THE SPECIFIC PEPTIDASE AND ESTERASE ACTIVITIES OF CHYMOTRYSIN*

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The experiments of Bergmann and Fruton on the substrate specificity of crystalline chymotrypsin demonstrate that this enzyme catalyzes the hydrolysis of tyrosyl and phenylalanyl peptides at the peptide bond which involves the carbonyl group of these aromatic amino acid residues (2–4). Typical substrates are N-substituted L-tyrosylglycinamides which are split into N-substituted L-tyrosine and glycaminamide. Representative examples of this type of substrate are benzoyl-L-tyrosylglycinamide, carbobenzoxy-L-tyrosylglycinamide, and glycyl-L-tyrosylglycinamide. The proteolytic coefficients for the hydrolysis of these substrates in 0.05 M substrate concentration are of the order of $1 \times 10^{-4}$ to $1 \times 10^{-2}$. Whereas in these peptides the susceptible peptide bond occupies an internal position, substrates containing the susceptible bond in a terminal position (amides) are likewise hydrolyzed by chymotrypsin. A representative example of this type of substrate is glycyl-L-tyrosinamide, which is split into glycyl-L-tyrosine and ammonia, the proteolytic coefficient being about $6 \times 10^{-4}$ (4). Phenylalanyl analogues of these substrates are hydrolyzed at a considerably lower rate.

According to the published data (2–4), it is irrelevant for hydrolysis whether the N-substituted L-tyrosylglycinamides have a free or masked terminal amino group. Thus, glycyl-L-tyrosylglycinamide and its phenylalanine analogue, which contain a positively charged amino group, are typical substrates, as are the uncharged molecules, benzoyl-L-tyrosylglycinamide and carbobenzoxy-L-tyrosylglycinamide. However, it has been reported that masking of the free amino group of glycyl-L-tyrosinamide by a carbobenzoxy (4) group practically eliminates the hydrolysis of the resulting peptide by chymotrypsin. Analogously, while L-tyrosinamide and L-phenylalaninamide are slowly hydrolyzed by this enzyme, at a rate approximately one-twentieth of that of the corresponding N-glycyl derivatives (4), benzoyl- (3) or carbobenzoxy-L-tyrosinamide (4) and their respec-

* Part of a thesis to be submitted by Mr. Seymour Kaufman to the Graduate School of Duke University in partial fulfillment of the requirements for the degree of Doctor of Philosophy. A preliminary account of this work has already been published (1).

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tive L-phenylalanine analogues have been reported to be entirely resistant to enzymatic splitting (4). Since chymotryptic activity is allegedly directed toward the peptide group which involves the carbonyl group of the aromatic amino acid residues, regardless of whether it occupies an internal or terminal position, it appears that, contrary to the published data, N-substituted tyrosinamides should be typical substrates for this enzyme. The results of the present investigation substantiate this prediction, and, moreover, show that, in aqueous methanol, N-acylated derivatives of L-tyrosinamide are hydrolyzed more rapidly than are any of the substrates previously described by Bergmann and Fruton.

Because of the very limited kinetic data that are available for the peptide hydrolysis by chymotrypsin, a comprehensive investigation of the kinetics of hydrolysis of the typical substrate, glycyl-L-tyrosinamide, and of the new substrate described herein, i.e. benzoyl-L-tyrosinamide, was undertaken. This included determination of the influence of substrate concentration on reaction kinetics (5), determination of the enzyme-substrate dissociation constant, \( K_m \), and for representative peptide and ester (vide infra) substrates, determination of the energetic constants of the reaction. Preliminary data on the influence of pH on hydrolysis rates are likewise included in this paper.

In a previous report from this laboratory on the specific esterase activity of trypsin (6), preliminary data on a like specificity of chymotrypsin were included. Since then, a specific esterase activity of another proteolytic enzyme, i.e. crystalline carboxypeptidase, has been established (7). As a logical sequence to these studies, the specific esterase activity of chymotrypsin has been investigated in greater detail. The most active, specific, esters which so far have been found are benzoyl-L-tyrosine ethyl ester and acetyl-L-tyrosine ethyl ester. Quantitative measurements on the kinetics and energetics of the hydrolysis of these esters have been made and compared to analogous data for the hydrolysis of one of the parent peptides. A search for specific inhibitors for chymotrypsin has been in progress. Preliminary experiments are included in this report, although they yielded negative results.

**EXPERIMENTAL**

*Enzymes and Methods*—The preparation of chymotrypsin and the methods for measuring amidase and esterase activities have already been described (6). Enzyme and substrate solutions were freshly prepared for each experiment. Unless otherwise stated, measurements were performed in the presence of phosphate buffer, pH 7.8, at 25°. Buffer concentration was 0.1 \( \text{m} \) in the absence of methanol, and 0.045 to 0.050 \( \text{m} \) in measurements carried out in the presence of methanol.
Substrates—Glycyl-L-tyrosinamide acetate (GTA) was prepared essentially according to the directions of Fruton and Bergmann (4). Reduction of carboxbenzoxymglycyl-L-tyrosinamide by catalytic hydrogenation in the presence of acetic acid (4) yielded only small amounts of GTA. Extensive experimentation showed that good yields of GTA could be obtained if the acid was withheld till after completion of the catalytic reduction. After conversion to the acetate salt and recrystallization (4), yields of 75 per cent were obtained.

Calculated, N 14.1; found, N 14.2

Benzoyl-L-tyrosinamide (BTA) was synthesized according to Bergmann and Fruton (3). It was recrystallized twice from absolute ethanol. M.p. found, 210–211°; reported (3), 198–199°.

Calculated, N 9.9; found, N 9.8

Acetyl-L-tyrosine ethyl ester (ATEE). Acetyl-L-tyrosine was prepared according to du Vigneaud’s method (8). Esterification was carried out in the usual manner. The ester was recrystallized from aqueous methanol. M.p. 79–80°.

\[ C_{13}H_{17}NO_4 \] (251.0). Calculated. C 62.08, H 6.78, N 5.48
Found. " 62.07, " 6.75, " 5.45

Benzoyl-L-tyrosine ethyl ester (BTEE) was prepared from benzoyl-n-tyrosine (3) in the usual manner and recrystallized twice from aqueous ethanol. M.p. 120–121°; mixed melting point with benzoic acid, 90–93°

Calculated, N 4.4; found, N 4.3

1-Phenyl-2-acetaminobutanone-3 (PAAB) was prepared according to the directions of Levene (9). It was recrystallized twice from toluene. M.p. found, 96–97°; reported (9), 98–99°.

Calculated, N 6.8; found, N 6.6

Benzoyl-L-tyrosine hydrazide (BTH) was synthesized according to Bergmann and Fruton (3). M.p. found, 251–253°; reported (3), 250–255°.

Results

Reaction Kinetics

It has already been shown (5) that interpretation of kinetic data of hydrolysis by proteolytic enzymes by first order reaction kinetics is of limited validity, the shift in equilibrium between free and combined enzyme during the course of the reaction causing marked deviations from the first order equation.

1 This substrate was prepared by Mr. John E. S PCIe of this laboratory.
If the reaction is pictured as occurring in three steps, of which the first is a reversible combination of enzyme $E$ and substrate $S$ to form the complex $ES$

$$E + S \xrightleftharpoons[k_1]{k_2} ES \xrightarrow{k'} ES^* \rightarrow E + P \quad (1)$$

where $e$ is the total enzyme concentration, $p$ the concentration of the complex $ES$, and $a$ the concentration of the substrate, then the velocity of the forward reaction is given by

$$v_1 = k_1 (e - p)(a) \quad (2)$$

and that of the reverse reaction by

$$v_2 = k_2 (p) \quad (3)$$

$ES$ then passes to an activated state, corresponding to that of the highest energy barrier, denoted by $ES^*$, at a rate given by

$$v' = k' (p) \quad (4)$$

and decomposes subsequently into free enzyme and reaction products, $P$. Although theory requires that the activation and decomposition reactions be considered as reversible, for purposes of simplification, only the experimentally measurable steps shown here have been considered.

Since at equilibrium

$$v_1 = v_2 + v' \quad (5)$$

it follows that

$$K_m = \frac{(e - p)(a)}{p} = \frac{k_2 + k'}{k_1} \quad (6)$$

The rate of disappearance of the substrate is given by

$$v' = k'(p) = -\frac{da}{dt} = \frac{k'ea}{K_m + a} \quad (7)$$

which on integration yields

$$k'et = 2.3 K_m \log \frac{a_0}{a} + (a_0 - a) \quad (8)$$

Here $e$ is the total enzyme concentration in mg. of enzyme N per cc., $t$ is the time in minutes, $a_0$ is the initial substrate concentration, and $a$ the substrate concentration at time $t$, the latter two in moles per liter.

Since the specific constant, $k'$, characterizing the rate of disappearance of the substrate (equation (8)), is given by the sum of a first order term and of a zero order term, it is evident that the reaction will approach first
order kinetics as $K_m$ increases, or specifically, as $k_1$ decreases in comparison to $k_2 + k'$ (equation (6)). Conversely, the reaction course will approach zero order kinetics as $K_m$ decreases, or specifically, as $k_1$ increases in comparison to $k_2 + k'$.

Since it was found in the present work that, with all substrates that were tested, the apparent first order reaction constant decreased with increasing substrate concentration, equation (8) was used to evaluate reaction rates. The value of $K_m$ used in this equation was determined from the relation of Lineweaver and Burk (10) as previously described (5).

For comparison with the work of Bergmann and Fruton (2-4), apparent proteolytic coefficients were likewise calculated from the initial, linear portions of the curves obtained when the experimental data were plotted according to the first order equation. Maximum proteolytic coefficients, $C_{\text{max}}$, were calculated as previously described (5), with use of the relation:

$$C_{\text{max}} = \frac{1}{2.3} \frac{k'}{K_m} \tag{9}$$

**Peptidase Activity**

Glycyl-L-tyrosinamide Acetate (GTA)—The enzymatic activity of the preparation of chymotrypsin was tested against GTA, for which quantitative data are available in the literature (4). In 0.05 M substrate solution, 0.1 M phosphate buffer, pH 7.8, 25°, the apparent proteolytic coefficient was $C = k/e = 0.0097 \pm 0.0011$ (five determinations at varying enzyme concentrations). This value is significantly higher than that obtained by Fruton and Bergmann under similar experimental conditions, i.e. $C = 0.0065$.

The apparent proteolytic coefficient increased with decreasing substrate concentration, $C$ being $0.0121 \pm 0.0016$ in 0.025 M concentration of GTA, and 0.0129 in 0.0125 M concentration of this substrate. $K_m$ was deter-

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2 This equation represents an approximation, since it presupposes first order reaction kinetics to apply as substrate concentration approaches zero. It is valid only if $k_2$, the specific reaction constant for the dissociation of the enzyme-substrate complex, is negligibly small in comparison to $k'$, since $K_m = (k_2 + k')/k_1$ (see also footnote 1 of reference (5)).

3 $k$ denotes the first order reaction constant as defined in Bergmann's work, i.e. $k = (1/t) \log_{10} a_0/a$, where $a_0$ and $a$ denote, respectively, initial substrate concentration and substrate concentration at time $t$.

4 It was found that GTA was spontaneously hydrolyzed at pH 7.8, even when stored at 4°. The extent of spontaneous hydrolysis was negligible during the experimental period of 2 to 3 hours; however, blank corrections were made in each enzymatic experiment. The crystals which were collected after storage of a stock solution of GTA in the cold, melted with decomposition at 265-275°. Abderhalden (11) reports a melting point of 295° for glycyl-L-tyrosine.
ACTIVITY OF CHYMOTRYPSIN

minded from a plot according to equation (15) of reference (5) where \( v \) was taken as the moles per liter of substrate hydrolyzed during the first 5 minutes of the reaction. This plot is shown in Fig. 1 together with similar plots for other substrates. \( K_m \) was calculated as 0.122. Substitution of this value into equation (8) yielded for each rate experiment a linear relation from which \( k' \) was determined to be 4.1 to 4.3 \( \times \) 10^{-3} when \( a_0 \) was varied from 0.05 \( \text{M} \) to 0.0125 \( \text{M} \). Substitution of these values into equation (9) yielded \( C_{\text{max.}} = 0.015 \).

![Figure 1](http://www.jbc.org/)

**Fig. 1.** A plot of the Lineweaver-Burk (10) equation for the determination of enzyme-substrate dissociation constants, \( K_m \). The curves refer to the substrates indicated; for details, see the text and Table I.

Since all of the other substrates that were studied in this work were relatively insoluble in water but soluble in dilute methanol, the rate of enzymatic hydrolysis of GTA was measured as a function of the methanol concentration, in order to provide a common basis of comparison of hydrolysis rates. It was found that in 0.025 \( \text{M} \) solutions of GTA the apparent proteolytic coefficient decreased logarithmically with increasing methanol concentration, as shown in Fig. 2, along with similar data for BTEE. The slopes of the straight lines were practically identical for the peptide (GTA) and for the ester (BTEE), i.e. 0.0279 for the former and 0.0276 for the latter. Since the presence of methanol introduces a constant second order term, it appears that the activity of chymotrypsin is essentially undiminished. This finding is in qualitative agreement with the work...
of Risley et al. (12) in which it was found that trypsin exhibits considerable activity in ethanol solutions up to 30 per cent. The quantitative relation established by the present measurements renders it possible to compare the hydrolysis rates of substrates, which are relatively insoluble in water but soluble in methanol-water mixtures, with the hydrolysis rate of a "standard" water-soluble substrate for chymotrypsin, i.e. GTA.

Benzoyl-L-tyrosinamide (BTA)—BTA, previously reported to be entirely resistant to chymotryptic hydrolysis (3), was found to be hydrolyzed more rapidly than GTA. Because of its limited solubility in water, measurements were performed in 30 per cent methanol, 0.05 M phosphate buffer, pH 7.8. In 0.0266 M substrate solutions, the apparent proteolytic coefficient was \( C = 0.0453 \) or about 20 times higher than the value obtained for GTA by extrapolation of the data plotted in Fig. 2 to 30 per cent methanol \( (C = 0.0023) \). The apparent proteolytic coefficient increased with decreasing substrate concentration, being \( C = 0.0498 \) in 0.0186 M substrate solution, and \( C = 0.0597 \) in 0.0106 M substrate solution. \( K_m \), determined from the data plotted in Fig. 1, was 0.0424. Substitution of this value into equation (8) gave values of \( k' = 6.47 \pm 0.31 \times 10^{-8} \) (six
measurements at varying enzyme and substrate concentrations). From these values, \( C_{\text{max}} \) was calculated as 0.066.

**Esterase Activity**

**Benzoyl-L-tyrosine Ethyl Ester (BTEE)**—This specific ester was hydrolyzed by chymotrypsin considerably faster than any of the specific peptides previously described. Hydrolysis apparently followed first order reaction kinetics; however, an unusually large substrate concentration dependence of the apparent proteolytic coefficients was noted. Because of the uncertainty of the initial substrate concentration in potentiometric measurements of esterase activity (6), recourse was had to the determination of \( a_0 \) by interpolation according to a method previously described (6). It follows from the properties of the equation of first order reaction kinetics that for any consecutive pair of time values, such that

\[
1 - \frac{z_2}{z_1} = 2 \frac{t_2}{t_1},
\]

\[
a_0 = \frac{x_1}{(2x_1 - x_2)} \quad (10)
\]

where \( x_2 \) and \( x_1 \) are the moles per liter of substrate hydrolyzed at times \( t_2 \) and \( t_1 \). In order to test the accuracy of this method on substrates for chymotrypsin, the rate of hydrolysis of BTA by chymotrypsin was determined, with the use of an initial, analytically determined, substrate concentration of 0.025 M. The initial substrate concentration was also calculated from various portions of the curve shown in Fig. 3, with the results given in the legend to Fig. 3. It will be noted that, within the region corresponding to 43 to 88 per cent of hydrolysis, the calculated value of \( a_0 \) agrees within 3 per cent with the analytical value.

When this method of calculation was applied to the kinetics of esterase activity of chymotrypsin, the apparent proteolytic coefficient for the hydrolysis of BTEE was found to vary from about \( C = 18 \) in 0.025 M initial substrate concentration to about \( C = 83 \) in 0.005 M initial substrate concentration, as shown in the upper curve of Fig. 4. The lower of these two values is still some 8000 times higher than the extrapolated value for the same concentration of GTA in 30 per cent methanol \( (C = 0.0023) \). From the plot shown in Fig. 1, \( K_m \) was determined as 0.0039. Substitution of this value into equation (8) yielded \( k' = 8.15 \pm 0.57 \times 10^{-1} \) (eight determinations at varying enzyme and substrate concentrations). The relative constancy of \( k' \) over the same range of substrate concentration in which \( C \) varied about 4.5-fold is shown in Fig. 4. \( C_{\text{max}} \) (calculated according to equation (9)) was 91.

**Acetyl-L-tyrosine Ethyl Ester (ATEE)**—A relatively small dependence of the apparent proteolytic coefficient on initial substrate concentration was noted, \( C \) being 20.3, 23.7, and 25.3, respectively, for substrate concentrations of 0.023, 0.0136, and 0.008 M. From the plot shown in Fig. 1,
$K_m$ was calculated as 0.074. Substitution of this value into equation (8) yielded a value of $k' = 4.49 \pm 0.12$ (three determinations). The strict adherence of the hydrolysis rate to equation (8) is shown for a typical experiment in Fig. 5 in which the right-hand side of equation (8) is plotted against time. The relation between initial substrate concentration and $C$ and $k'$, respectively, is shown in Fig. 6, which demonstrates the constancy of $k'$ over the same range in which $C$ markedly increases. $C_{\text{max.}}$ (equation (9)) was calculated as 26, which is close to the value of $C$ determined from the lowest substrate concentration. A summary of all similar adherence to equation (8) was found for all substrates that were studied in this work.
kinetic constants determined at 25° for the two peptide substrates and the two ester substrates is given in Table I. Interpretation of these data will be given in the discussion of this paper.

**Fig. 4.** A plot demonstrating the large substrate concentration dependence of apparent proteolytic coefficients, $C$, for the hydrolysis of BTEE by chymotrypsin, as compared to the substrate concentration independence of the specific reaction constant $k'$ (calculated from equation (8)). For further details, see the text and Table I.

**Influence of Temperature on Reaction Rates**

According to the concept of Michaelis and Menten (13) it has been assumed in this work that enzymatic hydrolysis proceeds in at least two steps: The first of these is the formation of a stable enzyme-substrate
complex, governed by the affinity constant, or its inverse function, the dissociation constant, $K_m$. The second step involves the activation of the stable complex and subsequent decomposition into the reaction products and free enzyme, and is governed by the specific reaction constant, $k'$.\(^6\)

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**Fig. 5.** The rate of hydrolysis of ATEE by chymotrypsin, calculated according to equation (8). $F$ denotes the right-hand side expression of equation (8), and is plotted along the axis of the ordinate against time, which is plotted along the axis of the abscissa. The slope of the straight line is equal to $k'e$.

Since $K_m$ defines a state of equilibrium, determination of the influence of temperature on $K_m$ yields the classical thermodynamic constants, $\Delta H$, $\Delta F$, and $\Delta S$, the heat of the reaction, the free energy, and the entropy.

\(^6\) Since $K_m$ as defined herein (equation (6)) contains explicitly $k'$, its inverse function is not an exclusive measure of the affinity of the enzyme for the substrate. The true affinity constant, $k_1/k_2$, is indeterminate in those as in any kinetic studies which are based on measurements of the rate of disappearance of the substrate.
of the reaction, respectively. The following relations have been used to evaluate these constants.

$$\log \frac{K_m''}{K_m'} = \frac{\Delta H}{2.3R} \left( \frac{1}{T_1} - \frac{1}{T_2} \right)$$  \hspace{1cm} (11)

$$\Delta F = -2.3RT \log K_m$$ \hspace{1cm} (12)

$$\Delta S = \frac{1}{T} (\Delta H - \Delta F)$$ \hspace{1cm} (13)

**Fig. 6.** A plot showing the substrate concentration dependence of the apparent proteolytic coefficient, $C$, for the hydrolysis of ATEE by chymotrypsin, as compared to the substrate concentration independence of the specific reaction constant, $k'$ (calculated from equation (8)). For further details see the text and Table I.

Calculations of $\Delta F$ and $\Delta S$ have been referred to a standard temperature of 298° K.

The relation between the specific reaction constant, $k'$, and temperature may be formally expressed by the Arrhenius equation

$$\log \frac{k'}{k'_1} = \frac{\Delta E}{2.3R} \left( \frac{1}{T_1} - \frac{1}{T_2} \right)$$ \hspace{1cm} (14)
where $\Delta E$ denotes the critical increment of temperature ($\Delta E = \Delta H^* + RT$). From this value, the heat of activation, $\Delta H^*$, and the free energy, $\Delta F^*$, and the entropy of activation, $\Delta S^*$, may be determined (14) by the equations

$$k' = \frac{kT}{h} \exp \frac{-\Delta H^*}{RT} \exp \frac{\Delta S^*}{R} = 6.25 \times 10^{12} \exp \frac{-\Delta H^*}{RT} \exp \frac{\Delta S^*}{R}$$

(15)

$$\Delta F^* = \Delta H^* - T\Delta S^*$$

(16)

**Table I**

**Kinetic Constants for Hydrolysis of Specific Peptide and Ester Substrates by Chymotrypsin at pH 7.8, 25°C**

$a_0$ is the initial substrate concentration; $C$ is the proteolytic coefficient calculated on the assumption of first order reaction kinetics, $C_{\text{max}}$, is the maximum proteolytic coefficient calculated from equation (9). For the determinations of $K_m$ and $k'$, see the text.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Methanol per cent</th>
<th>$a_0$ (M)</th>
<th>$C$</th>
<th>$C_{\text{max}}$</th>
<th>$K_m$</th>
<th>$k'$</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTA</td>
<td>0</td>
<td>0.0097</td>
<td>0.122</td>
<td>0.0041</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0.0121</td>
<td>0.0424</td>
<td>0.0065</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0.0129</td>
<td>0.015</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.0023</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BTA</td>
<td>30</td>
<td>0.0453</td>
<td>0.0424</td>
<td>0.0065</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.0496</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.0597</td>
<td>0.066</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BTEE</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.0597</td>
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</tr>
<tr>
<td>ATEE</td>
<td>30</td>
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<td></td>
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<tr>
<td></td>
<td>30</td>
<td>0.60</td>
<td>83.5</td>
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</tr>
<tr>
<td></td>
<td>30</td>
<td>0.30</td>
<td>40.3</td>
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<tr>
<td></td>
<td>30</td>
<td>0.80</td>
<td>25.3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* By extrapolation, by means of the equation, log $(C \times 10^4) = 1.15 - 0.0276$ (per cent methanol). For details, see the text and Fig. 2.

where $k$ denotes the Boltzmann constant, $h$, the Planck constant, $R$, the gas constant, and $k'_0$ the absolute specific reaction constant in sec.~$^{-1}$, corrected to an enzyme concentration of 1 mole per liter (molecular weight of chymotrypsin assumed to be 36,000), at a standard temperature of 298° K.

Measurements of the effects of temperature on $K_m$ and $k'$ have been carried out with the substrates BTA and BTEE. The temperatures were 9.5°, 18°, and 25° for BTA, and 7.5°, 15.5°, and 25° for BTEE. All since $k'$ is the specific reaction constant per mg. of enzyme N per cc., per minute, the relation between $k'_0$ and $k'$ is simply $k'_0 = (k'/60) (36,000/6.25)$, assuming a molecular weight for chymotrypsin of 36,000.
measurements were performed in the presence of 30 per cent methanol, and 0.05 phosphate buffer, pH 7.8. The results are shown in Fig. 7 in which log $k'$ and log $K_m$, respectively, are plotted along the axis of ordinates and $1/T \times 10^3$ along the axis of abscissas. From these sets of data, energetic constants were calculated by equations (11) to (16), and

are tabulated in Table II. Interpretation of these data will be deferred for the discussion of this paper.

Influence of pH

Comparative measurements of the effect of pH on the peptidase and esterase activities of chymotrypsin were made with BTA and BTEE as substrates. The buffers employed were borate buffer between pH 9.2 and pH 8.2, phosphate buffer between pH 8.2 and pH 7.0, and acetate buffer at pH 6.2. Buffer concentration was 0.045 M for BTA and 0.050 M for BTEE. Substrate concentration was 0.0125 M for BTA, while
# Table II

**Thermodynamic Constants for Hydrolysis of BTA and BTEE by Chymotrypsin**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (X 10^{-2})</th>
<th>$\Delta H$ (calories)</th>
<th>$\Delta S$ (calories E.U.)</th>
<th>$k'$ (sec^{-1})</th>
<th>$k_0$ (calories)</th>
<th>$\Delta H^*$ (calories)</th>
<th>$\Delta S^*$ (calories)</th>
<th>$k_0'$$\Delta H^* + \Delta S^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTA</td>
<td>4.24</td>
<td>10,900</td>
<td>+30.6 6.47</td>
<td>0.62†</td>
<td>14,600</td>
<td>14,000</td>
<td>-13.0</td>
<td>17,900 16,000</td>
</tr>
<tr>
<td>BTEE</td>
<td>3.90</td>
<td>8,400</td>
<td>+17.8 8.15</td>
<td>78.0‡</td>
<td>9,200</td>
<td>8,600</td>
<td>-21.4</td>
<td>15,000 11,700</td>
</tr>
</tbody>
</table>

† Calculated for $T = 298^\circ$, by means of equations (11) to (16).
‡ $k_0' = \frac{k'}{60} \times \frac{36,000}{6.25}$.

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**Fig. 8.** The pH dependence of the hydrolysis of BTA and of BTEE by chymotrypsin. For the buffer solutions employed, see the text. • denotes BTA, and ○ denotes BTEE. The initial substrate solutions of BTA were 0.0125 M, while the values obtained for BTEE were corrected to a common substrate concentration of 0.01 M. The apparent proteolytic coefficient determined at pH 7.8 was taken as unity and the results obtained at all other pH values were expressed as fractional values (axis of the ordinate).

Measurements on BTEE were corrected from the plot shown in Fig. 4 to a common substrate concentration of 0.01 M.‡ The results of these

* This was necessary because of the uncertainty of the initial substrate concentra-
measurements are shown in Fig. 8 in which the apparent proteolytic coefficients for the hydrolysis of BTA and BTEE, respectively, relative to $C = 1$ at the pH optimum, are plotted against pH. A sharp pH maximum at pH 7.8 is evident. As an approximation, the pH dependencies for the hydrolysis of the peptide and the ester are alike, suggesting that both activities are a function of the same active groups on the enzyme surface. A complete description of the effect of pH requires determination of both $K_m$ and $k'$ in relation to pH.

Inhibitors

It has already been shown that certain structural analogues of reaction products serve as inhibitors for a proteolytic enzyme (5). In the present work, 1-phenyl-2-acetaminobutanone-3 (PAAB) and benzoyl-L-tyrosine hydrazide (BTH) were prepared and tested as structural analogues of specific substrates. As can be seen from the structural formula, PAAB is related to acetyl-L-phenylalanine methyl ester (the analogue of the powerful substrate ATEE), the only difference being that the carbomethoxy group, $\text{--COOCH}_3$, has been replaced by the methylketone group, $\text{--COCH}_3$. However, PAAB failed to exert any inhibitory activity whatsoever on the chymotryptic hydrolysis of BTA and BTEE when present in a substrate-inhibitor ratio of 6:1.

BTH, in which the amide group, $\text{--CONH}_2$, of BTA is replaced by a hydrazide group, $\text{--CONHNH}_2$, was likewise found to be ineffective as an inhibitor. This search for an inhibitory analogue of specific peptide and ester substrates of chymotrypsin is being continued.

\[
\text{\begin{center}
\begin{tabular}{c}
\text{CH}_3 \text{--NH--CH--C--CH}_3 \\
\text{O} \quad \text{CH}_3 \text{O}
\end{tabular}
\end{center}
}\]

PAAB (1-phenyl-2-acetaminobutanone-3)

\[^{9}\text{Such determinations have been made for the system carboxypeptidase-carboxyglycyl-L-phenylalanine (Elkins-Kaufman, E., and Neurath, H., unpublished experiments), and have shown that both $K_m$ and $k'$ vary with pH. Analogous measurements for the chymotryptic hydrolysis of BTA and BTEE will be reported later.}\]
DISCUSSION

The results of the present investigation on the substrate specificity of chymotrypsin are in accord with two of the postulates of Bergmann and coworkers (2-4): (1) Chymotrypsin catalyzes the hydrolysis of peptides containing the aromatic amino acids, tyrosine and phenylalanine. (2) Hydrolysis occurs at the peptide bond which involves the carbonyl group of these amino acids. The definition of additional structural requirements, particularly as they pertain to the α-amino group of tyrosine, necessitates reconsideration of the data of Fruton and Bergmann.

Fruton and Bergmann (4) reported that L-tyrosinamide (TA) and L-phenylalaninamide (PA), the simplest conceivable substrates previously known, are hydrolyzed at a slow rate, corresponding to apparent proteolytic coefficients of about $C = 3 \times 10^{-4}$. If the amino group is converted into a secondary peptide group, and an additional amino group is introduced 2 carbon atoms further removed from the α-carbon atom of tyrosine, as in GTA (4), the rate of hydrolysis is increased about 30-fold ($C = 0.01$ in 0.05 M substrate concentration). If, however, the terminal amino group is absent, as in BTA, the rate of hydrolysis is increased further, i.e. 20-fold with respect to GTA, and 600-fold with respect to TA, demonstrating the antagonistic influence of a positive charge on the combination between enzyme and substrate.

The data of Fruton and Bergmann on carbobenzyoxglygyl-n-tyrosinamide are in apparent contradiction to these conclusions (4). This substrate has been reported to be relatively resistant to chymotryptic hydrolysis in aqueous solution, the apparent proteolytic coefficient, calculated from a single rate value, being about $C = 4 \times 10^{-5}$. Since it has already been shown that carbobenzyoxglygyl-L-phenylalanine ethyl ester is a specific substrate for chymotrypsin (6), the hydrolysis of carbobenzyoxglygyl-L-tyrosinamide was reinvestigated. In 0.025 M substrate concentration and 30 per cent methanol, the apparent proteolytic coefficient was about $C = 6.2 \times 10^{-4}$; which is about 3 times higher than the value obtained for GTA under similar experimental conditions.

It may be concluded, therefore, that the free α-amino group of tyrosyl peptides interferes with their hydrolysis by chymotrypsin, particularly when it is in close proximity to the susceptible peptide bond. Conversely, masking of this group by carbobenzyoxylation, acetylation, or benzoylation increases, in the order named, the susceptibility of the resulting peptide to enzymatic hydrolysis.

The higher susceptibility of specific esters to chymotryptic hydrolysis, particularly the structurally simplest substrate for chymotrypsin is the methyl ester of β-phenyllactic acid (Snoke, J. E., unpublished experiments).
in comparison to that of specific peptides, is evidenced by the data given in Table I, and is in accord with previous comparative measurements on esterase and peptidase activities of other proteolytic enzymes (6, 7). Since the kinetic measurements have been evaluated on the basis of a two-step enzymatic reaction (equation (1)), the effect of substrate structure on both of these steps, i.e. formation of the stable enzyme-substrate complex and decomposition of the latter into reaction products and free enzyme, has to be considered. Since the constant, $K_m$, determines the equilibrium between the formation of this complex and its decomposition, comparison of $K_m$ values for different substrates will not yield an explicit measure of the relative affinities of the enzyme for various substrates. However, the maximum proteolytic coefficient, $C_{\text{max}}$, represents the specific reaction constant per unit enzyme concentration in a range of substrate concentration sufficiently low to render this combination the rate-determining step (see equation (9) and reference (5)).

In these low ranges of substrate concentration, first order reaction kinetics apply, and comparison of $C_{\text{max}}$ values for various substrates will give a measure of their relative affinities. On this basis of comparison, the specific ester, BTEE, is some 8000 times more susceptible to chymotryptic hydrolysis than is the corresponding peptide, BTA, whereas the ester ATEE is only about one-fourth as susceptible as is the corresponding benzoyl derivative, BTEE.

The specific reaction constant, $k'$, is the rate of activation and decomposition of the enzyme-substrate complex per mg. of enzyme N per cc., in moles per liter per minute. Accordingly, $k'_0$ is the rate of activation and decomposition per mole per liter of enzyme-substrate complex per second (see equation (7)), and may be related to the susceptibility of the adsorbed substrate to activation and hydrolysis. According to the data given in Tables I and II, this activation and decomposition reaction proceeds about 120 times faster for the ester BTEE than for the corresponding peptide, BTA, whereas the acetylated ester, ATEE, decomposes even more readily than the benzoylated ester, BTEE. Additional interpretations are rendered available by considerations of the energetic data discussed below.

The substrate concentration dependence of apparent proteolytic coefficients varies in magnitude from substrate to substrate (Table I) and is highest for the substrate with the lowest $K_m$ value. This is in accord with expectations, since reactions tend to approach zero order kinetics as the equilibrium between free and combined enzyme shifts to the right of equation (1) (see also equation (8)). Conversely, first order reaction kinetics predominate as the rate of combination between enzyme and substrate becomes the rate-determining step (high $K_m$ values). The overlapping and crossing of the curves obtained when the proteolytic coefficient for
each substrate is plotted against substrate concentration (see Table I and Figs. 4 and 6) demonstrate the ambiguity which results when apparent proteolytic coefficients for the hydrolysis of two or more substrates by the same enzyme are compared at a single substrate concentration. Thus, in 30 per cent methanol and 0.025 M substrate concentration, ATEE appears to be more readily hydrolyzed than BTEE, whereas the maximum proteolytic coefficients are actually in the inverse order.

The thermodynamic data given in Table II afford an analysis of the mechanism of enzymatic hydrolysis of specific peptide and ester substrates for chymotrypsin. The equilibrium between the reactants, the stable enzyme-substrate complex $ES$ and the activated complex $ES^*$, is expressed by the equilibrium constant, $K_m$. The formation of the stable complex is accompanied by a decrease in entropy of $\Delta S = -30$ e.u. for the peptide (BTA), and of $\Delta S = -17$ e.u. for the ester (BTEE). The entropy decrease may be accounted for in part by the loss of rotational motion of the substrate molecules when they combine with the enzyme. Values within the range of $\Delta S_{rot} = -9.3$ to $-15.5$ e.u. have been calculated for specific peptides (15). This would account for about one-half of the total entropy loss reported in this paper. Alternatively, if it is assumed that both enzyme and substrate are hydrated, part of the entropy change may be ascribed to the "freezing out" of solvent molecules during complex formation. The higher entropy change calculated for the peptide as compared to the ester may be correlated either with a higher degree of orientation of the substrate or else with a larger number of "frozen" solvent molecules or with their higher orientation. The corresponding decrease in free energy is $\Delta F = -1900$ calories for the peptide and $\Delta F = -3300$ calories for the ester. The order of magnitude of these thermodynamic constants is comparable to that of those few other enzyme systems for which the temperature dependence of $K_m$ has been determined (16).

Since $k'_0$ is the specific reaction constant per mole of enzyme-substrate complex, the thermodynamic data given in the right-hand half of Table II are independent of the energetic changes incurred in the formation of the stable complex, and are solely characteristic of the activation process. This process is accompanied by an entropy decrease of $\Delta S^* = -13.0$ e.u. for the peptide and of $\Delta S^* = -21.4$ e.u. for the ester. These values are somewhat higher than those calculated by Butler (17) from data obtained under less strictly defined experimental conditions for the hydrolysis of proteins and peptides by trypsin and chymotrypsin.11 The

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11 Since Butler's data (17) were calculated from first order reaction constants, they are not strictly comparable to the present calculations. Comparison could be made if Butler's measurements had been performed in a range of substrate concentration
entire entropy decrease observed here may be numerically accounted for by the loss of entropy of the reacting water molecules required for the hydrolysis of the peptide or ester bonds (about $-19 \text{ E.U.}$ (15)).

The free energy change for the reaction, $E + S \ldots \rightarrow ES^*$ (equation (1)), $-\Delta F + \Delta F^*$, is approximately $-1900 + 17,900 = 16,000$ calories for the peptide hydrolysis, and $-3300 + 15,000 = 11,700$ calories for the ester hydrolysis. The free energy gain resulting from the exergonic combination of enzyme and substrate compensates in both cases for only a small part of the free energy loss of the endergonic activation of the complex.

The free energy change of activation is considerably smaller than that calculated for the acid hydrolysis of benzoylglycine and acetylglycine, e.g. about 30,000 calories (15). While this is in agreement with accepted theories of the mechanism of enzymatic activation, thermodynamic data for the enzymatic and non-enzymatic hydrolysis of the same substrate under otherwise identical conditions are needed to establish strict comparison, since the free energy of activation for the hydrolysis of tyrosyl peptides may differ considerably from that of N-substituted glycine. Moreover, the change in dielectric constant of the solvent by the addition of methanol undoubtedly affects the free energy of activation (18).

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in which the first order reaction constant is independent of initial substrate concentration, in which case the calculated entropy change would be equal to $-\Delta S + \Delta S^*$ of the present data. The values given by Butler seem to be in error since the factor $RT/N_h = 5.7 \times 10^{13}$ and not $5.7 \times 10^{14}$ as used by Butler. When this correction is applied to the chymotryptic hydrolysis of benzoyl-L-tyrosylglycinamide, $\Delta S^*$ is $-13.1$ E.U. instead of the published value of $-17$ E.U. Similar corrections apply to all other thermodynamic constants given in that paper.

Determinations of the thermodynamic constants of enzyme-catalyzed reactions recorded in the literature (16) have usually yielded approximated values for the overall activation reaction, the two-step nature of the reaction being neglected. The algebraic sum of $-\Delta F + \Delta F^*$ given in the last column of Table II is the approximate value for the over-all activation reaction.

The thermodynamic constants for the hydrolysis of carbobenzoxyglycyl-L-phenylalanine by carboxypeptidase at pH 7.5 are of the same order as those given here for the chymotryptic hydrolysis of BTA (Elkins-Kaufman, E., and Neurath, H., manuscript in preparation).
SUMMARY

1. The substrate specificity of chymotrypsin has been reinvestigated, with particular emphasis on the role of a free terminal amino group of tyrosyl peptides. Benzoyl-L-tyrosinamide (BTA) has been found to be the most specific peptide substrate for chymotrypsin.

2. Specific tyrosyl esters, such as benzoyl-L-tyrosine ethyl ester (BTEE) and acetyl-L-tyrosine ethyl ester (ATEE), are hydrolyzed considerably faster than the most specific peptide substrate previously described, e.g. glycyl-L-tyrosinamide (GTA).

3. The specific ester and peptide substrates are hydrolyzed by chymotrypsin in the presence of methanol, the logarithm of the apparent proteolytic coefficient decreasing linearly with increasing methanol concentration up to 30 per cent.

4. The kinetics of the chymotryptic hydrolysis of GTA, BTA, BTEE, and ATEE have been evaluated on the assumption of a two-step enzymatic reaction, i.e. the formation of a stable enzyme-substrate complex and the subsequent activation and decomposition of the complex. $K_m$, the enzyme-substrate dissociation constant, and $k'$, the specific rate constant for the activation and decomposition reactions, have been determined for the chymotryptic hydrolysis of each of these substrates.

5. The influence of temperature on $K_m$ and $k'$ for the chymotryptic hydrolysis of BTA and BTEE has been determined and thermodynamic constants for the equilibrium reaction and the activation rate have been evaluated. The mechanism of enzymatic hydrolysis of these specific peptide and ester substrates has been analyzed in terms of the thermodynamic data.

6. Preliminary measurements of the pH dependence of chymotryptic hydrolysis of a specific peptide (BTA) and of a specific ester (BTEE) indicate a sharp maximum at pH 7.8, the pH-activity curves being similar for both types of substrates.

7. Preliminary, although negative, results are recorded for the inhibition of chymotrypsin by two structural analogues of specific substrates.

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