the expression for b we can neglect $k_3^{tot} K_m$ with respect to $(k_2^{tot} + k_3^{tot}) S_0$ in the case of *p*-nitrophenyl acetate and then

$$b = \frac{(k_2^{tot} + k_3^{tot}) S_0}{K_m + S_0} \qquad (5)$$

so that a Lineweaver-Burk plot of $1/b$ versus $1/S$ will give $(k_2^{tot} + k_3^{tot})$ and $K_m/(k_2^{tot} + k_3^{tot})$ from the intercept and slope, respectively, or, if one can neglect k_3^{tot} with respect to k_2^{tot}:k_2^{tot} and K_m/k_2^{tot}.

(b) *Measurement of the Steady-State.*—The rate in the steady state is represented by the equation

$$(dP_1/dt) = \frac{E_0 S_0 k_2^{tot} k_3^{tot}}{(k_2^{tot} + k_3^{tot}) S_0 + k_3^{tot} K_m} \qquad (6)$$

so that by plotting dt/dP_1 versus $1/S_0$, we obtain

$$K_m^{app} = \frac{k_3^{tot}}{k_2^{tot} + k_3^{tot}} K_m \quad \text{and}$$

$$1/k_3^{app} = 1/k_2^{tot} + 1/k_3^{tot}$$

i.e. the slope will be $\frac{1}{E_0}\frac{K_m}{k_2^{tot}}$ and the intercept $\frac{1}{E_0}\left(\frac{1}{k_2^{tot}} + \frac{1}{k_3^{tot}}\right)$.

Condition II: $E_0 >> S_0$.—When the enzyme concentration is much greater than the substrate concentration conditions exist opposite to those of a normal enzymatic reaction (condition I). However, condition II offers the opportunity of an interesting and straightforward kinetic method for the determination of the pre-steady-state kinetics of the *p*-nitrophenyl acetate–α-chymotrypsin system. This condition has not been exploited before, although mention of its possible potential has been made (Straus and Goldstein, 1943; Goldstein, 1944; Reiner, 1959). The kinetics of an enzymatic system when enzyme concentration is greater than substrate concentration predict the *saturation of the substrate by the enzyme* leading to a limiting maximal velocity at high enzyme concentration. There are at least two reports in the literature in which such an interpretation may be given, involving the condensing enzyme (Ochoa *et al.*, 1951) and the enzyme transacetylase (Stadtman *et al.*, 1951). However, in neither of these reactions is it known that the enzyme concentration is in fact greater than the substrate concentration, for the absolute concentration of neither enzyme is known. Therefore the report in the present paper is the first authentic example of an enzymatic reaction which has been studied under conditions in which enzyme concentration is much greater than substrate concentration.

One advantage of this method is the fact that one does not have to state any condition as to the magnitude of k_3, owing to the fact that the turnover reaction cannot affect in any manner the concentration of the enzyme, which must remain constant and equal to the original concentration during the whole reaction. Using the complete kinetic scheme, it may be shown that under the condition that $E_0 >> S_0$

$$dP_1/dt = \frac{k_2^{tot} E_0}{K_m + E_0} (S_0 - P_1)$$

so that the first-order experimental rate constant, k_{exp_1}, is given by

$$k_{exp_1} = \frac{k_2^{tot} E_0}{K_m + E_0} \qquad (7)$$

which is of the same form as equation (5) with E_0 replacing S_0 for the condition where $k_2^{tot} >> k_3^{tot}$

$$b = \frac{k_2^{tot} S_0}{K_m + S_0} \qquad (5')$$

Both of the equations allow the determination of k_2^{tot} and K_m by the use of inverted (Lineweaver-Burk) plots: either $1/k_{exp_1}$ *versus* $1/E_0$ (eq. 7) or $1/b$ versus $1/S_0$ (eq. 5′).

Condition III: $E_0 \lesssim S_0 \ll K_m$ and $k_2 \gg k_3$—Under this set of conditions it has been shown that the experimental second-order rate constant, k_{exp_2}, of the enzymatic reaction is represented by the equation (Bender *et al.*, 1962)

$$k_{exp2} = \frac{k_2 + k_2'H/K_2}{K_b(1 + H/K_1)} = \frac{k_2 K_1/K_b H + k_2'/K_a}{1 + K_1/H}$$

Then, under the condition that $k_2' = 0$

$$k_{exp2} = \frac{k_2}{K_b(1 + H/K_1)} = \frac{k_2^{tot}}{K_m} \qquad (8)$$

Experimental

Materials.—α-Chymotrypsin was a Worthington three-times-crystallized product. Enzyme solutions were made up in acetate or phosphate buffers; they were centrifuged for 30 minutes at 15,000 rpm and their molarity was determined by spectrophotometric titration with *N-trans*-cinnamoylimidazole at 335 mμ (Schonbaum *et al.*, 1961). The Worthington three - times - crystallized product gives a titration value of about 85% of that calculated on a weight basis, assuming a m.w. of 24,800. *p*-Nitrophenol (Eastman Kodak indicator grade) was recrystallized twice from ethanol-water in the presence of charcoal and twice from distilled water. The colorless needles so obtained melted at 114.5–115°. *p*-Nitrophenyl acetate was a gift of Dr. K. A. Connors. After several recrystallizations from chloroform–hexane it melted at 79.5–80.0° and was almost colorless. *N-trans*-Cinnamoylimidazole was prepared from cinnamoyl chloride and imidazole in dry benzene according to the procedure of Schonbaum *et al.* (1961) and recrystallized several times from *n*-hexane immediately before use, m.p. 134.0–134.5°. Acetate, phosphate, and citrate buffers were prepared from doubly distilled water and Mal-

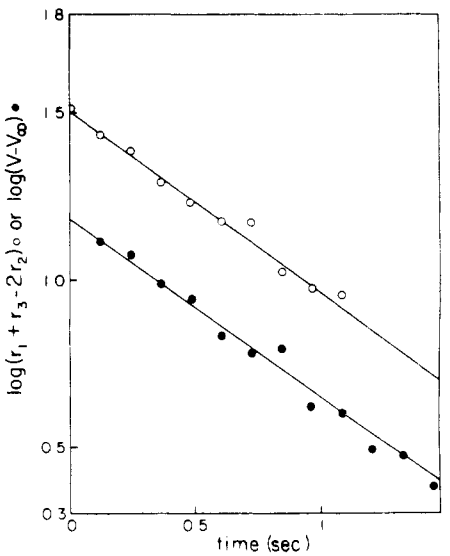

FIG. 1.—Determination of the first-order rate constant for the acylation of α-chymotrypsin by *p*-nitrophenyl acetate at *p*H 7.8 at 25.0° in 4% acetonitrile-water. $S_0 = 10.23 \times 10^{-4}$ M; $E_0 = 5.78 \times 10^{-5}$ M.

linckrodt A. R. grade products. Acetonitrile (Eastman Kodak spectral grade) was distilled several times over phosphorus pentoxide. Buffers were prepared according to Kolthoff and Rosenblum (1937).

Physical Constants of p-Nitrophenol.—In zero-order kinetics the measured rate depends essentially on the extinction coefficient of the observed reaction product, *i.e.*, *p*-nitrophenol. It was then necessary to determine an accurate value of this parameter under our experimental conditions. In preliminary experiments it was shown that small amounts (<2%) of acetonitrile and of α-chymotrypsin do not modify the spectrum of either *p*-nitrophenol or *p*-nitrophenolate ion, except for the small *p*H change involved in the addition of these substances to a buffered phenol solution. The spectrum of un-ionized *p*-nitrophenol was measured in 0.032 M hydrochloric acid. λ_{max} 317.5 mμ, ϵ_{max} 9630. The absorption of *p*-nitrophenolate ion was measured in sodium hydroxide solutions: 0.01 N sodium hydroxide, λ_{max} 400 mμ, ϵ_{max} 18,320: 1.0 N sodium hydroxide, λ_{max} 402 mμ, ϵ_{400} 18,380. In order to check these values, which are different from some literature values (ϵ_{400} 21,000 [Martin *et al.*, 1959; Doub and Vandenbelt, 1947], and ϵ_{400} 18,330 [Biggs, 1954]), a carefully purified sample of *p*-nitrophenol acetate was completely hydrolyzed by sodium hydroxide. An extinction coefficient at 400 mμ of 18,000 was found, in good agreement with the value obtained from the phenol itself.

The pK_a of *p*-nitrophenol was determined by measuring the apparent extinction coefficient in phosphate buffers of different *p*H. The *p*H of the solutions was measured simultaneously, with a Radiometer 4C *p*H-meter, standardized against *p*H 4.01 phthalate buffer. The pK_a was found to be 7.04.

Kinetic Measurements.—The rate of the fast reactions was measured with a stopped-flow mixing device mounted on a Beckman DU spectrophotometer, built by Dr. R. Thomas in this laboratory. The instrument was similar to one described by Stewart and Ouellet (1959). Beckman DU monochromator, light sources, and photomultiplier were used. The output of the photomultiplier was fed into a Brush Model RD5615 high-gain amplifier and recorded on a Brush Model RD 232100 direct writing oscillograph. Measurements were recorded 10 milliseconds after mixing. The stopped-flow apparatus was attached directly to the Beckman housing and was thermostated by means of circulating water. Ester in acetonitrile-water solution and enzyme in an appropriate buffer solution were mixed in the instrument and the intensity of the transmitted light at 400 mμ was recorded *vs.* time.

Figure 1 illustrates the determination of the rate constant of acylation by the modified Guggenheim plot of Gutfreund and Sturtevant (1956) and by use of the log $(V_t - V_\infty)$ *vs.* t plot (Equation 4). It is seen that the two methods are about equal to one another in precision.

Slow reactions were measured on a Cary 14PM recording spectrophotometer. The temperature of the buffer solution (3.00 ml) was maintained in the instrument provided with a thermostatted cell compartment. The ester solution in acetonitrile (50 μl) was added and the spontaneous hydrolysis was measured for a few hundred seconds. Then 50 μl of the standardized enzyme solution was added and the absorbance of the solution was recorded at 400 or 330 mμ (the maximum of *p*-nitrophenoxide and near the maximum of *p*-nitrophenol) using various time scales. At low *p*H, where the spontaneous hydrolysis was negligible, the ester solution was added to a buffered enzyme solution. One-cm quartz cuvets were used, and the reference cell contained the corresponding buffer.

RESULTS

Determination of the True Michaelis Constant (K_m) and the Rate Constant of Acylation (k_2) of the Hydrolysis of p-Nitrophenyl Acetate by α-Chymotrypsin

Condition I: $E_0 \ll S_0$.—Two sets of experiments were carried out to determine the Michaelis constant and the rate constant of acylation of α-chymotrypsin by *p*-nitrophenyl acetate. The results are summarized in Table I and Figure 2. They may be compared with those of Gutfreund and Sturtevant (1956) for the same reaction. The value for k_2 is in fair agreement with that given by Gutfreund and Sturtevant (1956), but the value for K_m is very different from their value. The reason for the apparent discrepancy is the fact that 1.6 and 4.0% acetonitrile were used in

TABLE I

THE HYDROLYSIS OF p-NITROPHENYL ACETATE BY α-CHYMOTRYPSIN; FIRST-ORDER KINETICS[a]

Solvent	pH	$E_0 \times 10^5$ (M)	$S_0 \times 10^5$ (M)	k_2 (sec.$^{-1}$)	$K_m \times 10^3$ (M)	k_2/K_m (M^{-1} sec.$^{-1}$)
			$E_0 \ll S_0$[a]			
4% Acetonitrile[b]	7.8	5.8	62–280	3.72 ± 0.58	1.9 ± 0.4	1960 ± 190
1.6% Acetonitrile[c]	7.8	5.6	48–156	3.96 ± 0.08	1.12 ± 0.16	3530 ± 450
20% Isopropyl[d] alcohol	7.8 (?)	~4.6	25–500	3.3 ± 0.3	7.7 ± 0.8	429 ± 80
1.6% Acetonitrile	5.91	1.8	9.8–21	0.18 ± 0.012[e]	0.37 ± 0.03[e]	487 ± 15[e]
			$E_0 \gg S_0$			
1.6% Acetonitrile	6.47	8.5–20	1.97	0.81 ± 0.13	0.72 ± 0.22	1130 ± 210
1.6% Acetonitrile	5.91	4–90	0.5–1	0.18 ± 0.012[e]	0.37 ± 0.03[e]	487 ± 15[e]
1.6% Acetonitrile	5.38	6.9–71	0.5			186[f]
1.6% Acetonitrile	5.05	7.4–83	0.93			78.5[f]
1.6% Acetonitrile	4.18	4–111	0.5			14.6[f]

[a] 25.0 ± 0.5°. [b] Phosphate buffer 0.033 M. [c] Tris-hydrochloric acid buffer 0.05 M. [d] Gutfreund and Sturtevant (1956). [e] Calculated using both $E_0 \gg S_0$ and $S_0 \gg E_0$ values. [f] Obtained from a plot of k_{exp} *vs.* E_0 at low values of E_0.

the present experiments, whereas Gutfreund and Sturtevant used 20% isopropyl alcohol. These results illustrate the generalization that the rate constants of acylation (and of deacylation) are independent of solvent, whereas K_m is very sensitive to changes in solvent composition. The former generalization is confirmed by the set of experiments in two different concentrations of acetonitrile in which k_2 is essentially independent of solvent composition (the difference in ionic strength in these two experiments was independently shown not to affect the kinetics). Organic solvents, including acetone and dioxane, previously have been shown to have a profound effect on K_m but essentially no effect on the turnover rate constant (Applewhite *et al.*, 1958). The present report extends this observation; solvent effects will be the subject of a future communication.

Condition III: $E_0 \leqslant S_0 \ll K_m$.—If E_0 and S_0 are much smaller than K_m and are approximately equivalent to one another, the measured second order rate constant is given by equation (8). Table II summarizes the second-order kinetics of acylation of p-nitrophenyl acetate by α-chymotrypsin at a number of different pH values and with a number of different solvents.

TABLE II

THE HYDROLYSIS OF p-NITROPHENYL ACETATE BY α-CHYMOTRYPSIN; SECOND-ORDER KINETICS[a]

pH	Acetonitrile (%)	$E_0 \times 10^6$ (M)	$S_0 \times 10^6$ (M)	$k_{exp_1} = k_2^{tot}/K_m$ (M^{-1} sec.$^{-1}$)
7.82	0.33	30.50	27.72	4680
7.82	1.61	7.85	6.72	3630
7.82	1.61	7.85	6.72	3940
7.82	1.61	20.42	6.72	3800
6.97	1.61	9.42	6 82	2350
7.21	1.61	10.02	6.82	2850
7.94[b]	10	41.3	41.3	563

[a] 25.0 ± 0.5°. [b] Bender and Nakamura (1962).

Condition II: $E_0 \gg S_0$.—It was shown in the introduction that when $E_0 \gg S_0$, the experimental first-order rate constant obtained by measuring the rate of formation of P_1 is represented by equation (7). Equation (7) implies that a plot of $1/k_{exp_1}$ *versus* $1/E_0$ (a Lineweaver-Burk type plot) should be linear and yield the values of k_2^{tot} and K_m from the intercept and slope respectively. It is seen in Figure 3 that the data of the α-chymotryspin-catalyzed hydrolysis of p-nitrophenyl acetate at pH 5.91 do indeed conform to such a relationship. Furthermore, Figure 3 indicates also that exactly the same Lineweaver-Burk plot is obtained whether one plots $1/k_{exp_1}$ *versus* $1/E_0$ (equation 7) or plots $1/k_{exp_1}$ *versus* $1/S_0$ (equation 5′). The same plot is thus obtained over a 50-

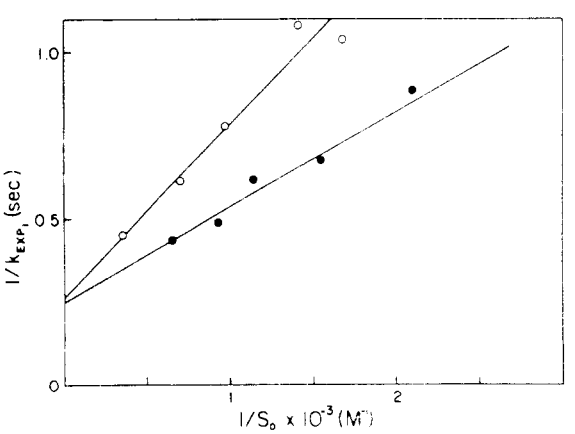

FIG. 2.—Lineweaver-Burk plot of the presteady-state portion of the hydrolysis of p-nitrophenyl acetate by α-chymotrypsin at pH 7.8 and 25.0°. E_0 = 5.6 – 5.8 × 10^{-5} M. ○, 4% acetonitrile-water; ●, 1.6% acetonitrile-water.

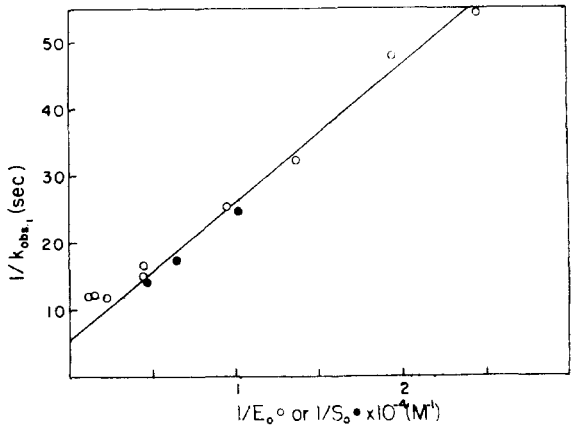

FIG. 3.—Lineweaver-Burk plot of the presteady-state portion of the hydrolysis of *p*-nitrophenyl acetate by α-chymotrypsin at *p*H 5.91 in 1.6% acetonitrile-water at 25.0°. O, $E_0 \gg S_0$; ●, $S_0 \gg E_0$.

fold change in E_0 and a 42-fold change in S_0. A *p*H of 5.91 was chosen because of experimental considerations involving spectra, stability of the substrate and the ratio S_0/K_m. The results of kinetic experiments employing $E_0 \gg S_0$ at various *p*H levels are summarized in Table I together with the data from experiments in which $E_0 \ll S_0$.

This demonstration of the compliance of an enzymatic reaction to a saturation phenomenon in the presence of either excess enzyme or excess substrate is the first of its kind and gives further experimental proof of the usual enzymatic hypothesis involving enzyme-substrate complex formation. Furthermore this procedure offers the possibility of a new and kinetically simple method for the determination of the pre-steady-state of an enzymatic reaction as exemplified by the present system.

At *p*H values below 5.91, however, the experimental first-order rate constants determined with E_0 much greater than S_0 do not fit a Lineweaver-Burk plot. In fact, if one plots k_{exp} versus E_0, one does not obtain a "saturation" curve at all but rather a straight line which at high E_0 values can even turn upwards, indicating possibly that an enzyme activation is occurring. Since this extraneous reaction occurs with E_0 much greater than S_0 at low *p*H and since experimental difficulties do not permit the use of conditions in which E_0 is much less than S_0 in the acid region, the data in Table I are of limited scope.

At lower E_0 values, however,

$$k_{exp_1} = k_2^{tot} E_0 / K_m$$

so that the slope of the straight line of the plot of k_{exp} *versus* E_0 gives us k_2^{tot}/K_m. These values are entirely consistent with the k_2^{tot}/K_m values obtained from second-order kinetics at higher *p*H values.

Determination of the Apparent Michaelis Constant and the Rate Constant of Deacylation (k_3) of the Hydrolysis of p-Nitrophenyl Acetate by α-Chymotrypsin

The zero-order (turnover) rate of the hydrolysis of *p*-nitrophenyl acetate by α-chymotrypsin in 1.6% acetonitrile-water has been determined by measuring the rate of appearance of *p*-nitrophenoxide ion at 400 mμ, and correcting it for the spontaneous hydrolysis of the substrate. The substrate concentration was always at least ten times as high as the enzyme concentration. In the introduction it was pointed out that a plot of $dt/dP_1 = 1/V$ *versus* $1/S_0$ in the steady state (equation 6) leads to a straight line whose intercept yields k_3^{tot}. The slope, furthermore, enables one to calculate k_2^{tot}/K_m. Such a Lineweaver-Burk plot for the steady state is shown in Figure 4 for data at *p*H 7.8 and 7.7. From the slope and the intercept of Figure 4, $K_m^{app} = 1.59 \times 10^{-6}$ M at *p*H 7.8. From the values of k_2 and K_m obtained by the stopped-flow method at the same *p*H and $k_3 = 5.6 \times 10^{-3}$ obtained in this plot, $K_m^{app} = k_3^{tot} K_m/(k_2^{tot} + k_3^{tot}) = 1.58 \times 10^{-6}$ M.

The agreement, which is fortuitously good, shows that the proposed kinetic scheme is correct in quite a wide concentration range of substrate and enzyme.

Although the Lineweaver-Burk plot is adhered to over a considerable range of substrate concentration, at higher substrate concentrations the experimental points fall below the line (Fig. 4), indicating that another mechanism is starting to play a role. In order to analyze this phenomenon the hydrolysis of *p*-nitrophenyl acetate at higher substrate concentrations was measured. Substrate concentrations were used which were high enough to saturate the enzyme to an extent that dP_1/dt = constant for the normal reaction.

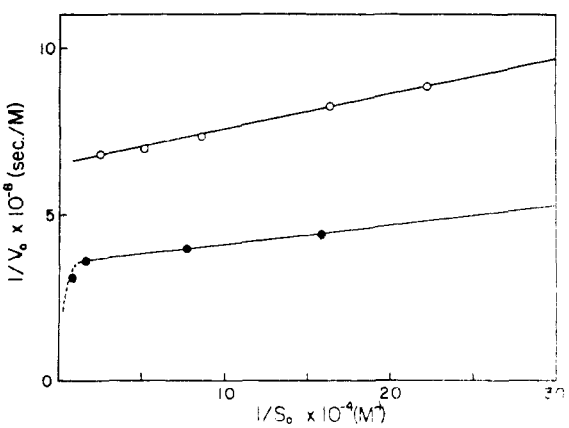

FIG. 4.—Lineweaver-Burk plot of the steady-state portion of the hydrolysis of *p*-nitrophenyl acetate by α-chymotrypsin at 25.0° in 1.6% acetonitrile. O, *p*H 7.7, $E_0 = 3.126 \times 10^{-7}$ M. ●, *p*H 7.8; $E_0 = 5.0 \times 10^{-7}$ M.

Under these conditions

$$V_0 = dP_1/dt = S_0E_0/[(S_0/k_3^{tot}) + (K_m/k_2^{tot})]$$

since k_3^{tot} can be neglected with respect to k_2^{tot}. When $S_0/k_3^{tot} \gg K_m/k_2^{tot}$, an *apparent* saturation is realized, leading to

$$dP_1/dt = k_3^{tot}E_0$$

At pH 7.8, the *apparent* saturation will be greater than 90% at $S_0 = 1.6 \times 10^{-5}$ M. At higher substrate concentrations the *normal* reaction rate can be considered to be substrate independent. Experiments were carried out at apparent saturation of the enzyme using several values of S_0 and a constant value of E_0 and pH. If one plots V versus S_0 for such a set of experiments a straight line is obtained (Fig. 5), whose intercept

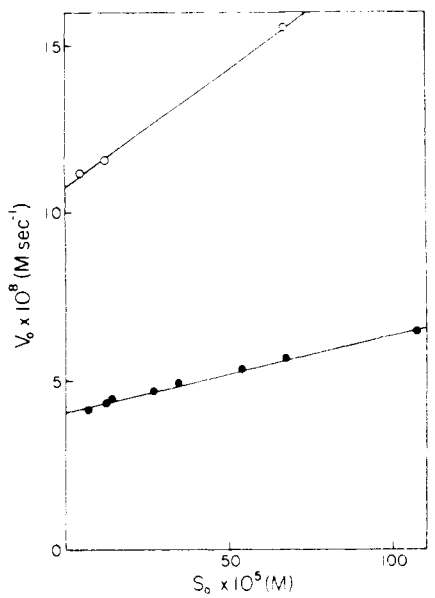

FIG. 5.—The steady-state portion of the hydrolysis of p-nitrophenyl acetate by α-chymotrypsin at high S_0 in 1.6% acetonitrile-water at pH 7.8 and 25.0°. The velocity is corrected for the spontaneous hydrolysis of p-nitrophenyl acetate by α-chymotrypsin at high S_0 in 1.6% acetonitrile-water at pH 7.8 and 25.0°. The velocity is corrected for the spontaneous hydrolysis of the substrate. ○, $E_0 = 2.04 \times 10^{-5}$; ●, $E_0 = 0.785 \times 10^{-5}$ M.

on the V axis and whose slope are both proportional to E_0. Moreover, the intercepts divided by E_0 give a value of k_3^{tot} of 5.3×10^{-3} sec.$^{-1}$, in good agreement with that determined from the Lineweaver-Burk plot in Figure 4 (5.6×10^{-3} sec.$^{-1}$ at pH 7.8). These facts suggest that at high S_0 values the steady state rate may be represented by an equation of the form

$$dP_1/dt = k_3^{tot}E_0 + k_{III}E_0S_0 \qquad (9)$$

The value for k_{III} can be calculated from the slope of the V versus S_0 plots. The results so obtained as well as the extrapolated k_3^{tot} values are reported in Table III.

TABLE III

THE HYDROLYSIS OF p-NITROPHENYL ACETATE BY α-CHYMOTRYPSIN ZERO-ORDER KINETICS AT HIGH SUBSTRATE CONCENTRATION[a]

pH	$k_3 \times 10^3$ (sec.$^{-1}$)	k_{III} (M^{-1} sec.$^{-1}$)
6.967[b]	2.2$_6$	0.86
7.208[c]	3.1$_6$	1.30
7.82[d]	5.3$_2$	3.33

[a] T = 25.0 ± 0.50°; 1.6% acetonitrile-water. [b] $E_0 = 0.942 \times 10^{-5}$ M; $S_0 = 5.88 - 217.4 \times 10^{-5}$ M. [c] $E_0 = 1.002 \times 10^{-5}$ M; $S_0 = 26.26 - 217.3 \times 10^{-5}$ M. [d] $E_0 = 0.784 - 2.04 \times 10^{-5}$ M; $S_0 = 4.73 - 106.7 \times 10^{-5}$ M.

DISCUSSION

The results of this kinetic investigation of the hydrolysis of p-nitrophenyl acetate by α-chymotrypsin in general support the description of the reaction provided by earlier investigators. New kinetic methods have been employed which allow easier and more detailed investigation of the reaction. These include the investigation of the presteady state under first-order conditions in which $E_0 \gg S_0$ and under second-order conditions in which $E_0 \cong S_0 \ll K_m$. It has been demonstrated that this enzymatic process is subject, in addition to the usual saturation process, to a saturation of the substrate by the enzyme. This demonstration indicates that there is nothing unique about the saturation of the enzyme by the substrate, but rather that it is only a member of a family of kinetically observable saturation phenomena. Saturation of the substrate by the catalyst can be and has been observed in nonenzymatic reactions (Laidler, 1950).

The use of second-order kinetic conditions at low enzyme and substrate concentrations allows one to observe the reaction free of the complications of enzyme activation or extraneous substrate reactions which are encountered in this relatively simple enzymatic process.

It has been demonstrated that the kinetic methods outlined above as well as the more conventional kinetic methods give consistent results when applied to the α-chymotrypsin-catalyzed hydrolysis of p-nitrophenyl acetate. Table IV summarizes the values of the parameter k_2/K_m determined by a number of different approaches. It is seen that within the range of substrate and enzyme concentration where these determinations were made, all results are within experimental error of one another. Since these values of k_2/K_m were determined by such diverse kinetic methods, *e.g.* first-order kinetics involving $E_0 \gg S_0$ (presteady state), first-order kinetics involving $E_0 \ll S_0$ (presteady state), second-order kinetics with $E_0 \cong S_0$ (presteady state), and

TABLE IV

VALUES OF k_2/K_m FOR THE HYDROLYSIS OF *p*-NITROPHENYL ACETATE BY α-CHYMOTRYPSIN OBTAINED FROM VARIOUS KINETIC METHODS[a]

Kinetics	E_0/S_0	State	k_2/K_m
First-order	$E_0 \ll S_0$	Presteady	3530 ± 450
First-order	$E_0 \gg S_0$	Presteady	3800[c] ± 660
Second-order	$E_0 \cong S_0$[b]	Presteady	3790[d] ± 105
Zero-order	$E_0 \ll S_0$	Steady	3390 ± 280

[a] *p*H 7.8, 1.6% acetonitrile-water, 25.0°. [b] Each concentration is much less than K_m. [c] Extrapolated value from data at lower *p*H levels. [d] Mean of three runs.

zero-order kinetics with $E_0 \ll S_0$ (steady state), it can be said with some confidence that the various kinetic approaches employed in this investigation are mutually consistent and are in fact measuring the same reaction. It should be pointed out that, over most of the *p*H range studied, chymotrypsin may exist as a dimer particularly at high enzyme concentration (Steiner, 1954). The kinetic results do not show any difference between the enzyme in the monomeric or dimeric form, indicating that the dimerization, if any, does not involve the active site, as reflected in the present reaction. It should be pointed out, however, that there is an effect of dimerization in the α-chymotrypsin-catalyzed hydrolysis of methyl hippurate (Martin and Niemann, 1958).

From the effect of *p*H on the various rate constants, it is possible to calculate the *p*H-independent rate constants, k_2 and k_3, and the apparent ionization constants, K_1, K_2, and K_3, of equation (1) and in addition to calculate the *p*H-independent rate constant k_{III} and the apparent ionization constant K_{III} pertaining to the additional reaction occurring at high substrate concentration. When $k_2' = 0$—which seems to be the case here—the *p*H dependence of k_2/K_m gives K_1 (eq. 8); the *p*H dependence of k_2^{tot} gives K_2; ($k_2^{tot} = k_2/(1 + H/K_2)$; and the *p*H dependence of k_3^{tot} gives K_3; ($k_3^{tot} = k_3/(1 + H/K_3)$. The results of the calculations are given in Table V. It is seen from Table V that the pK values of the enzyme, enzyme-substrate complex, and acyl-enzyme intermediate (pK_1, pK_2, and pK_3 respectively) are all close to 7. This general finding is in agreement with the results reported previously (Neurath and Hartley, 1959). The values of pK_2 and pK_3 as determined in this investigation are quite close to one another, and the difference is only slightly greater than the experimental error associated with these determinations. The value of pK_1 has not been determined previously; it is seen to be somewhat smaller than the other values. The value of pK_{III} is surprisingly higher than all the other values found. The occurrence of this value indicates that this extraneous reaction (to be discussed below) is not operative except at relatively high *p*H values and could easily be overlooked. The constant K_m can be

TABLE V

*p*H-INDEPENDENT RATE CONSTANTS AND APPARENT IONIZATION CONSTANTS IN THE HYDROLYSIS OF *p*-NITROPHENYL ACETATE BY α-CHYMOTRYPSIN[a]

Rate Constant	Value	Corresponding Ionization Constant	pK
k_2/K_m	4060 M^{-1} sec.$^{-1}$	K_1	6.8[b]
k_2/K_m	4060 M^{-1} sec.$^{-1}$	K_1	6.7[c]
k_2	4.8 sec.$^{-1}$	K_2	7.14
k_3	6.8×10^{-3} sec.$^{-1}$	K_3	7.28
k_{III}	6.6 M^{-1} sec.$^{-1}$	K_{III}	7.82

[a] 1.6% acetonitrile-water at 25.0°. [b] Calculated from k_2/K_m obtained from second-order kinetics in 1.6% acetonitrile. [c] Calculated from k_2/K_m obtained from first- and second-order kinetics.

seen from Table I to be *p*H dependent. This *p*H dependence can be analyzed in terms of K_a and K_b of equation (1). Such an analysis leads to a value of $K_a = 0.35 \times 10^{-3}$ M and $K_b = 1.1 \times 10^{-3}$ M in 1.6% acetonitrile-water.

The hydrolysis of *p*-nitrophenyl acetate by α-chymotrypsin has been widely held to be a simple prototype of all chymotrypsin-catalyzed reactions. It should be pointed out at this time that several complications have been observed in this reputedly simple reaction. One such complication has been noted previously by Hartley and Kilby (1954), namely, that diisopropylphosphoryl-chymotrypsin, which is supposedly completely inactive, will nevertheless hydrolyze *p*-nitrophenyl acetate. Interestingly enough this hydrolysis was reported to have a second-order rate constant which is practically equivalent to that of the hydrolysis of *p*-nitrophenyl acetate by imidazole (Bender and Turnquest, 1957). Because of this fact and because chymotrypsin contains two imidazole moieties, only one of which may be blocked by the diisopropylphosphoryl group, it was postulated that this apparently nonspecific hydrolysis of *p*-nitrophenyl acetate by diisopropylphosphoryl-chymotrypsin occurs *via* an imidazole group completely separate from the active site of the enzyme. Presumably this second site is also operative in chymotrypsin itself.

It was therefore not completely unexpected that the zero-order kinetics of the steady state reaction (turnover) at high substrate concentration were found to be anomalous. It was shown in the Results section that the rate of reaction under these conditions may be represented by two terms, one of which is dependent on enzyme concentration and the other of which is dependent on both enzyme and substrate concentrations (equation 9). The latter term could be equated to the (second-order) reaction of an imidazole outside of the active site with *p*-nitrophenyl acetate. However, the rate constant for this term, k_{III}, is 6.60 M^{-1} sec.$^{-1}$, whereas that found for imidazole in a model system is 0.46 M^{-1} sec.$^{-1}$. Therefore it appears that the nonspecific imidazole

reaction can account for only 10% of the extraneous reaction and one must look elsewhere for the main component of this reaction. Another explanation for these results is a substrate activation of the normal enzymatic reaction. Such a phenomenon has been observed in the α-chymotrypsin-catalyzed hydrolysis of methyl aceturate (Wolf and Niemann, 1959) and may be thought to be a reasonable possibility here because substrate activation should occur to a greater extent with nonspecific substrates (which may be fixed at the active site by a second substrate molecule) such as *p*-nitrophenyl acetate than with specific substrates. Using equation (9) and the *pK* values listed in Table V, it is possible to reconcile some of the existing discrepant data in the literature on the turnover (or catalytic) rate constant (k_{cat}). For example, Gutfreund and Sturtevant (1956a) reported $k_{cat} = 25.3 \times 10^{-3}$ sec.$^{-1}$ at 25° and $S_0 = 2.5 \times 10^{-3}$. Using our values and equation (9), we find 23.3×10^{-3} sec.$^{-1}$ in good agreement. Hartley and Kilby (1954) found $k_{cat} = 15. \times 10^{-3}$ sec.$^{-1}$ at *p*H 7.6 and $S_0 = 3 \times 10^{-3}$ M. Again using our values and equation (9), we find 12.1×10^{-3} sec.$^{-1}$ in good agreement. The present data do not, however, agree with the k_{cat} value of Spencer and Sturtevant (1959).

A second extraneous reaction was observed at *p*H values below 5.91 in the presence of excess enzyme (up to 10^{-3} M) (see Table VI). Here an apparent enzyme activation occurs since a Lineweaver-Burk plot is not observed, but rather the reaction rate increases at somewhat greater rate than does the enzyme concentration. No analogy for this phenomenon exists at the moment, since no other examples of enzymatic reactions are known which have been investigated under conditions in which the enzyme concentration was much greater than the substrate concentration. Extensive data at *p*H 4.18 can be fitted to the equation

$$k_{exp_1} = \frac{k_2 E_0}{K_m + E_0} + k_{1V} E_0^2$$

where the first term is the expressed reaction involving a saturation phenomenon and the second term involving E_0^2 corresponds to the enzyme activation. The two terms have been successfully separated with use of the data in Table VI and the values of k_2 and k_2/K_m obtained from a Lineweaver-Burk plot. The values are completely consistent with the data obtained at higher *p*H levels. At *p*H 5.91, the first term of the above equation dominates completely; at *p*H 5 both terms are of comparable importance whereas at *p*H 4.18 the second term becomes of major importance. No reasonable chemical explanation can be given at this time. Dimerization undoubtedly occurs under these conditions; however, dimerization has no effect at *p*H 5.91. Therefore it appears that this phenomenon may be associated with the protonation of the enzyme in some undefined manner.

TABLE VI

THE HYDROLYSIS OF *p*-NITROPHENYL ACETATE BY CHYMOTRYPSIN AT LOW *p*H[a]

*p*H	$E_0 \times 10^4$ (M)	$S_0 \times 10^3$ (M)	$k_{obs} \times 10^2$ (sec.$^{-1}$)
5.38	7.14	5.0	13.3
	5.35		10.1
	3.55		6.4
	1.76		3.9
	0.87		1.7
	0.69		1.2
5.05	8.29	9.29	6.2
	7.45		5.79
	6.61		5.09
	5.36		4.18
	4.1		3.37
	0.745		0.60
4.18	11.1	5.0	4.18
	8.87		2.42
	6.64		1.62
	4.41		1.00
	2.18		0.44
	1.85		0.34
	1.40		0.25
	1.07		0.17
	0.84		0.13
	0.40		0.058

[a] 1.6% acetonitrile-water at 25.0°.

ACKNOWLEDGMENT

The authors are pleased to acknowledge valuable theoretical and experimental assistance from Dr. B. Zerner.

APPENDIX

Dixon and Neurath (1957) carried out kinetic studies of the hydrolysis of *p*-nitrophenyl acetate by α-chymotrypsin in which they observed the second-order kinetics of the presteady state reaction. Since their results appear to be different from those presented in this paper, an analysis of their results is in order. It will be seen that there is no discrepancy between their results and the present results. Their second-order reactions were carried out under the following conditions: $S_0 > E_0$; $K_m \gg E_0$ while $S_0 \cong K_m$ (and $k_2 \gg k_3$). On the other hand, our conditions were $S_0 \cong E_0 \ll K_m$. The general expression for second-order kinetics (Bender *et al.*, 1962) is

$$dP_1/dt = \frac{k_2(S_0 - P_1)(E_0 - P_1)K_m}{(K_m + S)(K_m + E)} \quad (10)$$

Under the conditions of the experiments in the present paper, S and E may be neglected with respect to K_m, and therefore one observes pure second-order kinetics. However, under the conditions of Dixon and Neurath, S is not negligible with respect to K_m and therefore pure second-order conditions do not hold. The integrated form of equation (10) (with $K_m \gg E_0$) is

$$k_2 t = ln \frac{E_0}{(E_0 - P_1)} + \frac{K_m}{S_0 - E_0} ln \frac{E_0(S_0 - P_1)}{S_0(E_0 - P_1)}$$

and it is seen that in addition to the second-order term a first-order term exists as well. If, however, one follows equation (10) at early stages of the reaction, as Dixon and Neurath did, the apparent second-order rate constant is (with $S_0 \cong S$)

$$k_{2\text{exp}} = k_2/(K_m + S_0)$$

which permits the determination of the individual rate constants, k_2 and K_m, by using an inverse form of the equation

$$1/k_{\text{exp}} = K_m/k_2 + S_0/k_2$$

The data of Dixon and Neurath conform to this equation. The values of k_2 and K_m determined from the slope and intercept of this plot are possible values. However, since these experiments were conducted at 10° and since this method demands further checking the results of this analysis must be considered tentative.

Note Added in Proof

Investigation of the acylation of α-chymotrypsin by *p*-nitrophenyl acetate at *p*H levels from 7.94 (the maximum in this study) to 9.2 indicates that a diminution in rate occurs, leading to an overall bell-shaped *p*H-rate profile for the acylation rate constant (k_2'). The implications of this result will be discussed in a future publication.

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