THE SPECIFIC ESTERASE ACTIVITY OF TRypsIN

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The discovery of relatively simple peptide derivatives as substrates for proteolytic enzymes has introduced a new approach to the problem of the specificity of this group of hydrolytic enzymes. Although crystalline trypsin is known to catalyze the hydrolysis of internal peptide bonds of proteins, its action toward internal peptide bonds of synthetic substrates has been demonstrated for only one case (1). Since all other synthetic substrates for trypsin, described in the extensive work of Bergmann and coworkers (2), are amides of lysine and arginine derivatives and are split enzymatically into ammonia and the corresponding amino acid derivatives, the action of trypsin toward these substrates may be more adequately described as an amidase activity.

In the course of an investigation of the inhibition of crystalline pancreatic proteolytic enzymes by specific low molecular weight compounds the discovery was made that crystalline trypsin is likewise a powerful catalyst for the hydrolysis of certain amino acid esters. The most specific substrates of this type are α-benzoyl-L-arginine methyl ester (BAME) and α-toluene-sulfonyl-L-arginine methyl ester (TSAME). It was also found that, contrary to previous reports (3), trypsin catalyzes the amide hydrolysis of α-toluene-sulfonyl-L-argininamide and does so even more rapidly than it catalyzes the hydrolysis of the classical substrate, α-benzoyl-L-argininamide. The structural specificity of these esters for trypsin is approximately analogous to that of the corresponding amides, although a certain measure of cross-reactivity of one of these (BAME) with chymotrypsin exists. Both the esterase and amidase activities are apparently mediated by the same active surface configurations of the enzyme, as is evidenced by studies of various modes of partial and complete enzyme inactivation.

The results of these kinetic studies are presented in this paper.

EXPERIMENTAL

Preparations

Enzymes—Three lots of crystalline trypsin and chymotrypsin were prepared according to the method of Kunitz and Northrop (4); activation of trypsinogen was carried out by the procedure of McDonald and Kunitz.
Although difficulty was encountered in crystallizing trypsinogen, highly active, wholly crystalline trypsin was obtained by applying the method of McDonald and Kunitz to amorphous trypsinogen. All active enzyme preparations were recrystallized once. A portion of one preparation of trypsin was further purified by trichloroacetic acid precipitation (4). Crystalline \( \gamma \)-chymotrypsin was prepared from the chymotrypsin filtrates of two preparations according to the method of Kunitz (6).

One trypsin preparation was dialyzed in the cold for 72 hours against 0.001 \( \text{n} \) sulfuric acid and was subsequently lyophilized.

**Substrates**—\( \alpha \)-Benzoyl-\( L \)-argininamide hydrochloride (BAA), \( \alpha \)-benzoyl-\( L \)-arginine methyl ester hydrochloride, \( \alpha \)-\( p \)-toluenesulfonyl-\( L \)-argininamide (TSAA), and \( \alpha \)-\( p \)-toluenesulfonyl-\( L \)-arginine methyl ester hydrochloride were prepared essentially according to the method of Bergmann, Fruton, and Pollok (3). A slightly improved yield of benzoylarginine was achieved by adding benzoyl chloride and 10 per cent sodium carbonate to the aqueous solution of arginine monohydrochloride at rates such that the reaction mixture was kept just alkaline to phenolphthalein. By this procedure 21.2 gm. of arginine monohydrochloride yielded 24 gm. of \( \alpha \)-benzoylarginine.

Although BAME has not been prepared in the crystalline state, it was converted to a stable, although highly hygroscopic, glass by the following procedure: The solvent was removed \textit{in vacuo} from a methanolic solution of approximately 8 gm. of BAME until a solid foam formed. The foam was dissolved in 30 ml. of water and the pH adjusted from 0.75 to 8.75 with 5 \( \text{n} \) NaOH. A resulting slight precipitate was filtered off and the solution reacidified to about pH 3 with 5 \( \text{n} \) HCl. The water was removed \textit{in vacuo} and two portions of absolute methanol were added and distilled \textit{in vacuo} to remove the last traces of water. The resulting oil was then dissolved \textit{in vacuo} in 60 ml. of absolute methanol and the NaCl was filtered off. The solution was again distilled \textit{in vacuo} and the remaining oil was heated to 100\(^\circ\) for 10 minutes. The product is a friable, white frothy glass which was stored in a vacuum desiccator. On dry days this material could be weighed without difficulty. Although the product was not suitable for analysis, its identity was proved by conversion of a portion of it to BAA.

Carbobenzyloxygycyl-\( L \)-tyrosine ethyl ester and \( N \)-carbobenzyloxy-\( O \)-acetyl\( L \)-tyrosylglycine ethyl ester were prepared according to the method of Bergmann and Fruton (7).

\( \epsilon \)-Carbobenzyloxy-\( L \)-lysine methyl ester hydrochloride was prepared according to Bergmann, Zervas, and Ross (8), and carbobenzyloxygycyl-\( D \)-phenylalanine ethyl ester was a preparation previously described (9, 10).

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\(^1\) We are indebted to Dr. C. E. Graham of The Wilson Laboratories, Chicago, for the crude pancreatic protein precipitates used in these isolations.
Tyrosine ethyl ester hydrochloride and L-phenylalanine ethyl ester hydrochloride were prepared in the usual manner.

Glycine ethyl ester hydrochloride, ethyl butyrate, and butyl acetate were Eastman preparations and were used without purification.

Methods

Amidase Activity—The amidase activity of the proteolytic enzymes was measured by a modification of the Conway micro diffusion technique (11). 0.75 ml. of 2 per cent boric acid solution was substituted in the inner chamber of the Conway plate for the strong acid solution used by Conway.\(^2\) By this means the ammonia evolved could be titrated directly and difficulties encountered in the back titration in air with dilute strong base were avoided.

The reaction mixtures used for determination of amidase activity were made by mixing equal volumes of 0.1 \(M\) substrate in phosphate buffer and of enzyme solution in phosphate buffer. In earlier runs \( \frac{1}{15} \) phosphate buffer was used, but later this concentration was raised to 0.1 \(M\) to insure pH control. Because of the well known instability of trypsin in the pH range in which it is active, the enzyme was dissolved in the buffer immediately before it was added to the substrate solution.

All amidase activities were determined at 25° with shaking. 0.2 ml. samples were withdrawn at intervals for analysis and were introduced into the Conway plates. After each sample was mixed with 1 ml. of saturated \(K_2CO_3\) solution used to volatilize the ammonia, the plates were allowed to stand for at least 1 hour before being titrated with approximately 0.01 \(N\) HCl and 1 drop of Tashiro's indicator. Thus, 0.01 ml. of acid corresponded to approximately 1 per cent hydrolysis. Since the indicator color varied with the volume of the system at the end-point, water was added to the plates in which the extent of hydrolysis was small in order to bring the final volume for all titrations close to a constant volume.

Time was measured from the time of the completion of addition of the trypsin solution to the time at which the saturated \(K_2CO_3\) solution touched the sample. It was assumed that the enzyme was inactivated immediately by the \(K_2CO_3\) solution and that any temperature effect due to the difference between the bath temperature and room temperature could be neglected, since the difference was not large and also since the elapsed time was never more than 1 minute. The validity of the first assumption seemed to be confirmed by the fact that the blanks were always within a few thousandths of 0.025 ml.

The horizontal burettes used for these titrations were made by drawing out the ungraduated portions of Kimble Exax 1 ml. measuring pipettes and

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\(^2\) This modification was suggested by Miss Elaine Elkins of this laboratory.
fitting the undrawn ends with Clay-Adams pipette suction units. The burettes were subsequently calibrated by weighing to within 0.1 mg. the water delivered at 0.1 ml. intervals. In no case was the error large enough to warrant the application of a correction.

The accuracy of the method was tested with solutions of ammonium sulfate. With 0.05 and 0.005 N solutions of ammonium sulfate as standards, the titration values could be reproduced to within 0.3 per cent.

Preliminary experiments indicated that ammonia is not evolved by saturated K$_2$CO$_3$ solution from BAA, TSAA, trypsin, or chymotrypsin.

Blank determinations were made by placing 0.1 ml. each of substrate and enzyme solutions a short distance apart on Conway plates and by tipping the plates so that the solutions were mixed with the saturated K$_2$CO$_3$ solution before they were mixed with each other.

Esterase Activity—For the determination of the esterase activity precise pH control in the presence of the liberated carboxyl groups necessitated the use of such large quantities of buffer that the conventional amino acid titrations were rendered very difficult. Recourse was had to a direct electrometric titration of the liberated carboxyl groups with approximately 0.2 M NaOH with a Beckman model G pH meter as a null indicator. Low concentrations of buffer were used to render the system stable against great variations in pH but still sensitive to changes in hydrogen ion activity.

Substrate solution, buffer solution, and enough water to bring the final volume to approximately 10 ml. were mixed and equilibrated in a 25° bath. The glass electrodes were introduced and 0.2 M NaOH was added from the horizontal burette previously described until the pH rose to a value 0.2 to 0.5 unit higher than the pH selected as the null point for the determination. The enzyme solution was then added and zero time taken as the time when the pH fell to the null point value. A small increment of 0.2 M NaOH was then added and the rise in pH noted. In nearly all cases the rise in pH was less than 0.2 unit. The time was again recorded when the pH fell to the null value. This process was continued until the reaction stopped or until a sufficient portion of the reaction curve had been determined to establish the nature and rate of the reaction. The setting of the pH meter was checked against a standard buffer after each run.

In most of the experiments stirring was accomplished by means of a slow stream of nitrogen bubbled through the reaction system through a syringe needle, but in the experiments involving a high concentration of enzyme, higher temperatures, or a volatile solvent, stirring was done by hand with a small glass rod.

This method of measurement gave rise to some uncertainty as to the concentration of the substrate at the moment the zero time reading was

*We are indebted to Dr. W. A. Perlzweig for suggesting these useful devices.
made. This is of no consequence for reactions which follow zero order kinetics. The treatment of the data for first order reactions is illustrated in a later section of this paper.

A stock solution of enzyme was made up daily, adjusted to pH 2.5 with HCl, and kept in the refrigerator when not in use.

Nitrogen determinations on the enzyme solutions were made by the semimicro-Kjeldahl method.

Results

Benzoylargininamid

Specific Activity of Trypsin—In order to test the present method of analysis and also to characterize the preparations of trypsin used in these studies, the activity of each preparation toward BAA was determined.

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>$K^* \times 10^{-4}$</th>
<th>Activity remaining (per cent)</th>
<th>$K_2 \times 10^{-4}$</th>
<th>Calculated residual activity ($K_2 = 1.14 \times 10^{-4}$) (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.02</td>
<td>100</td>
<td>1.17</td>
<td>58.6</td>
</tr>
<tr>
<td>61\frac{1}{2}</td>
<td>4.09</td>
<td>58.1</td>
<td>1.12</td>
<td>42.3</td>
</tr>
<tr>
<td>119\frac{1}{2}</td>
<td>3.00</td>
<td>42.7</td>
<td>1.12</td>
<td>26.8</td>
</tr>
<tr>
<td>240</td>
<td>1.90</td>
<td>27.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Throughout this paper $K$ will refer to the first order reaction constant defined by $K = 2.303/t \log (C_0/(C_0 - \alpha))$, where $C_0$ is the initial substrate concentration and $\alpha$ the amount of substrate hydrolyzed in time $t$.

† $K_2$ is the second order reaction constant expressed by Kunitz and Northrop (13) as $K_2 = 1/t(1/A - 1/A_0)$. In the present calculation $t$ is expressed in minutes and $A$ is the per cent of the initial activity, $A_0$, remaining at time $t$.

Measurements were made at four levels of enzyme concentration over an 8-fold range.

In every case the reaction was found to follow first order kinetics strictly and for each preparation the rate constant was directly proportional to the enzyme concentration. The proteolytic coefficients, $C$, for all lots of trypsin prepared in this laboratory were found to fall within the limits ($C = 3.2$ to $3.8 \times 10^{-2}$) reported by previous investigators (12).

The classical studies of Kunitz and Northrop (13) on the stability of crystalline trypsin have shown that at the pH of its maximum activity rapid inactivation occurs. In the present experiments the rate of autolysis of trypsin was redetermined with BAA as substrate. The stabilizing effect of the substrate was sufficient to maintain the activity of the enzyme at a constant value during each activity determination as evidenced by the adherence of the reaction to first order kinetics. 13.6 mg. of Prep-
paration TIIz were dissolved in 5 ml. of m/15 phosphate buffer, pH 7.80. 0.5 ml. was immediately withdrawn and added to an equal volume of 0.1 m BAA in the same buffer. Subsequent samples of the enzyme solution were removed at 1, 2, and 4 hour intervals and the activity against BAA was determined.

The results given in Table I show a decrease in the first order reaction constant, $K$, with time. In agreement with Kunitz and Northrop (13) this has been attributed to the spontaneous inactivation of trypsin and has been expressed as a second order reaction between native and denatured trypsin. With $K$ assumed to be proportional to activity and the remaining activities expressed as per cent of the initial activity at $t = 0$, second order reaction constants, $K_2$, have been calculated with the equation of Kunitz and Northrop (13). The relative constancy of $K_2$ together with the close agreement between calculated and observed residual activities testifies to the validity of this interpretation.

**Esterase Activity**

*Enzymatic Nature of Reaction*—The discovery of the amino acid esterase activity of trypsin was made in the course of a search for a specific low molecular weight inhibitor for trypsin. It was observed that the addition of BAME to the trypsin-BAA reaction system caused a marked decrease in the rate of splitting of BAA without causing any change in the order of reaction and also caused the pH of the system to fall. Qualitative experiments with weakly buffered solutions of BAME indicated that the decrease of pH upon the addition of trypsin was due to the splitting of BAME by trypsin. In one such experiment the pH fell from 7.5 to 4.3 in 35 minutes.

At pH 8 and 25°C the tryptic hydrolysis of BAME is a zero order reaction throughout about 95 per cent of its course. The results of measurements at four levels of enzyme concentration over an 8-fold range are shown in Fig. 1. A plot of the reaction rate against the weight of enzyme N in the reaction system is shown in the inset graph of Fig. 1. The deviation from linearity shown by the highest trypsin concentration in the inset plot is not considered significant, since the rate of this reaction approaches the limiting rate which can be accurately measured with the apparatus used.

The chemical nature of the reaction was established by allowing trypsin to act upon 560 mg. of BAME at pH 8 until the reaction was completed. The reaction system was dried *in vacuo* and extracted with absolute ethanol to separate benzoylarginine from the buffer salts. The ethanol was removed *in vacuo* and 339 mg. of α-benzoylarginine, corresponding to 72 per cent of the BAME used, was recovered following crystallization from water. The recovered benzoylarginine decomposed at 273–275°C as did an authentic

4 This investigation is still in progress.
sample, and its identity was further established by the isolation of benzoic acid following alkaline hydrolysis of a portion of the recovered material.

The following additional observations indicated that the hydrolysis of BAME is mediated by trypsin: (1) alkali-inactivated trypsin has no effect upon BAME; (2) the addition of increasing amounts of crystalline, salt-free soy bean trypsin inhibitor progressively reduced the rate of splitting of BAME and of BAA by trypsin; (3) the rate of autolysis of trypsin at pH 7.80 and 25° is the same when measured by its activity toward BAME and toward BAA. The identity of the autolysis rates of the two activities is shown in Fig. 2 in which, in analogy to the corresponding measurements of amidase activity, the tryptic activity was found to decrease with time according to second order kinetics (13). The experimental points may be

![Graph](http://www.jbc.org/)  
**Fig. 1.** Hydrolysis of BAME by various concentrations of trypsin measured by electrometric titration in 0.015 M phosphate buffer, pH 8.00, at 25°. The amount of trypsin N in mg. present in these systems is shown by ○ 0.0492, • 0.0246, △ 0.0123, ▲ 0.0062. The inset curve indicates the linear relation between the rate of hydrolysis of BAME and the amount of trypsin present.

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*We are deeply grateful to Dr. M. Kunitz for two samples of crystalline trypsin and for the crystalline soy bean trypsin inhibitor used in these experiments.

*Work is still in progress to determine whether the linear relation between decrease in tryptic activity and amount of trypsin inhibitor added, established by Kunitz (14), using protein substrates, holds equally for low molecular weight substrates.
seen to fall on the theoretical curve calculated from the data for BAA given in Table I.

As is shown in Table II, all preparations of trypsin catalyze the hydrolysis of BAME, and the ratio of the specific reaction rate for BAME to the specific initial slope of the trypsin-catalyzed hydrolysis of BAA is essentially a constant of all preparations of trypsin tested. This method of comparing the rates of reactions of different orders was selected as offering the most valid basis for comparison, since in the initial phase of the reaction, the first order reaction, most nearly approaches a zero order reaction.7

**Enzymatic Specificity of Reaction**—The enzymatic specificity of the hydrolysis of BAME was tested at pH 8.0 with crystalline chymotrypsin, γ-chymotrypsin, chymotrypsinogen, carboxypeptidase, and ribonuclease. Of these, eight times recrystallized chymotrypsinogen (0.13 mg. of N per ml. of reaction mixture), six times recrystallized carboxypeptidase (0.009 mg. of N per ml. of reaction mixture), and ribonuclease8 (1 mg. of crystalline enzyme per ml. of reaction mixture) had no measurable effect on 0.015 M BAME in 0.02 M phosphate buffer. In each case the subsequent addition

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7 It would be expected that, if the difference in reaction rates between BAA and BAME were due to a difference in the rate of desorption of the products from the enzyme surface, the addition of ammonium salts to the BAME system would decrease the rate of hydrolysis of BAME. This was not found to be true. Even in 0.1 M NH₄Cl the specific hydrolysis rate of BAME remained constant.

8 We are greatly indebted to Dr. Lawrence L. Lachat, Armour and Company, Chicago, for the ribonuclease, crystallized from alcohol, used in this experiment.
of a small amount of trypsin caused a rapid decrease in the pH of the reaction system.

Both chymotrypsin and γ-chymotrypsin, however, were found to hydrolyze BAME, the reaction following first order kinetics. The reaction curves for these hydrolyses are shown in Fig. 3 for one concentration of γ-chymotrypsin and three concentrations of chymotrypsin.

Since, by the direct titration method of measurement, the reaction starts before the zero time reading is made and since the initial concentration of BAME is not well defined, the following procedure was devised for calculating first order reaction constants. The experimental data were plotted,

<table>
<thead>
<tr>
<th>Trypsin preparation</th>
<th>Concentration mg. N in system</th>
<th>Slope of reaction curve mm per min.</th>
<th>$K_s$ mm per min. per mg. N</th>
<th>$K_{BAA-C4} E$</th>
<th>$K_s K_{BAA-C4} E^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>TII, lyophilized</td>
<td>0.0110</td>
<td>0.00274</td>
<td>0.249</td>
<td>3.92</td>
<td>64</td>
</tr>
<tr>
<td>TIII</td>
<td>0.0087</td>
<td>0.00212</td>
<td>0.244</td>
<td>4.25</td>
<td>65</td>
</tr>
<tr>
<td>CCl₃COOH purified T</td>
<td>0.0089</td>
<td>0.00212</td>
<td>0.238</td>
<td>4.16</td>
<td>57</td>
</tr>
<tr>
<td>Kunitz' Tₙ</td>
<td>0.0124</td>
<td>0.00282</td>
<td>0.228</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot; Lot 2</td>
<td>0.0099</td>
<td>0.00256</td>
<td>0.239</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* $K_s$ will be used throughout this paper to refer to specific zero order reaction constants, i.e. per mg. of enzyme N in the system.

† $K_{BAA-C4}/E$ expresses the initial slope of the BAA hydrolysis curve per mg. of trypsin N per ml. (E).

‡ Mean of several determinations.

TABLE II

Hydrolysis of BAME by Various Preparations of Trypsin

Temperature 25°, 0.015 m phosphate, pH 8.00. Approximate initial concentration of BAME 0.0063 m.
Fig. 3. The rate of hydrolysis of BAME by chymotrypsin and by $\gamma$-chymotrypsin at $25^\circ$. The curves are the theoretical first order reaction curves calculated from the following constants.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Enzyme</th>
<th>Enzyme concentration, mg. N per ml.</th>
<th>pH</th>
<th>Buffer</th>
<th>$K \times 10^{-4}$</th>
<th>$C_0$ (calculated)</th>
<th>$10^{-4}$ m.eq.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\gamma$-Chymotrypsin</td>
<td>0.0806</td>
<td>8.00</td>
<td>0.015 m phosphate</td>
<td>2.70</td>
<td>20.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chymotrypsin</td>
<td>0.3785</td>
<td>7.60</td>
<td>0.0185 &quot;</td>
<td>14.9</td>
<td>8.36</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td>0.196</td>
<td>7.80</td>
<td>0.0185 borate</td>
<td>6.37</td>
<td>7.98</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td>0.0482</td>
<td>7.60</td>
<td>0.0185 phosphate</td>
<td>2.22</td>
<td>8.92</td>
<td></td>
</tr>
</tbody>
</table>

calculated. The fit of these calculated curves to the experimental points is illustrated in Fig. 3.$^9$

$^9$ In several cases the tentative values of $C_0$ were found to increase as larger values of $t_1$ were selected. In these instances greater weight was given to the values of $C_0$ determined for larger values of $t_1$ because the initial points of the reaction curve were subject to a greater error in measuring times than were subsequent points. This
It may be seen qualitatively from Fig. 3 that the rate of hydrolysis of BAME by chymotrypsin increases with the enzyme concentration. The mean value for the proteolytic coefficient of chymotrypsin on BAME is \(1.6 \times 10^{-1}\) (\(C_0\) of BAME about 0.01 M) and of \(\gamma\)-chymotrypsin (single determination) \(1.5 \times 10^{-1}\) (\(C_0\) of BAME about 0.02 M). Since the corresponding mean value of \(K \times C_0/E\) for chymotrypsin is \(3.7 \times 10^{-3}\), it appears that trypsin splits BAME about 65 times as rapidly as does chymotrypsin (cf. Table II).

Effect of pH—It was observed that at pH values greater than 8 BAME undergoes spontaneous hydrolysis at 25°. This phenomenon was investigated as a preliminary to the measurement of the effect of pH on the enzymatic hydrolysis of BAME. The extent of the spontaneous hydrolysis of BAME at 25° and at pH 9, 10, and 11 is shown by the continuous curve and by the initial regions of the discontinuous curves in Fig. 4.

In subtracting the correction for spontaneous hydrolysis from the data obtained in the presence of trypsin, it was assumed that the correction increased linearly with time. Since the reaction in the presence of trypsin is relatively rapid compared to the spontaneous hydrolysis and since the enzymatic runs at pH 9 and 10 show no deviation from linearity except at the extreme end of the reaction, this assumption seems to be a valid first approximation.

Since the rate of the spontaneous hydrolysis is a function of the concentration of BAME (first order kinetics), while the rate of the enzymatic hydrolysis depends only upon the amount of trypsin present (zero order treatment neglects the dilution of the system by the base added in the course of the reaction. For a first order, non-enzymatic reaction this dilution would have no effect upon \(K\), but for an enzymatic reaction the additional reduction in the concentration of the substrate will tend to decrease slightly the tendency for enzyme-substrate complex formation. This change will be relatively small, since the dilution in no case amounted to more than 10 per cent of the initial volume and it has, therefore, been omitted from this calculation.

Because of this cross-reactivity between trypsin and chymotrypsin with BAME, the action of chymotrypsin on BAA was reinvestigated. A chymotrypsin concentration of 0.448 mg. of N per ml. of reaction mixture caused no hydrolysis of 0.05 M BAA. This observation is in agreement with the results of Bergmann, Fruton, and Pollok (3).

The discontinuous curve for pH 10 of Fig. 4 is of particular interest, since the plateau reached after the addition of trypsin defines a rather small region within which the value of \(C_0\), calculated from the initial portion of the curve, must fall if this treatment of the data is satisfactory. It will be observed that the agreement between the experimental and calculated values for \(C_0\) is quite good and that the values for \(K\) for the two curves at pH 10 show fair agreement despite the fact that \(C_0\) for the continuous curve has about twice the value of \(C_0\) for the discontinuous curve (see the legend to Fig. 4).
Fig. 4. Rates of hydrolysis of BAME under various conditions of pH, trypsin concentration, and BAME concentration. The curves shown for the first order reactions are the theoretical curves calculated from the values of \( K \) and \( C_0 \) shown below. Borate buffers were used in these systems.

<table>
<thead>
<tr>
<th>pH</th>
<th>Symbol</th>
<th>Trypsin concentration, mg. N in system</th>
<th>Buffer concentration</th>
<th>( K \times 10^{-2} )</th>
<th>( C_0 ) (calculated)</th>
<th>( 10^{-1} ) m.eq.</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.00</td>
<td>•</td>
<td>0</td>
<td>None</td>
<td>22.2</td>
<td>1.76</td>
<td></td>
</tr>
<tr>
<td>10.00</td>
<td>○</td>
<td>0</td>
<td>0.03</td>
<td>1.55</td>
<td>1.97</td>
<td></td>
</tr>
<tr>
<td>10.00</td>
<td>△</td>
<td>0</td>
<td>0.03</td>
<td>1.67</td>
<td>1.12</td>
<td></td>
</tr>
<tr>
<td>10.00*</td>
<td>△</td>
<td>0.0248</td>
<td>0.024</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.00</td>
<td>▲</td>
<td>0</td>
<td>0.03</td>
<td></td>
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<tr>
<td>9.00*</td>
<td>▲</td>
<td>0.0248</td>
<td>0.024</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* After trypsin was added.

The inset graph illustrates the first order kinetics of the hydrolysis of BAME at pH 11 in the absence of a buffer. \( K \) for the plotted line is \( 2.22 \times 10^{-1} \). \( C_0 \), calculated in milliequivalents \( \times 10^{-3} \), 1.76 (○), 1.70 (●), and 1.58 (△) in 0, 0, and 0.0195 mg. of trypsin N in system.
kinetics), the magnitude of the correction applied for spontaneous hydrolysis was somewhat arbitrary. It was chosen to apply to the initial concentration of BAME.

The pH-activity curve for the tryptic hydrolysis of BAME is shown in Fig. 5. The pH of maximum activity is about pH 8, the activity falling off rapidly below pH 7 and above pH 10. The rate of decrease in activity above pH 10 is intermediate between that observed with trypsin on BAA (3) and that observed with trypsin on casein (13). The inset in Fig. 4 illustrates the point that at pH 11 trypsin does not increase the rate of splitting of BAME over the rate of the spontaneous hydrolysis and that the same first order reaction constant applies to the hydrolysis of BAME in both the presence and absence of trypsin.

**Effect of Temperature**—The spontaneous hydrolysis of BAME was also studied at temperatures between 0.5° and 42°. Although no spontaneous hydrolysis of BAME could be observed at pH 8 at 25°, a slow but measurable reaction does occur at 42°. The spontaneous reaction was so slow at pH 10 and at pH 11 at 0.5° that only the initial slope of the reaction curve could be measured. At pH 10 at 25° and 42° and at pH 11 at 25° the reaction is clearly first order in character. At pH 11 at 42° the reaction rate is too high to be measurable by this method. From these analyses the
value of $Q_{10}$ for the spontaneous hydrolysis of BAME was found to be about 3.8.

The hydrolysis of BAME by trypsin was studied in 0.015 M phosphate buffer, pH 8.0, at 0.5°, 25°, and 42°. The reaction at 42° deviates slightly from zero order but the deviation is so small that the initial slope of this curve is probably strictly comparable to the slopes of the zero order curves measured at the lower temperatures. The corresponding reaction constants, $K_0$, are 0.0515, 0.273, and 0.765 (corrected for spontaneous hydrolysis) mM per minute per mg. of trypsin N. The values of $Q_{10}$ are 1.97 (0.5–25°) and 1.83 (25–42°). These values are somewhat lower and decrease less rapidly with increasing temperature than the values determined by Butler for the action of trypsin on BAA (15). Calculation of Butler's data yields $Q_{10}$ (6–15.2°) = 3.00, $Q_{10}$ (15.2–25.5°) = 2.36, and $Q_{10}$ (25.5–37.5°) = 1.82.

Substrate Specificity of Trypsin and Chymotrypsin toward Esters and Amides

The substrate specificity of trypsin and chymotrypsin was investigated with a variety of esters, amino acid esters, and peptide esters which are related to the specific substrates. Toluenesulfonylarginine methyl ester (TSAME) was found to be split by trypsin even more rapidly than is BAME. The pH activity relation for the action of trypsin on TSAME is shown in Fig. 5.

The reaction follows zero order kinetics between pH 6 and 8, but at pH 9 and 10 approaches first order. Reaction rates at the latter pH values were determined from the initial slopes of the reaction curves. TSAME was not found to undergo spontaneous hydrolysis at any pH studied. Comparison of the specific reaction constants shown in Fig. 5 indicates that trypsin hydrolyzes TSAME about 6 times as rapidly at pH 8 as it does BAME.

The results obtained with other esters are shown in Table III. The relative insolubility of some of these substrates in aqueous solutions necessitated the use of 50 per cent ethanol for enzymatic analyses. For comparison, the activity of trypsin toward BAME in 50 per cent ethanol was determined. To 12 ml. of a 50 per cent (by volume) buffered ethanolic solution, pH 8, of approximately 0.01 M BAME was added 0.2 ml. of a trypsin solution containing 0.135 mg. of N per ml. The reaction curve deviated slightly from a zero order curve, but from the slope of the main portion of the curve $K_0$ was calculated to be 0.19, a value not markedly lower than that found for the tryptic hydrolysis of BAME in aqueous solutions. This is in agreement with the findings of Risley, Buffington, and Arnow (16) who studied the digestion of proteins by trypsin in alcoholic solutions.
The observation that TSAME is split by trypsin led to a reinvestigation of the splitting of toluenesulfonylargininamide by trypsin (3). Preliminary experiments, with the Conway method, have indicated that trypsin splits TSAA about 1½ times as rapidly as it splits BAA.12 Estimates of the pro-

### TABLE III

Effect of Trypsin and Chymotrypsin on Various Esters

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Trypsin Concentration</th>
<th>Trypsin Rate*</th>
<th>Chymotrypsin Concentration</th>
<th>Chymotrypsin Rate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-Tyrosine ethyl ester HCl</td>
<td>0.0285</td>
<td>±</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine ethyl ester HCl</td>
<td>0.0285</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl butyrate†</td>
<td>0.0285</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Butyl acetate</td>
<td>0.0285</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Carbobenzoxy-O-acetyl-L-tyrosylglycine ethyl ester†</td>
<td>0.0363</td>
<td>±</td>
<td>0.0556</td>
<td>0§</td>
</tr>
<tr>
<td>L-Phenylalanine ethyl ester HCl</td>
<td>0.0399</td>
<td>±</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbobenzoxyglycyl-β-phenylalanine ethyl ester†</td>
<td>0.0399</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ε-Carbobenzoxy-L-lysine methyl ester HCl‡</td>
<td>0.0363</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbobenzoxyglycyl-L-tyrosine ethyl ester‡</td>
<td>0.0399</td>
<td>0</td>
<td>0.0185</td>
<td>K = 0.14§</td>
</tr>
<tr>
<td>α-Toluensulfonylarginine methyl ester HCl§</td>
<td>(See text)</td>
<td>+</td>
<td>0.0705</td>
<td>± †</td>
</tr>
</tbody>
</table>

For the water-soluble substrates the volume of the reaction system was 10 ml. Substrate concentration ¼/40 and buffer concentration ¼/66. The buffer used was phosphate, pH 8.0. The temperature was 25° for all runs.

* The rate is expressed as 0 if the pH remained steady for 15 minutes after the enzyme solution was added, and as ± if there was a fall in pH but at a rate too low to be measurable.

† Substrate concentration 0.0145 M due to limited solubility.

‡ Determination made in 50 per cent (by volume) ethanol, containing 0.02 M phosphate buffer, pH 8.00.

§ Substrate concentration 0.0156 M.

|| Owing to the limited quantity of this substrate available, quantitative studies were not made.

† γ-Chymotrypsin also failed to hydrolyze TSAME at an appreciable rate.

Theolytic coefficient toward TSAA are of the order of $6 \times 10^{-2}$. This evidence combined with the observation that chymotrypsin splits BAME

12 Toluensulfonylarginine was isolated from the enzymatic reaction mixtures of both TSAA and TSAME. The isolated samples decomposed at 247–248°. Bergmann, Fruton, and Pollok (3) report 256–257° as the decomposition point of a recrystallized sample.
rather rapidly, while it hydrolyzes TSAME only very slowly, suggests that TSAA may be a more specific substrate for trypsin than is BAA.

**DISCUSSION**

The experimental data presented in the preceding section appear to provide unequivocal proof for the tryptic hydrolysis of the esters of specific amino acid derivatives. Evidence for the chemical nature of the reaction is derived from the following considerations. The large release of titratable acid observed in weakly buffered reaction systems containing BAME or TSAME can only be attributed to the hydrolysis of the ester bond of these substrates. This has been confirmed by the isolation in 72 per cent yield of benzoylarginine from the hydrolysate of BAME. Although it has not been possible to characterize BAME by chemical analysis, its ready conversion into benzoylargininamide, together with the parallel results obtained with TSAME, a crystalline, chemically identified compound, leaves little doubt about the chemical identity of this substrate.

The conclusion that the hydrolysis of these esters is catalyzed by trypsin itself and by the same active surfaces which catalyze the hydrolysis of BAA is based upon the following evidence. (1) The enzymatic purity of the trypsin preparations used in this work is the same as that established by Hofmann and Bergmann (12) for a series of crystalline samples. (2) The specific zero order reaction constant, $K_0$, for the hydrolysis of BAME is independent of enzyme concentration and comparable values were obtained with various enzyme preparations, including one that had been subjected to precipitation by trichloroacetic acid, a procedure equivalent to several recrystallizations (4). (3) Since the specific zero order reaction constant toward BAME is approximately proportional to the proteolytic coefficient toward BAA, both measured activities appear to be properties of the same enzyme. This is further borne out by the identity of the rates of autolysis of trypsin measured by activities toward BAME and BAA (see Fig. 2). (4) When trypsin is inactivated by alkali or by the addition of soy bean inhibitor, the enzymatic activities toward BAME and BAA decrease in a parallel fashion. (5) The pH optima toward casein (13), BAA (3), BAME, and TSAME are the same. Although the pH-activity curves of trypsin toward BAME and TSAME are broader than that toward BAA, a qualitative resemblance among the three curves is apparent.

It is of significance that under standard conditions of measurement (pH 8.0, 25°, substrate concentration varying from 0.006 to 0.025 M) the esterase activity of trypsin follows a zero order reaction, whereas the amidase activity, even in higher substrate concentrations (0.05 M), follows first order reaction kinetics. Zero order reaction kinetics were observed for BAME at all pH values between pH 5 and 10, whereas with TSAME
deviations toward first order reaction kinetics were noted at pH 9 and pH 10. The molecularity of the reaction is evidently the same in both reaction systems (esters and amide). If zero order kinetics are ascribed to a complete saturation of the enzyme by the substrate at all ratios of enzyme-substrate concentration that have been studied, the higher rate and lower order of reaction in the case of the esters must indicate a considerably higher affinity of the enzyme for these substrates. It is unlikely that the rate of desorption of the reaction products is the rate-limiting step, since the readesorption of the accumulating products should then decrease the reaction rate. It is more likely that the rate of activation at the enzyme surface is a controlling factor and that the activation energy for the amides is higher than that for the esters. This conclusion is corroborated by the Arrhenius activation energy calculated from the rate measurements made at various temperatures. The corresponding values for the tryptic hydrolysis of BAME and of BAA are 11,200 calories per mole and 14,900 calories per mole (from Butler's data (15)). The higher temperature coefficient for the spontaneous hydrolysis of BAME, $Q_{10} = 3.8$, compared to that for the tryptic hydrolysis, $Q_{10} = 1.9$, is in agreement with analogous data for catalyzed and non-catalyzed reactions presented by Line-weaver (17).

A certain measure of enzymatic cross-reactivity is exhibited by trypsin and chymotrypsin toward BAME. The lower affinity of chymotrypsin for BAME is evidenced by the lower rate and higher order of the reaction (first order). In contrast, this cross-reactivity does not appear when the more specific TSAME is used as a substrate.

Both the amidase and esterase activities of trypsin show a high degree of substrate specificity. Of all the esters that have been tested only BAME and TSAME exhibit a measurable rate of tryptic hydrolysis, the latter being hydrolyzed about 6 times faster than the former. Analogously, the hydrolysis of toluenesulfonylargininamide, a substrate previously reported to be resistant to tryptic hydrolysis (3), somewhat exceeds in rate that of BAA. Although, because of the different orders of reaction, precise comparison is difficult, it is evident that the activity of trypsin toward BAME is about 60 times higher than that toward BAA (see Table II).

The negative results obtained with trypsin and $\epsilon$-carbobenzoxy-$\alpha$-lysine methyl ester are in agreement with the findings of Hofmann and Bergmann (18) on the effect of introduction of an $\epsilon$-carbobenzoxy group into an $\alpha$-substituted lysinamide. The resistance of N-carbobenzoxy-O-acetyl-$\alpha$-tyrosylglycine ethyl ester to chymotryptic hydrolysis is possibly caused by the introduction of the O-acetyl group. The hydrolysis of carbobenzoylglycyl-$\alpha$-tyrosine ethyl ester and of carbobenzoylglycyl-DL-phenylalanine ethyl ester by chymotrypsin indicates that trypsin is not the only pro-
teolytic enzyme endowed with specific amino acid esterase activity. Present evidence seems to indicate that those esters which are readily split by proteolytic enzymes are structural analogues of the typical amide substrates.

Since the present studies were limited to simple esters, it cannot be decided whether the observed esterase activity is simply related to the lower bond energy of esters as compared to amides or whether the structural details of the ester group are of essential importance. Further studies of this problem, involving the substitution of other radicals for the amide group, are in progress.

It has been suggested that in proteins certain terminal carboxyl groups form ester linkages with the hydroxyl groups of serine, threonine, or tyrosine (19). The biological significance of the esterase activity of trypsin and chymotrypsin for this and other processes remains to be evaluated.

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**SUMMARY**

Crystalline trypsin exhibits esterase activity toward specific esters of amino acid derivatives. Of these, α-benzoyl-arginine methyl ester and α-p-toluenesulfonyl-arginine methyl ester are most active, and are hydrolyzed about 60 times faster than are the corresponding amides. The catalysis of esters is mediated by the same active surface as the catalysis of the corresponding amides. Quantitative kinetic studies, including the effects of enzyme concentration, substrate concentration, pH, and temperature are reported.

Contrary to previous reports, it is found that α-p-toluenesulfonyl-argininamide is a typical substrate for trypsin and is hydrolyzed even more rapidly than is the corresponding α-benzoyl derivative.

Although BAME is also hydrolyzed by chymotrypsin, α-p-toluenesulfonyl-arginine methyl ester is not. The ethyl esters of carbobenzoxyglycyl-tyrosine and of carbobenzyoxglycyl-phenylalanine are readily hydrolyzed by chymotrypsin, indicating that the amino acid esterase activity may be a general attribute of proteolytic enzymes.

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