The Kinetics of the $\alpha$-Chymotrypsin-catalyzed Hydrolysis of $p$-Nitrophenyl Acetate in Organic Solvent-Water Mixtures*

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SUMMARY

The $\alpha$-chymotrypsin-catalyzed hydrolysis of $p$-nitrophenyl acetate has been studied at pH 7 and 25° in isopropanol alcohol-water and dioxane-water mixtures. It was found that the initial reaction in which the enzyme is acylated follows second order kinetics rather than Michaelis-Menten kinetics as previously reported. The second order rate constant for this reaction is markedly decreased by addition of either isopropanol alcohol or dioxane in a manner which can be formally attributed to either competitive or noncompetitive inhibition. The rate of the steady state reaction increases with substrate concentration in a manner which can be formally described in terms of substrate activation. After correction for this effect, the rate at substrate saturation is increased by organic solvents (by a factor of 3.5 in 20% (v/v) dioxane); the apparent Michaelis-Menten constant is also greatly increased (by a factor of 150 in 20% (v/v) dioxane).

The primary amine tris(hydroxymethyl)aminomethane, which was used as buffer, is shown to have a strong inhibitory effect on the pre-steady state reaction, and to be very much more effective than water as an acyl acceptor.

Although several of these effects can be expressed in reasonably quantitative terms, it is not possible to offer convincing explanations for them. Organic solvents such as dioxane may produce more or less extensive changes in the enzyme, perhaps conformational in character, which drastically alter its kinetic properties; they may also exert an influence by disrupting the water structure in the vicinity of the active site.

Several studies of the effects of various concentrations of organic solvents on the rates of enzyme-catalyzed hydrolyses have been reported. In a paper which appeared while the work reported here was in progress, Clement and Bender (1) summarized these studies and extended the information available on chymotrypsin-catalyzed reactions. Of particular interest in the present connection is the work of Clement and Bender on the hydrolysis of $p$-nitrophenyl acetate by $\alpha$-chymotrypsin in solvent mixtures containing dioxane, acetone, or acetonitrile. The use of $p$ nitrophenyl acetate as substrate gives the possibility of studying solvent effects on both the acylation of the enzyme and the hydrolysis (deacylation) of the acyl enzyme. We have studied the $\alpha$-chymotrypsin-catalyzed hydrolysis of $p$-nitrophenyl acetate in solvent mixtures containing up to 20% (v/v) dioxane or isopropyl alcohol, with the use of the stopped flow method for measurements on the acylation reaction and ordinary spectrophotometry to follow the steady state reaction.

KINETICS OF CHYMOTRYPsin CATALYZES

Hartley and Kilby (2) observed that there is a rapid initial liberation of nitrophenol in the $\alpha$-chymotrypsin-catalyzed hydrolysis of $p$-nitrophenyl acetate, and attributed this "burst" of nitrophenol to the formation of an acylated enzyme intermediate. Gutfreund and Sturtevant (3, 4) studied the kinetics of the reaction by means of the stopped flow technique and concluded that the simplest mechanism capable of explaining their results involved three steps: the formation of a Michaelis-Menten complex between enzyme and substrate; acylation of the enzyme; and hydrolysis, or, more generally, deacylation of the acyl-enzyme intermediate. Actually, as will be pointed out below, the data of Gutfreund and Sturtevant do not give an adequate experimental basis for the mechanism they assumed.

A considerable body of evidence has developed which supports the view that $\alpha$-chymotrypsin-catalyzed hydrolyses of simple carboxylic acid derivatives, including derivatives of L-phenylalanine, L-tyrosine, and L-tryptophan, proceed by way of a three-step mechanism formally similar to that proposed by Gutfreund and Sturtevant (3, 4). Much of this evidence is summarized by Keddy and Bender (5). Inagami and Sturtevant (6) have recently obtained results which further support this mechanism in the hydrolysis of amide substrates. Although experiments reported by Bernhard and Gutfreund (7) and by Barman and Gutfreund (8) suggest that in the case of specific substrates of the enzyme the second intermediate may not be an acyl-enzyme, there can be little doubt that in the case of $p$-nitrophenyl acetate as substrate an acyl-enzyme intermediate is involved. For this substrate the three-step mechanism may be formulated in the following way:

$$EH + RCO\text{O} \xrightarrow{k_1, k_{-1}} EH \cdot RCO\text{O}$$

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In these equations \( EH \) represents the enzyme, \( EH \cdot RCOX \) the Michaelis-Menten complex, and \( ECOR \) the acyl-enzyme intermediate. The various rate constants may be functions of \( pH \) and other experimental parameters. It is convenient not to include explicit representation of the acyl acceptor \( HY \), usually \( H_2O \), and to replace \( JG' \) by \( k_3 \), a rate constant which includes the appropriate expression for the kinetic effect of the (usually constant) acceptor concentration. Application of the method of Laplace transforms leads to the expression

\[
\frac{(HX)_t}{(S)_0} = \frac{k_{3(app)}}{k_3 + K_m(app)} \left( \frac{(S)}{K_m + K_m(app)} \right) t + \frac{k - k_3}{k}(1 - e^{-kt})
\]

where \( (HX) \) is the concentration of \( HX \) at time \( t \), \( (E)_0 \) and \( (S)_0 \) are the initial concentrations of enzyme and substrate, and the other symbols are defined by the equations

\[
K_m = (k_1 + k_2)/k_1 \quad (5)
\]

\[
K_m(app) = k_1 K_m/(k_1 + k_2) \quad (6)
\]

\[
1/k_{3(app)} = 1/k_3 + 1/k_3 \quad (7)
\]

\[
k = \frac{k}{(S)_0 + K_m + k_3} \quad (8)
\]

Gutfreund and Sturtevant (3, 4) used Equation 8 in the form

\[
k = \frac{(k_2 + k_3)(S)_0 + k_3 K_m}{(S)_0 + K_m}
\]

and incorrectly assumed that \( (k_2 + k_3)(S)_0 \gg k_3 K_m \). The minimal assumptions necessary to permit the approximations involved in deriving Equation 4 are (a) that the reversal of Steps 2 and 3 may be neglected, (b) that the concentration of substrate remains essentially constant, and (c) that \( (k_1 + k_3)^* \ll k_3(k_1 + k_2) \). The first two assumptions are probably valid in the initial stages of the forward reaction, provided \( (S)_0 \gg (E)_0 \). A series of kinetic experiments was performed in the presence of \( p \)-nitrophenol at concentrations up to \( 10^{-4} \), and it was found that this concentration of product was without effect on the initial steady state rate of the reaction. Assumption c is satisfied if both \( k_2 \) and \( k_3 \) are small compared to \( k_1 \), which in turn implies that \( K_m \) is a true equilibrium constant and makes it very likely that the equilibrium concentration of \( EH \cdot RCOX \) will be maintained. Kéady and Bender (9) applied the equilibrium assumption to obtain a solution for the differential equations of the three-step mechanism, which holds whether or not substrate is present in large excess over enzyme. It is interesting to note that

\[
\frac{k_{3(app)}}{K_m(app)} = \frac{k_2}{K_m} \quad (9)
\]

Thus rate measurements after the steady state concentration of \( ECOR \) has been established are capable of giving the ratio of the two kinetic parameters, \( k_2 \) and \( K_m \), pertaining to the pre-steady state portion of the reaction.

The exponential term in Equation 4 describes the buildup of the steady state concentration of \( ECOR \). The amplitude of the burst of \( HX \) is given by the expression

\[
\frac{(HX)_0}{(E)_0} = \left( \frac{k_{3(app)}}{k_3} \right) \left( \frac{(S)_0}{(S)_0 + K_m(app)} \right)
\]

The initial steady state rate of liberation of \( HX \) is

\[
r = \frac{k_{3(app)}(E)_0}{(S)_0} (\frac{(S)_0}{(S)_0 + K_m(app)})
\]

If we denote \( r_0/(E)_0 \) by \( k_{sa} \), it follows from Equations 6, 7, 8, and 11 that

\[
k_{sa} = \frac{(S)_0}{(S)_0 + K_m} = \frac{k + \sqrt{k(k - 4k_{sa})}}{2} \quad (12)
\]

It will be shown later that in the case of \( p \)-nitrophenyl acetate, \( k_{sa} \) increases with \( (S) \) more rapidly than indicated by Equation 11; Equation 12 holds in spite of this, provided that the values of \( k \) and \( k_{sa} \) are determined at the same \( (S)_0 \). It is evident that \( 4k_{sa} \leq k \), and that when the equality holds,

\[
k_{sa} = 2 \frac{(S)_0}{(S)_0 + K_m} = 2 k_{sa}
\]

EXPERIMENTAL PROCEDURE

Materials—Salt-free, three times crystallized \( \alpha \)-chymotrypsin was purchased from Worthington, and was used without further purification. Four different lots of the enzyme were used. One of these, E6035-6, was assayed by the method of Schwert and Takesnak (10), the enzyme concentration being determined from the absorption at 280 nm in molar HCl solution with the use of a factor of 0.53 to convert optical density to milligrams of enzyme per ml, and was found to have an activity of \( 5.63 \times 10^3 \) units per mg of enzyme. (One unit of activity causes a change in absorbance of the substrate, acetyl-\( L \)-tyrosine ethyl ester, of 0.001 per min under the assay conditions.) With this same sample of enzyme, secondary standard assay curves with \( p \)-nitrophenyl acetate as substrate at various concentrations were determined in the solvent systems used in the subsequent rate measurements, and enzyme concentrations throughout the work were determined by reference to these curves, with the use of 25,000 as the molecular weight of \( \alpha \)-chymotrypsin.

\( p \)-Nitrophenol (Allied Chemical, reagent grade) was used without further purification. \( p \)-Nitrophenyl acetate was prepared by the method of Chattaway (11); after recrystallization from ethanol the melting point was 77.3-77.9°. Acetyl-\( L \)-tyrosine ethyl ester (Mann) was used without further purification. Tris (Fisher primary standard), reagent grade materials, and glass-distilled \( H_2O \) were used in making up buffer solutions. Throughout this work the buffer system contained 0.4 M Tris, with HCl to give a \( \text{pH} \) of 7.0 ± 0.1, and 0.1 M NaCl. The \( \text{pH} \) was operationally defined as the reading of a glass electrode \( \text{pH} \) meter, which had been standardized by means of an aqueous, \( \text{pH} \) 7.00, standard buffer.

Reagent grade isopropyl alcohol was distilled before use. Dioxane was purified by a modification of the method of Lind and Fuoss (12). Reagent grade materials was refluxed for 12 hours over NaOH pellets, the pellets being replaced by fresh ones halfway through the refluxing. The liquid was dried by being shaken overnight with BaO and was then decanted onto 10% sodium-lead alloy. It was distilled through a Vigreux column under an atmosphere of \( N_2 \) just before use.

Solutions containing organic solvents were made up with a specified volume of pure organic solvent in a known total volume.
of solution. Thus a solution denoted as 10% (v/v) dioxane contained 10 ml of dioxane with sufficient water added to make a total volume of 100 ml.

Rate Measurements—Two different stopped flow machines were used in measurements of pre-steady state rates, one described by Spencer and Sturtevant (13), and the other by Day (14) and Sturtevant (15). Steady state rates were determined in a Cary model 14 spectrophotometer. In all experiments the reaction system was maintained at 25.0°±0.1°.

Since the rate measurements depended on observing the change in absorbance at 400 mμ due to the liberation of p-nitrophenol, it was necessary to determine the effective absorptivity at pH 7.0 in the various solvent mixtures employed. It was expected that the ionization of p-nitrophenol would be markedly affected by addition of organic solvents, largely because of the decrease in the dielectric constant. Table I gives the absorptivity values found.

Appropriate corrections for nonenzymic hydrolysis were made in all experiments.

It is difficult to give a fully objective estimate of the reliability of the rate constants reported in this paper and of the various quantities derived from them. For purposes of internal comparisons, both the pre-steady state and the steady state experimental rate constants should generally be reliable to ±10%. The derived kinetic parameters are probably subject to an uncertainty of ±20%, except for the values of $k_2/K_m$ deduced from the steady state data, which should have a considerably higher uncertainty because of the insensitivity of the calculation to the values selected.

### RESULTS AND DISCUSSION

**Kinetics of Pre-Steady State Reaction with p-Nitrophenyl Acetate as Substrate**—Measurements during the burst of p-nitrophenol give values for the first order rate constant, $k$. From Equation 8 it is seen that an Eadie (16) plot of $k - k_2$ against $(k - k_2)/(S)_o$ should give a straight line with slope $-K_m$ and intercept on the ordinate equal to $k_2$. For the data obtained in the present work with the use of the appropriate value of $k_4$ derived from steady state observations and listed in Table IV, plots of this type give, within the limits of experimental error,

![Graph](http://www.jbc.org./Downloaded from http://www.jbc.org/)

**Table I**

<table>
<thead>
<tr>
<th>Added organic solvent</th>
<th>Concentration (%) (v/v)</th>
<th>μm&lt;sup&gt;-1&lt;/sup&gt;cm&lt;sup&gt;-1&lt;/sup&gt;x10&lt;sup&gt;4&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>9.89</td>
</tr>
<tr>
<td>Isopropyl alcohol</td>
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<td>9.56</td>
</tr>
<tr>
<td>Isopropyl alcohol</td>
<td>10</td>
<td>9.08</td>
</tr>
<tr>
<td>Isopropyl alcohol</td>
<td>20</td>
<td>7.72</td>
</tr>
<tr>
<td>Dioxane</td>
<td>1</td>
<td>9.79</td>
</tr>
<tr>
<td>Dioxane</td>
<td>5</td>
<td>9.43</td>
</tr>
<tr>
<td>Dioxane</td>
<td>10</td>
<td>8.98</td>
</tr>
<tr>
<td>Dioxane</td>
<td>20</td>
<td>7.60</td>
</tr>
</tbody>
</table>

![Graph](http://www.jbc.org./Downloaded from http://www.jbc.org/)

Fig. 1. The rate of the pre-steady state phase of the α-chymotrypsin-catalyzed hydrolysis of p-nitrophenyl acetate as a function of substrate concentration. ○, 1% (v/v) isopropyl alcohol; ●, 5% (v/v) isopropyl alcohol. Conditions: 0.4 M Tris-HCl buffer, pH 7.0, 0.1 M NaCl, 25°.
The rate of the pre-steady state phase of the α-chymotrypsin-catalyzed hydrolysis of p-nitrophenyl acetate as a function of substrate concentration. ○, 10% (v/v) dioxane; ●, 20% (v/v) dioxane.

**Table II**

Kinetic data for acylation of α-chymotrypsin by p-nitrophenyl acetate in 0.4 M Tris-HCl buffer, pH 7.0, containing 0.1 M NaCl, at 25°C.

<table>
<thead>
<tr>
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<th></th>
<th></th>
<th></th>
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<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
<td>2700</td>
<td>2000</td>
<td>0.048</td>
<td>0.024</td>
</tr>
<tr>
<td>Dioxane</td>
<td>1.0</td>
<td>34</td>
<td>1380</td>
<td>1040</td>
<td>0.112</td>
<td>0.020</td>
</tr>
<tr>
<td>Dioxane</td>
<td>5.0</td>
<td>11</td>
<td>440</td>
<td>330</td>
<td>-0.003</td>
<td>0.010</td>
</tr>
<tr>
<td>Dioxane</td>
<td>10.0</td>
<td>9</td>
<td>160</td>
<td>180</td>
<td>0.001</td>
<td>0.011</td>
</tr>
<tr>
<td>Isopropyl alcohol</td>
<td>20.0</td>
<td>20</td>
<td>8</td>
<td>50</td>
<td>0.001</td>
<td>0.010</td>
</tr>
<tr>
<td>Isopropyl alcohol</td>
<td>1.0</td>
<td>9</td>
<td>2200</td>
<td>2000</td>
<td>0.028</td>
<td>0.022</td>
</tr>
<tr>
<td>Isopropyl alcohol</td>
<td>5.0</td>
<td>7</td>
<td>700</td>
<td>1050</td>
<td>0.204</td>
<td>0.086</td>
</tr>
<tr>
<td>Isopropyl alcohol</td>
<td>10.0</td>
<td>10</td>
<td>400</td>
<td>350</td>
<td>0.148</td>
<td>0.098</td>
</tr>
<tr>
<td>Isopropyl alcohol</td>
<td>20.0</td>
<td>9</td>
<td>155</td>
<td>240</td>
<td>0.009</td>
<td>0.079</td>
</tr>
</tbody>
</table>

* From steady state measurements with the use of Equation 15.
† From the intercept in Fig. 4.

lines of infinite slope, indicating $K_m$ to be considerably larger than the highest attainable concentration of the slightly soluble substrate. The reaction is thus experimentally second order with rate constant $k_2/K_m$. Typical second order plots for data in isopropyl alcohol-H$_2$O mixtures and dioxane-H$_2$O mixtures are given in Figs. 1 and 2. The quantity $k_2(S)_o/K_m$ was evaluated by means of Equation 12 from the measured values of $k$ and from the values of $k_2$ interpolated in the steady state data given in the next section. Table II summarizes the values of $k_2/K_m$ obtained in eight different solvent mixtures. In each experiment at least three separate kinetic runs on the same reactant solutions were analyzed and averaged. The last column in the table gives the variance of the rate constant from the least squared second order line; comparison of these figures with the intercepts given in Column 6 indicates that no significant deviation from purely second order kinetics for the acylation process was found.

Gutfreund and Sturtevant (3) concluded that their pre-steady state data for the p-nitrophenyl acetate hydrolysis gave definite indication of significant binding between enzyme and substrate. However, this conclusion was based on an analysis of the data in which $k_2$ was assumed to be negligible compared to $k$. If this incorrect assumption is not made, it turns out that their data follow apparent second order kinetics, with $(k - k_2)$ proportional to $(S)_o$ within the limits of experimental error.

Keady and Bender (9) have reported pre-steady state data for the α-chymotrypsin-catalyzed hydrolysis of p-nitrophenyl acetate in 1.6 and 4% acetonitrile. In their experiments, in which $(S)_o << (E)_o$, the assumption referred to above was legitimate, and the data, although not as extensive as might be desired, are definitely better fitted by Equation 8 with $K_m = 10^{-3}$ M than with $K_m >> (S)_o$. It appears that these data are actually the only ones available at present which can be interpreted to indicate Michaelis Menten kinetics for the acylation of α-chymotrypsin by p-nitrophenyl acetate.

In view of the fact that all experiments reported here were performed in the presence of 0.4 M Tris, which can participate in the deacylation step, it was of interest to learn whether Tris has any effect on the pre-steady state kinetics. Table III lists results obtained at Tris concentrations up to 0.7 M in 10% (v/v) dioxane, the total ionic strength being adjusted to 0.037 M with KCl. The burst rate constants given in Column 2 of Table III show no dependence on Tris concentration. However, when $k_2/K_m$ is calculated by means of Equation 12 (assuming $(S)_o << K_m$), with $k_2$ having the values given in the next section, the quantities listed in Column 3 of Table III are obtained. It thus appears that Tris has a small inhibitory effect on the pre-steady state reaction.

**Effect of Organic Solvents on Pre-steady State Reaction**—The values of $k_2/K_m$ given in Column 4 of Table II indicate that the addition of organic solvents has a dramatic effect on the rate of the pre-steady state reaction. Similar large changes were found

Stewart and Ouellet (17) made the same approximation in interpreting their data on the acylation of trypsin by p-nitrophenyl acetate, and concluded that the system is characterized by a finite $K_m$. More exact treatment of the data as published shows that they are adequately represented by second order kinetics.
by Clement and Bender (1). As shown in Fig. 3, \((K_m/K^2)^3\) varies linearly with solvent molarity for both dioxane and isopropyl alcohol. The common intercept gives a value of 2700 \(\text{m}^{-1} \text{sec}^{-1}\) for \(k_2/K_m\) in H\(_2\)O. The data of Clement and Bender show this same behavior, as illustrated by Fig. 4, in which the square root of the ratio \(R = (k_2/K_m)_{\text{H}_2\text{O}}/(k_2/K_m)_{\text{solv}}\) is plotted as a function of solvent molarity. Their data for the dioxane-H\(_2\)O system are in very close agreement with ours, in spite of the fact that a very different buffer system was employed. It is interesting that acetone and acetonitrile give approximately equal slopes much smaller than that given by dioxane.

One possible interpretation of the observed behavior of \(k_2/K_m\) is based on the assumption that the organic solvents are non-competitive inhibitors bound at two identical but independent sites on the enzyme, so that the concentration of active enzyme is given by

\[
(E)_{\text{act}} = (E)_0 \left[ 1 + \frac{(I)}{K_1} + \frac{(I)^2}{K_2K_1} \right],
\]

where \(K_1\) and \(K_2\) are, respectively, the dissociation constants of \(EI\) and \(EI_2\), and \(K_2 = 4K_1\). According to this view the effect of the solvent is exerted solely on \(k_2\). It can, of course, equally well be assumed that the organic solvents affect only \(K_m\) in a manner corresponding to competitive inhibition. It need not be added that other interpretations can be devised. Indeed, Clement and Bender (1) found that their data were well accounted for on the basis of a more complex treatment, combining the effects of competitive inhibition and of changing dielectric constant on electrostatic interactions.

Kinetics of Steady State Reaction with p-Nitrophenyl Acetate as Substrate—According to Equation 11, the rate of the steady state reaction should be proportional to \((E)_0\) at constant \((S)_0\). Several sets of observations verified this expectation. Equation 11 also predicts that Eadie plots of \(k_\text{obs}\) against \(k_\text{obs}/(S)_0\) should be straight lines. Typical plots of observed values of \(k_\text{obs}\) are given in Fig. 5, and show pronounced deviations from the expected behavior. These deviations can be formally accounted for on the basis of substrate activation (cf. Trowbridge, Krebbri, and Laskowski (18)). Kędz and Bender (5) also observed anomalies in the hydrolysis of p-nitrophenyl acetate by \(\alpha\)-chymo-

### Table III

**Dependence of rate constant for acylation of \(\alpha\)-chymotrypsin by p-nitrophenyl acetate on Tris concentration in 10% (v/v) dioxane-H\(_2\)O**

<table>
<thead>
<tr>
<th>Tris concentration M</th>
<th>(k) (mean ± average deviation) sec(^{-1})</th>
<th>(k_2/K_m)</th>
<th>(k_2/K_m) calculated by Equation 8: O, dioxane; •, isopropyl alcohol. (K_m/k_1) calculated by Equation 15: ♦, isopropyl alcohol.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.040</td>
<td>0.224 ± 0.011</td>
<td>212</td>
<td></td>
</tr>
<tr>
<td>0.055</td>
<td>0.237 ± 0.005</td>
<td>222</td>
<td></td>
</tr>
<tr>
<td>0.10</td>
<td>0.239 ± 0.000</td>
<td>219</td>
<td></td>
</tr>
<tr>
<td>0.20</td>
<td>0.235 ± 0.016</td>
<td>225</td>
<td></td>
</tr>
<tr>
<td>0.30</td>
<td>0.221 ± 0.002</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>0.40</td>
<td>0.228 ± 0.002</td>
<td>178</td>
<td></td>
</tr>
<tr>
<td>0.50</td>
<td>0.239 ± 0.002</td>
<td>182</td>
<td></td>
</tr>
<tr>
<td>0.60</td>
<td>0.242 ± 0.002</td>
<td>181</td>
<td></td>
</tr>
<tr>
<td>0.70</td>
<td>0.226 ± 0.003</td>
<td>158</td>
<td></td>
</tr>
</tbody>
</table>

* For set of experiments at fixed Tris concentration.
In 0.4 M Tris-HCl buffer, pH 7.0, and 0.1 M NaCl, at 25°C, O, 5% (v/v) dioxane; •, 0% (v/v) isopropyl alcohol. Experiments at equal or nearly equal values of \( (S)_0 \) are averaged to avoid crowding the figure.

Trypsin, which they attributed to substrate activation. If Equation 3 is replaced by Equations 3a, 3b, and 3c,

\[
\text{ECOR} + \text{HY} \rightarrow k'_1 \rightarrow \text{EH} + \text{RCOY} \tag{3a}
\]

\[
\text{ECOR} + \text{RCOX} \rightarrow k_1 \rightarrow \text{ECOR-RCOX} \tag{3b}
\]

\[
\text{ECOR-RCOX} + \text{HY} \rightarrow k'_4 \rightarrow \text{EH} + \text{RCOY} + \text{RCOX} \tag{3c}
\]

and if it is assumed that equilibrium with respect to the acyl-enzyme and RCOX (Equation 3b) is maintained, the equations derived earlier hold with \( k_1 \) replaced by \( k_{\text{tot}} \), where

\[
k_{\text{tot}} = k_3 + \frac{k_4}{K_{(S)_0}} \tag{14}
\]

In particular, the equation

\[
\frac{1}{k_{\text{tot}}} = \frac{1}{k_4} - \frac{(S)_0 + K_n}{k_4(S)_0} \tag{15}
\]

or a similar one, involving Equation 12, can be utilized to evaluate \( k_{\text{tot}} \). In view of the fact that \( K_m \gg (S)_0 \), it would be expected that \( K_4 \gg (S)_0 \). It is found that in each of the solvent systems we have employed, a value of \( k_4/K_n \) can be selected such that \( k_{\text{tot}} \) calculated by Equation 15 (assuming \( K_m > (S)_0 \)) is a linear function of \( (S)_0 \) within the limits of experimental error. This is illustrated in Figs. 6 and 7, in which O represents the values of \( k_{\text{tot}} \) and • those of \( k_{\text{tot}} \) calculated with the optimum value of \( k_4/K_n \). The values for \( k_4/K_n \) for the nine solvent systems employed are listed in Table IV, together with values of \( K_m(\text{app}) \) calculated by means of Equation 6, taking \( k_4 > k_3 \), and using the values of \( k_4/K_n \) selected as described above.

The values selected for \( k_4/K_n \) are listed in Column 5 of Table II, for comparison with the values derived from the pre-steady state measurements. The steady state values show qualitatively the same dependence (cf. Fig. 3) on solvent concentration as do the burst values, although with a considerably different slope in the case of the isopropyl alcohol mixtures. Although there is admittedly a large uncertainty in the selection of \( k_4/K_n \), according to the criterion of obtaining a linear plot of \( k_{\text{tot}} \) with respect to \( (S)_0 \), some of the discrepancies appear to exceed experimental uncertainties. No explanation for this can be proposed.

### Steady State Rates with Partially Inhibited Enzyme

It is conceivable that the abnormal variation of steady state rate with p-nitrophenyl acetate concentration is due to the presence of two or more catalytic species having different kinetic properties. As a partial check on this possibility, experiments in 5% dioxane were performed with \( \alpha \)-chymotrypsin which had been partially inactivated by treatment with diisopropyl fluorophosphate (18, 20). The relative activity of the inhibited enzyme was determined with acetyl-L-tyrosine ethyl ester as substrate at pH 8 and 25°C, with a pH-stat. With each sample of inhibited enzyme, \( (S)_0 \) was varied from 0.2 to 5.0 m. The results obtained are summarized in Table V. In no case was any trend of the ratio of inhibited to normal rate observed as \( (S)_0 \) was varied, even with enzyme retaining only 5% of its original activity. Thus, discounting the unlikely possibility that all enzyme species present react with diisopropyl fluorophosphate at exactly the same rate, these results lend support to the view that the apparent substrate activation is a property of the pure enzyme.

### Effect of Tris Concentration on Steady State Rate

A series of experiments was performed to give an estimate of the relative effectiveness of water and Tris as acyl acceptors. These experiments were carried out in 10% (v/v) dioxane, with \( (S)_0 \) and \( (E)_0 \) constant at 1.0 mm and 53 μm, respectively. The ionic strength was held constant at 0.0637 m by addition of KCl. The molar absorptivity of p-nitrophenol was found to increase with Tris concentration, \( (\text{Tris}) \), under these experimental conditions according to the equation

\[
(\text{Tris}) \times 10^4 = 8.22 + 1.65 \times (\text{Tris}) \tag{19}
\]

and the observed rates were appropriately corrected. The values of \( k_{\text{tot}} \) are plotted as ○ in Fig. 8. \( k_{\text{tot}} \) is conveniently calculated by means of the alternative form of Equation 15,

\[
\frac{1}{k_{\text{tot}}} = \frac{1}{k_4} - \frac{2}{k_3} \tag{17}
\]

since the data in Table III show that under the conditions of these experiments \( k \) is substantially constant with the value 0.235 sec⁻¹. Values of \( k_{\text{tot}} \) calculated by means of Equation 17 are plotted as • in Fig. 8. It appears that \( k_{\text{tot}} \) increases linearly with Tris concentration up to 0.4 M. If it is assumed that the effect of apparent substrate activation is the same for deacylation by H₂O and by Tris, the values of \( k_{\text{tot}} \) up to 0.4 M Tris can be fitted by the equation

\[
k_{\text{tot}} = k'_1(1 + \alpha(S)_0) (\text{H}_2\text{O}) + k'_2(1 + \alpha(S)_0) \tag{18}
\]

with \( k'_1(1 + \alpha(S)_0) = 1.8 \times 10^{-4} \text{m}^{-1} \text{sec}^{-1} \), and \( k'_2(1 + \alpha(S)_0) = 0.11 \text{m}^{-1} \text{sec}^{-1} \). In these expressions, \( \alpha = k_3/k_4K_n \). So far as the effect of the added nucleophile is concerned, this equation is equivalent to one given by Bender et al. (21). According to this interpretation, Tris is 600 times more effective as an acyl acceptor than is H₂O; if it is only the unprotonated form of Tris which acts as an acceptor, this factor becomes about 6000.
Fig. 6. Illustration of obtaining from steady state rate data a linear plot of $k_{\text{tot}}$ (Equation 15) with respect to $(S)_0$ by selection of an appropriate value of $k_s/K_m$ for the pre-steady state reaction. Conditions: 0.4 M Tris-HCl buffer, pH 7.0, 0.1 M NaCl, 25°C, 5% (v/v) isopropyl alcohol. O, $k_{\text{ass}}$; $\bullet$, $k_{\text{tot}}$ with $k_s/K_m = 1060$ M$^{-1}$ sec$^{-1}$; $\bigcirc$, $k_{\text{tot}}$ with $k_s/K_m = 700$.

Fig. 7. Illustration of obtaining from steady state rate data a linear plot of $k_{\text{tot}}$ (Equation 15) with respect to $(S)_0$ by selection of an appropriate value of $k_s/K_m$ for the pre-steady state reaction. Conditions: 0.4 M Tris-HCl buffer, pH 7.0, 0.1 M NaCl, 25°C, 5% (v/v) dioxane. O, $k_{\text{ass}}$; $\bullet$, $k_{\text{tot}}$ with $k_s/K_m = 330$. 
Chymotrypsin-catalyzed Hydrolysis of p-Nitrophenyl Acetate

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**TABLE IV**

Rate constants (according to Equation 14) for steady state hydrolysis of p-nitrophenyl acetate by α-chymotrypsin at 25°, pH 7.0, 0.4 M Tris-HCl buffer, 0.1 M NaCl

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Organic solvent concentration</th>
<th>No. of experiments</th>
<th>$k_2$</th>
<th>$k_2/K_a$</th>
<th>$K_w( app )$</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$O</td>
<td></td>
<td>8</td>
<td>0.0132</td>
<td>16.5</td>
<td>0.66</td>
</tr>
<tr>
<td>H$_2$O-dioxane</td>
<td>1</td>
<td>23</td>
<td>0.0244</td>
<td>5.4</td>
<td>2.35</td>
</tr>
<tr>
<td>H$_2$O-dioxane</td>
<td>5</td>
<td>32</td>
<td>0.0382</td>
<td>4.1</td>
<td>11.6</td>
</tr>
<tr>
<td>H$_2$O-dioxane</td>
<td>10</td>
<td>29</td>
<td>0.0471</td>
<td>3.25</td>
<td>29.4</td>
</tr>
<tr>
<td>H$_2$O-dioxane</td>
<td>20</td>
<td>28</td>
<td>0.0474</td>
<td>0.96</td>
<td>94.8</td>
</tr>
<tr>
<td>H$_2$O-isopropyl alcohol</td>
<td>1</td>
<td>26</td>
<td>0.0181</td>
<td>13.1</td>
<td>0.91</td>
</tr>
<tr>
<td>H$_2$O-isopropyl alcohol</td>
<td>5</td>
<td>21</td>
<td>0.0301</td>
<td>7.25</td>
<td>2.87</td>
</tr>
<tr>
<td>H$_2$O-isopropyl alcohol</td>
<td>10</td>
<td>14</td>
<td>0.0387</td>
<td>6.87</td>
<td>6.38</td>
</tr>
<tr>
<td>H$_2$O-isopropyl alcohol</td>
<td>20</td>
<td>16</td>
<td>0.0387</td>
<td>1.61</td>
<td>16.1</td>
</tr>
</tbody>
</table>

**TABLE V**

Initial steady state rates for hydrolysis of p-nitrophenyl acetate by α-chymotrypsin partially inhibited by diisopropyl fluorophosphate

Conditions: 5% (v/v) dioxane, 0.4 M Tris, 0.1 M NaCl, pH 7.0, 25°. (S)$_I$ varied from 0.2 to 5.0 m in each series.

<table>
<thead>
<tr>
<th>Series</th>
<th>No. of experiments</th>
<th>% activity (ATE$^a$ assay)</th>
<th>Ratio of inhibited rate to normal rate, mean value $\times 10^5$ ± standard deviation $\times 10^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>23.7</td>
<td>22.5 ± 1.1</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>23.6</td>
<td>22.1 ± 0.9</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>15.7</td>
<td>17.4 ± 0.7</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>7.1</td>
<td>6.88 ± 0.3</td>
</tr>
<tr>
<td>5</td>
<td>7</td>
<td>5.6</td>
<td>5.31 ± 0.3</td>
</tr>
</tbody>
</table>

$^a$ ATE, acetyl-L-tyrosine ethyl ester.

Since the concentration of Tris has been shown to have a substantial effect on both the burst and steady state kinetics, the question arises as to how much weight can be attached to comparisons based on kinetic data obtained at a fixed Tris concentration. Although this question cannot be answered directly, the solvent effects observed in the present work are so large that it seems quite unlikely that conclusions significantly different from those presented here would be obtained from experiments...
performed at a different Tris concentration, or in the presence of some other buffer. In this connection it is significant that the slope of a plot of \((k_0/K_s)^3\) with respect to dioxane molarity is practically the same in 0.4 M Tris buffer and in 0.1 M phosphate buffer (cf. Fig. 4).

**Effect of Organic Solvents on Steady State Rate**—In considering the effect of organic solvents on the rate of the steady state reaction, no allowance will be made for the decrease of the concentration of \(H_2O\) as the concentration of organic solvent is increased. The justification for this simplification lies in the fact that the water concentration decreases less than 20% at most, and in the fact that the bulk of the deacylation process involves Tris, which is present at constant concentration.

Of the two rate constants listed in Table IV, \(k_1\) can be identified as the rate constant for deacylation in the absence of substrate activation, and \(k_0/K_s\) as the second order rate constant which determines the extent of substrate activation. The former is markedly increased by the addition of organic solvents, as shown by the plots in Fig. 9, with the acceleration reaching an apparent saturation at about 1.5 M for both solvents. This acceleration by organic solvents has been noted before by several authors, but never to the pronounced degree observed here. Previously reported accelerations of maximum rates have been of the order of 23 to 30%, whereas in the present systems accelerations up to 350% are observed. The relatively small increase in the maximum rate produced by additions of dioxane reported by Inagami and Sturtevant (22) for the benzoyl-\(L\)-arginine ethyl ester-trypsin system were tentatively explained as being due to an inhibitory action of water. The same explanation cannot reasonably be adopted in the present case because of the much larger effects which must be accounted for. It is conceivable that a large part of the effect of added organic solvents results from an increase in the relative efficiency of Tris as acyl acceptor, although this seems unlikely. In view of the fact that isopropyl alcohol exerts a considerably smaller effect than dioxane, it seems unlikely that the ability of the alcohol to act as acceptor has much influence on the kinetics.

The values of \(k_1/k_4\) given in Table IV are plotted in Fig. 10. It is seen that the apparent substrate activation is greatly decreased by the addition of organic solvents, by a factor of 17 in the case of 20% dioxane. No satisfactory explanation for these observations is available.

The possibility exists that some of the observed solvent effects may be due to enhancement of the well known tendency of \(\alpha\)-chymotrypsin to polymerize (23-26). \(\alpha\)-Chymotrypsin dissolved in Tris buffer containing 20% (v/v) dioxane at a concentration of 10 mg per ml, 10 times higher than used in any of the kinetic experiments, was studied in a Spinco model E ultracentrifuge, and was found to have a sedimentation coefficient of 2.32 S. Under the same conditions but in the absence of dioxane, the sedimentation coefficient was found to be 1.47 S. If the value in the presence of dioxane is transformed to the value for aqueous solution with the formula

\[
 s_B = s_D \frac{\eta_B(1 - \varepsilon_B)}{\eta_D(1 - \varepsilon_D)} \tag{19} 
\]

where the subscripts \(B\) and \(D\) refer to aqueous buffer and buffer containing dioxane, \(\eta\) is the viscosity, \(\rho\) the density, and \(\varepsilon\) the partial specific volume of the enzyme, then the value 1.53 S is obtained. The agreement with the value obtained in aqueous buffer makes it appear unlikely that significant polymerization takes place under the conditions of our experiments. In the calculation the values \(\eta_D = 1.330\) and \(\rho_D = 1.016\) given by Lind and Fuoss (12) for 22.2% (w/w) dioxane-\(H_2O\) were used, and it was assumed that no serious error results from applying values for \(H_2O\) and dioxane-\(H_2O\) to the present system containing buffer components. Also implicit in the calculation is the assumption of spherical shape for the enzyme molecules in both solvents. For \(\varepsilon\) the value 0.721 given for chymotrypsinogen by Schwert (27) was used, and it was assumed to be independent of solvent.

It may be noted that isopropyl alcohol has been shown to produce a small increase in the fluorescence emission of \(\alpha\)-chymotrypsin (28), presumably resulting from a conformational change induced by the solvent. It is quite possible that solvent-induced conformational changes are responsible for many of the effects noted in this paper.

**Acknowledgment**—The authors are indebted to Mr. Arthur Gregory for expert technical assistance.

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The Kinetics of the $\alpha$-Chymotrypsin-catalyzed Hydrolysis of $p$-Nitrophenyl Acetate in Organic Solvent-Water Mixtures
Larry Faller and Julian M. Sturtevant


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