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# Kinetics of the Pepsin-Catalyzed Hydrolysis of *N*-Acetyl Dipeptides*

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ABSTRACT: A study was carried out to determine what effect the presence of phenylalanyl and tyrosyl residues and their position in a dipeptide have upon the binding and hydrolysis of the molecule by pepsin. Values for $K_M$ and $k_3$ for *N*-acetyl-L-phenylalanyl-L-phenylalanine ($0.16 \times 10^{-3}$ M, 0.86 $min^{-1}$), *N*-acetyl-L-tyrosyl-L-phenylalanine ($2.0 \times 10^{-3}$ M, 0.3 $min^{-1}$), *N*-acetyl-L-phenylalanyl-L-tyrosine ($2.2 \times 10^{-3}$ M, 5.1 $min^{-1}$), and *N*-acetyl-L-tyrosyl-L-tyrosine ($6.1 \times 10^{-3}$ M, 0.88 $min^{-1}$) were determined as were values of $K_I$ for *N*-acetyl-L-phenylalanine ($23 \times 10^{-3}$ M) and *N*-acetyl-L-tyrosine ($41 \times 10^{-3}$ M) and an approximate value of $K_I$ for *N*-acetylglycylglycine (*ca.* 0.5 M). An analysis of the data led to the following conclusions. (1) Phenylalanyl residues are bound to pepsin more strongly than tyrosyl residues, supporting the theory that a part of the binding region of the active center is hydrophobic. (2) Dipeptides are bound to pepsin principally through their side chains. (3) Binding of acetyl dipeptides involves both side chains. (4) The nature and position of the amino acids in dipeptides affect $k_3$ values. Further, the rate-limiting step in pepsin-catalyzed reactions is probably the formation of an intermediate in which pepsin is covalently bonded to part(s) of the dipeptide rather than the subsequent hydrolysis of this intermediate.

The value of $K_I$ for *N*-acetyl-L-phenylalanine obtained by direct measurement is approximately ten times greater than previously reported values inferred from first-order kinetics of the hydrolysis of *N*-acetyl-L-phenylalanyl-L-tyrosine. These kinetics have been confirmed. However, they are interpreted to indicate inhibition by both products, L-tyrosine and *N*-acetyl-L-phenylalanine, rather than inhibition only by *N*-acetyl-L-phenylalanine with a $K_I$ equal to the $K_M$ of the substrate as previously inferred. A pepsin preparation made by the conversion of pepsinogen to pepsin at pH 2 was found to have approximately the same kinetic constants as crystalline pepsin. The significance of this finding is discussed.

Recent reviews on the hydrolytic action of pepsin (Bovey and Yanari, 1960; Herriott, 1962; Tang, 1963) indicate that at low pH values around 2 the enzyme rapidly hydrolyzes substrates with aromatic amino acids on one or both sides of the susceptible peptide bond. Available kinetic data (Baker, 1954; Jackson *et al.*, 1965; Silver *et al.*, 1965) are too fragmentary to assess the influence that particular aromatic amino acids and their position in a peptide have on the susceptibility of the substrate to peptic hydrolysis. This influence could be on either binding or catalysis or both. A comparison of the dissociation constants and molecular activity coefficients of appropriate dipeptide substrates could possibly resolve this question.

An appropriate group of compounds for such a study comprises the *N*-acetyl dipeptides of phenylalanine and tyrosine in all four possible amino acid combinations (*e.g.*, Ac-Phe-Phe,[1] Ac-Phe-Tyr, Ac-Tyr-Phe, and Ac-Tyr-Tyr). The Michaelis constant, $K_M$, and the molecular activity coefficient, $k_3$, of each of these compounds were determined. In comparisons

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[1] Abbreviations used: Ac-Phe-Phe, *N*-acetyl-L-phenylalanyl-L-phenylalanine; Ac-Phe-Tyr, *N*-acetyl-L-phenylalanyl-L-tyrosine; Ac-Tyr-Phe, *N*-acetyl-L-tyrosyl-L-phenylalanine; Ac-Tyr-Tyr, *N*-acetyl-L-tyrosyl-L-tyrosine; Ac-Phe, *N*-acetyl-L-phenylalanine; Ac-Tyr, *N*-acetyl-L-tyrosine; Ac-Gly-Gly, *N*-acetylglycylglycine; $S_0$, initial substrate concentration; $S_t$, substrate concentration at time $t$; $v_0$, initial velocity of the reaction; $I$, inhibitor concentration; $e$, enzyme concentration; $\alpha$, the factor that relates the dissociation constant of EIS with that of EI (Webb, 1963).

of substrate binding it was assumed[2] that $K_M$ is approximately equal to $K_S$. A dissociation constant, $K_I$, for Ac-Phe, Ac-Tyr, and Ac-Gly-Gly was also determined to help evaluate the binding contributions of individual parts of the aromatic dipeptides.

The value for $K_I$ of Ac-Phe as determined in this laboratory by direct measurement is tenfold greater than previously reported ones (Baker, 1954, 1956; Silver *et al.*, 1965) obtained inferentially from time-course studies of the hydrolysis of Ac-Phe-Tyr. This discrepancy is beyond the range of experimental error. Inasmuch as the $K_I$ values for Ac-Phe and Ac-Tyr are to be used in the interpretation of the binding data of the dipeptides it was necessary to reevaluate the indirect method as a procedure for obtaining $K_I$ data.

## Materials

*Substrates.* All substrates were products of Cyclo Chemical Corp. who reported the following properties: Ac-Phe-Tyr (lot M-2570), mp 220–223° (lit. (Silver *et al.*, 1965) mp 220–222 and (Baker, 1951) 230°), 7.46% nitrogen (theory, 7.57%), homogeneity in two solvent systems of paper chromatography, neutralization equivalent value of 362 (theory, 370.4); Ac-Phe-Phe (lot M-2026), 7.87% nitrogen (theory, 7.91%), neutralization equivalent value of 358 (theory, 354.4); Ac-Tyr-Phe (lot M-3026), mp 245–247°, 7.50% nitrogen (theory, 7.57%), homogeneity in three solvent systems of paper chromatography, neutralization equivalent value of 381 (theory, 370.4); Ac-Tyr-Tyr (lot M-3027), mp 238° (lit. (Baker, 1951) mp 241°), 7.09% nitrogen (theory, 7.25%), homogeneity in three solvent systems of paper chromatography.

*Inhibitors.* All inhibitors also were obtained from Cyclo Chemical Corp. The properties of Ac-Phe (lot M-1113) have been described previously (Jackson *et al.*, 1965). Ac-Tyr (lot H-1018) had mp 152–154° (lit. (du Vigneaud and Meyer, 1932) mp 152–154° cor), $[\alpha]_D$ +47.5° in water (lit. (du Vigneaud and Meyer, 1932) value, $[\alpha]_D^{26}$ +47.5°), and homogeneity in two solvent systems of paper chromatography. Ac-Gly-Gly (lot K-5344) had 15.7% nitrogen (theory, 16.1%), neutralization equivalent value of 179 (theory, 174.2), and homogeneity in two solvent systems of thin layer chromatography. This preparation of Ac-Gly-Gly was recrystallized four times from absolute ethanol.

*Enzyme.* In all but one experiment a three-times crystallized preparation of pepsin (Pentex Co., Inc.; lot D-3709) was used. As previously reported (Jackson *et al.*, 1965) this preparation is assumed to contain 83% active enzyme with a molecular weight of 34,500.

In one experiment (*cf.* Table I) the $K_M$ of Ac-Phe-Tyr was also determined using pepsin made from a pepsinogen preparation obtained from Worthington Biochemical Corp. (lot 6007–10). The properties of the pepsinogen sample have been described (Schlamowitz *et al.*, 1963). For this experiment pepsin was made by allowing pepsinogen to stand at pH 2 for 20 min at room temperature. A small amount of insoluble material was removed by centrifugation.

TABLE I: Reaction Constants for Pepsin with Synthetic Substrates and Inhibitors.

| Compound | $K_I$[a] (M × $10^3$) | $K_M$[a] (M × $10^3$) | $-\Delta F_0$[b] (kcal/mole) | $k_3$ ($min^{-1}$) |
|---|---|---|---|---|
| Ac-Tyr-Tyr | | 6.1 | 3.1 | 0.88 |
| Ac-Tyr-Phe | | 2.0 | 3.8 | 0.3 |
| Ac-Phe-Tyr | | 2.2 | 3.8 | 5.1 |
| | | (2.3)[c] | (3.7) | (4.1) |
| Ac-Phe-Phe | | 0.16 | 5.4 | 0.86 |
| Ac-Tyr | 41 | | 2.0 | |
| Ac-Phe | 23 | | 2.3 | |
| Ac-Gly-Gly | *Ca.* 0.5 M | | *Ca.* 0.4 | |

[a] The data are reliable to ±5% except where indicated. [b] Standard free energies of association are calculated using the expression $-\Delta F_0 = 2.303RT \log K$, where $R = 1.986$ cal × $deg^{-1}$ × $mole^{-1}$, $T = 310°$ K, and $K$, the association constant, = the reciprocal of $K_M$ or $K_I$. [c] The figures in parentheses were obtained using a pepsin made from crystalline pepsinogen. The other data reported in the table were obtained using a crystalline pepsin.

## Methods

*Determination of $K_M$, $k_3$, and $K_I$.* The methods for determining $K_M$ and $K_I$ were similar to those previously described (Jackson *et al.*, 1965). For each aromatic acetyl dipeptide, the initial velocity of the pepsin-catalyzed hydrolysis was measured at different initial concentrations of substrate. The Michaelis constant and the molecular activity coefficient were calculated from a plot of $S_0/v_0$ *vs.* $S_0$. For each *N*-acetyl-amino acid and for Ac-Gly-Gly, the inhibition constant, $K_I$, was determined from plots of $1/v_0$ *vs.* I at two different initial substrate concentrations. The substrate used was Ac-Phe-Tyr. To ensure that velocities approximated initial reaction rates, the extent of hydrolysis at 37° and pH 2 was kept to less than 20%. Assays were carried out by the ninhydrin procedure and were done in duplicate or triplicate.

*Kinetics of the Extensive Hydrolysis of N-Acetyl-L-phenylalanyl-L-tyrosine by Pepsin.* This study was carried out in the following manner. A series of tubes containing 1.5 ml of stock enzyme solution and 0.2 ml of stock substrate solution was incubated at 37° and pH 2 for 0–121 min. The extent of hydrolysis

[2] Although it appears likely that $K_M$ is equal to $K_S$ (Jackson *et al.*, 1965) it remains an assumption that awaits verification by direct measurements of $k_1$ and $k_2$ for pepsin–substrate systems. In the meantime the values of $\Delta F_0$ (Table I), calculated from the $K_M$ values must be considered provisional.

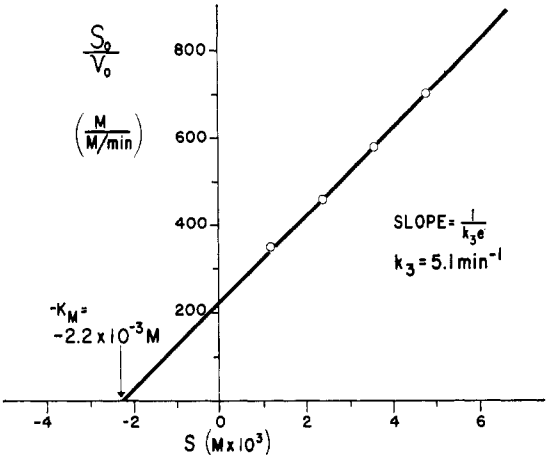


FIGURE 1: Determination of Michaelis constant, $K_M$, and molecular activity coefficient, $k_3$, for the hydrolysis of *N*-acetyl-L-phenylalanyl-L-tyrosine by pepsin at pH 2 and 37°. The concentration of pepsin was $2 \times 10^{-6}$ M.

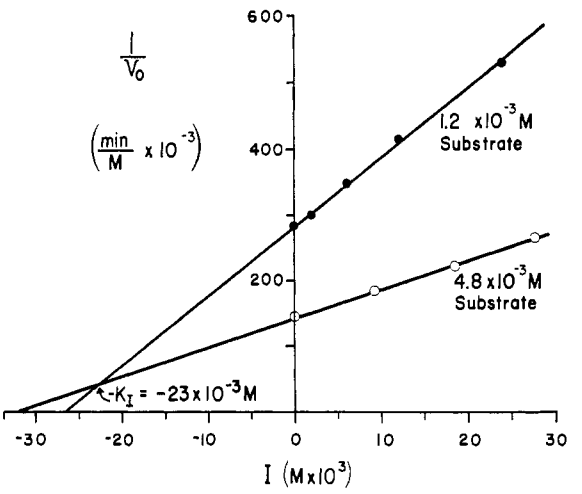


FIGURE 2: Determination of the $K_I$ of *N*-acetyl-L-phenylalanine at pH 2 and 37°. The substrate was *N*-acetyl-L-phenylalanyl-L-tyrosine. The concentration of pepsin was $2 \times 10^{-6}$ M.

achieved in 121 min was 91%. The reactions were stopped by the addition of 0.05 ml of an alkaline solution that raised the pH to 9.6. Appropriate aliquots were diluted to 3.5 ml and assayed by the ninhydrin procedure. After correction for ninhydrin-reacting material present at the start, the amount of hydrolysis was calculated from a standard absorbance curve of tyrosine. No correction was made for production of additional ninhydrin color owing to products of self-digestion of pepsin nor for reduction in ninhydrin color owing to loss of enzyme activity during incubation. These effects were small and tended to offset each other. Results are plotted as $\log S_0/S_t$ *vs.* $t$.

## Results

*$K_M$ and $k_3$ for the Peptic Hydrolysis of Aromatic N-Acetyl Dipeptides.* The values of these kinetic constants for the hydrolysis of the four aromatic *N*-acetyl dipeptides by crystalline pepsin were obtained from plots of $S_0/v_0$ *vs.* $S_0$. Figure 1, a plot for the hydrolysis of Ac-Phe-Tyr, is representative of the plots obtained for all the substrates. Substrate concentrations both higher and lower than $K_M$ were used in all cases. The $K_M$ was obtained from the intercept on the abscissa and $k_3$ from the slope of the experimental line. Values of the kinetic constants are tabulated in Table I. The values of $K_M$ for Ac-Tyr-Tyr and Ac-Phe-Tyr agree with those reported by Baker (1954). Also in Table I is the standard free energy of combination of pepsin with each substrate, calculated on the assumption[2] that $K_M$ is equal to $K_S$. This assumption entails the view that $k_3$ is much smaller that $k_2$ in the expression $K_M = (k_2 + k_3)/k_1$ so that $K_M = K_S$. The basis for believing this to be the case has been set forth (Jackson *et al.*, 1965).

Crystalline pepsin prepared commercially from gastric hog mucosa has been reported to contain three fractions separable by chromatography at pH 5.7 while pepsin prepared at pH 2 from crystalline pepsinogen is homogeneous (Rajagopalan *et al.*, 1966). Therefore, $K_M$ and $k_3$ of Ac-Phe-Tyr were also determined using a pepsin prepared from pepsinogen at pH 2. These values, which are close to those obtained using the commercial pepsin, are tabulated in Table I.

*$K_I$ for Inhibitors of Peptic Hydrolysis.* Values of $K_I$ for Ac-Phe, Ac-Tyr, and Ac-Gly-Gly were determined from plots of $1/v_0$ *vs.* $I$. The $K_I$ and $\Delta F_0$ for each inhibitor are recorded in Table I. Figure 2 shows the graph obtained for Ac-Phe. Inhibitor concentrations from 0- to $1.2K_I$ were used. The graph for Ac-Tyr was similar. The value on the abscissa corresponding to the point of intersection of the experimental lines is equal to $-K_I$. The data for Ac-Phe and Ac-Tyr are consistent with kinetics for mixed inhibition described by the equation

$$\frac{1}{v_0} = \frac{1}{k_3 e K_I}\left(\frac{1}{\alpha} + \frac{K_M}{S_0}\right) I + \frac{1}{k_3 e}\left(\frac{S_0 + K_M}{S_0}\right)$$

where $\alpha = 4.6$ in the case of Ac-Tyr inhibition and $\alpha = 1.9$ in the case of Ac-Phe inhibition. For the substrate concentrations used, these two values clearly distinguish this inhibition from noncompetitive inhibition where $\alpha = 1$ and competitive inhibition where $\alpha = \infty$.

Since the highest concentration of Ac-Gly-Gly that could be used was 0.1 M, it was not possible to obtain an accurate measurement of the $K_I$ which is much greater than 0.1 M. Our best estimate for the $K_I$ value from the graphic data of $1/v_0$ *vs.* I is 0.5 M. Concentrations of Ac-Gly-Gly higher than 0.1 M could not be used because of high blanks. Even after four

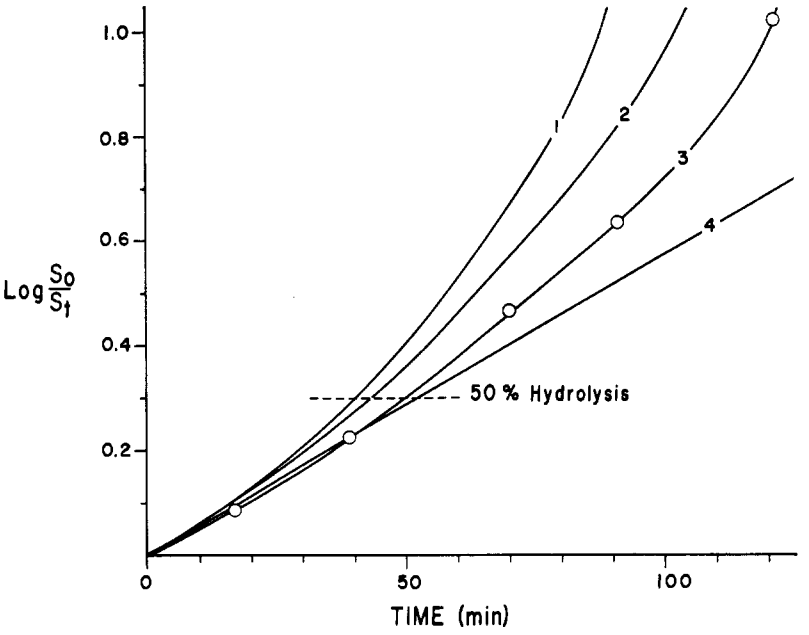


FIGURE 3: Kinetics of the complete hydrolysis of *N*-acetyl-L-phenylalanine at pH 2 and 37°. Curve 1, the line expected if neither product inhibited the reaction; curve 2, the line expected if only the product *N*-acetyl-L-phenylalanine inhibited the reaction; curve 3, the observed data; curve 4, the line expected if only one of the products inhibited the reaction competitively and had a $K_I$ equal to $K_M$. The following values were used to plot the theoretical curves: $K_M$, $2.2 \times 10^{-3}$ M; $k_3$, 5.1 $min^{-1}$; $S_0$, $9.6 \times 10^{-3}$ M; $e$, $3.12 \times 10^{-5}$ M; $K_I$ for curve 2, $23 \times 10^{-3}$ M; $\alpha$ for curve 2, 1.9.

recrystallizations, trace amounts of ninhydrin-reacting material remained. This plus the color contributed as a result of a slight extent of spontaneous acid hydrolysis made the use of concentrations greater than 0.1 M impractical.

*Kinetics of the Extensive Hydrolysis of N-Acetyl-L-phenylalanyl-L-tyrosine.* Our findings from the study of the inhibition of the hydrolysis of Ac-Phe-Tyr by Ac-Phe differ in two important respects from those of Baker (1954, 1956) and Silver *et al.* (1965). First, the $K_I$, $23 \times 10^{-3}$ M, for Ac-Phe is approximately tenfold greater than values inferred by these other workers. Second, the inhibition is not competitive but mixed.

An explanation for these differences was sought in the methods used to determine $K_I$. In the present study $K_I$ was obtained directly from measurements of initial velocities in the absence and presence of inhibitor. The type of inhibition was deduced from the values of $\alpha$ obtained from the plots of $1/v_0$ *vs.* $I$. In the studies reported by the other investigators $K_I$ values and the type of inhibition were inferred from the experimentally observed apparent first-order kinetics of extensive hydrolysis of Ac-Phe-Tyr. They based their conclusion on the apparent agreement of their data with the fact that an enzymic reaction will follow first-order kinetics under conditions where inhibition by products is restricted to competitive inhibition by only one product and where that product has a $K_I$ equal to the $K_M$ of the substrate (Haldane, 1930). The inferred $K_I$ values from Baker's (1954) and Silver's *et al.* (1965) work are in the range $1.5–4.6 \times 10^{-3}$ M.

As part of the reevaluation of this latter method, it was necessary to confirm the experimental observations of Baker (1954) and Silver *et al.* (1965). This was done by following the kinetics of hydrolysis of Ac-Phe-Tyr ($9.6 \times 10^{-3}$ M) until 91% of the initial substrate had reacted. The results plotted as log $S_0/S_t$ *vs.* $t$ (Figure 3, curve 3) approximately simulate the first-order kinetics reported by the earlier workers. An elaboration on possible reasons for the differences in $K_I$ values obtained by the two methods is given in the discussion.

## Discussion

The primary purpose of this study was to assess what influence the presence and the relative position of phenylalanine and tyrosine in a dipeptide have on the susceptibility of the CONH bond to peptic hydrolysis. A study of the kinetic and thermodynamic data given in Table I indicates that the binding of the dipeptide to pepsin and the catalytic hydrolysis of the compound are both affected.

With reference to influences on binding it is observed that the binding energy[2] of the dipeptides ($-\Delta F_0$ of association) increases in the following order: Ac-Tyr-Tyr < Ac-Tyr-Phe = Ac-Phe-Tyr < Ac-Phe-Phe. Thus, when a tyrosine residue is replaced by a phenylalanine residue in either position of the dipeptide,

TABLE II: Equations for the Kinetics of Assumed Reaction Mechanisms for the Hydrolysis of *N*-Acetyl-L-phenylalanyl-L-tyrosine by Pepsin.

| Mechanism | Kinetic Equations |
|---|---|
| Michaelis–Menten kinetics without inhibition by either product (Figure 3, curve 1) | $\text{Log}\ \frac{S_0}{S_t} = \left\{\frac{k_3 e}{2.303K_M}\right\} t - \frac{(S_0 - S_t)}{2.303K_M}$ |
| Michaelis–Menten kinetics with mixed inhibition by *N*-acetyl-L-phenylalanine (Figure 3, curve 2) | $\text{Log}\ \frac{S_0}{S_t} = \left\{\frac{k_3 e \alpha K_I}{2.303(\alpha K_M K_I + \alpha K_M S_0)}\right\} t - \frac{(S_0 + \alpha K_I - \alpha K_M)(S_0 - S_t)}{2.303(\alpha K_M K_I + \alpha K_M S_0)} + \frac{(S_0)^2 - (S_t)^2}{2.303(\alpha K_M K_I + \alpha K_M S_0) \times 2}$ |
| Michaelis–Menten kinetics with competitive inhibition by one of the products whose $K_I = K_M$ (Figure 3, curve 4) | $\text{Log}\ \frac{S_0}{S_t} = \left\{\frac{k_3 e}{2.303(K_M + S_0)}\right\} t$ |

there is an increase in the binding energy. For example, it may be seen that the binding energy of Ac-Phe-Phe is about 75% greater (2.3 kcal) than that of Ac-Tyr-Tyr. This increase in binding energy upon replacing a hydroxyl group with a hydrogen atom supports the theory that a region of the active center is hydrophobic (Tang, 1963). It should also be noted that the absolute amount of change in binding energy that occurs when a tyrosine residue is substituted by a phenylalanine residue is not a fixed increment but depends upon the nature of the remaining part of the molecule.

The binding data provide further evidence for the view (Bovey and Yanari, 1960; Jackson *et al.*, 1965) that aromatic dipeptides are bound to the enzyme through their side chains and that binding involves both side-chain residues. The first point is substantiated by the fact that the binding energy of Ac-Gly-Gly, which simulates closely the non-side-chain portion of these aromatic dipeptides, is very small. Hence, the major contribution to the binding of aromatic dipeptides must come from their side chains. That both side-chain residues are involved is shown by the fact that the binding energy of any of the acetyl dipeptides is considerably greater than that of either Ac-Phe or Ac-Tyr.

The values of $K_M$ for Ac-Phe-Tyr and Ac-Tyr-Phe show that the binding of these dipeptides is not influenced by the relative position of their side chains. Although the binding of the two dipeptides is equal, it is not yet possible to decide whether the binding regions on pepsin for the residues on each side of the CONH bond are equivalent.

With reference to the influence of phenylalanine and tyrosine on the catalytic hydrolysis of the acetyl dipeptides, the values of $k_3$ in Table I indicate that the position and the nature of the amino acids in the compound are both important. The effect of position is shown by the dipeptides Ac-Phe-Tyr and Ac-Tyr-Phe which contain the same amino acids yet differ by a factor of 17 in their $k_3$ values. As regards the nature of the amino acid, a comparison of the $k_3$ values for the following pairs of dipeptides: Ac-Phe-Phe and Ac-Tyr-Phe, Ac-Phe-Tyr and Ac-Tyr-Tyr, shows that higher values were obtained when phenylalanine rather than tyrosine was at the amino end of the dipeptide. Similarly a comparison of Ac-Phe-Tyr with Ac-Phe-Phe and of Ac-Tyr-Tyr with Ac-Tyr-Phe shows that higher $k_3$ values were obtained when tyrosine was the residue on the carboxyl end.

The $k_3$ values also furnish some information about the nature of the rate-limiting step in the catalytic hydrolysis of the dipeptide $R_2R_1$. Starting with the Michaelis–Menten complex (Enz·$R_2R_1$), this mechanism may be divided into two stages: first, the conversion of the dissociable complex into an intermediate in which the pepsin molecule is connected by a covalent linkage to either one (Enz-$R_2$ or Enz-$R_1$) or both ($\text{Enz}<^{R_2}_{R_1}$) of the amino acid residues of the dipeptide; and second, the hydrolysis of this intermediate to yield the products of the reaction and regenerate the pepsin molecule. If the intermediate is either Enz-$R_2$ or Enz-$R_1$, then it can be said that the rate-limiting step involves the formation of this intermediate rather than its subsequent hydrolysis. If the reverse were true, then one would have expected to find the same $k_3$ values for all dipeptides having the same carboxyl-terminal amino acid (for the case of Enz-$R_1$) and for all dipeptides having the same amino-terminal amino acid (for the case of Enz-$R_2$). Examination of the data shows that this was not the case. If the intermediate is $\text{Enz}<^{R_2}_{R_1}$, one cannot exclude the possibility that as a result of mutual influences of the R groups the first phase of the hydrolysis may have become rate limiting.

The values of $K_M$ and $k_3$ in Table I were obtained with a commercial preparation of crystalline pepsin. Since it has been reported that crystalline pepsin preparations are heterogeneous at pH 5.7 while pepsin prepared by activation of pepsinogen is homogeneous (Rajagopalan *et al.*, 1966), values of $K_M$ and $k_3$ for the hydrolysis of Ac-Phe-Tyr by a pepsin preparation made from the conversion of pepsinogen to pepsin at pH 2 were determined. These values, given in Table I, approximate very closely the ones obtained with the commercial preparation. The results suggest either that only one form of pepsin exists at pH 2 or that all the active fractions in crystalline pepsin have the same kinetic constants at pH 2. Neumann and Sharon (1960) have reported results that favor the former view inasmuch as they have shown that a "pH-3 pepsin" which does not have transpeptidation activity toward synthetic substrates can be readily converted to a "pH-2 pepsin" which does have such activity by lowering the pH to 2 with HCl.

Our study of the kinetics of extensive hydrolysis of Ac-Phe-Tyr was carried out to reevaluate this as a method for obtaining the $K_I$ of Ac-Phe. Our experimental results essentially confirm the experimental observations of the other investigators; however, our interpretations differ. The results are compared in Figure 3 with those expected for other kinetic mechanisms, the equations for which are shown in Table II. The experimental curve closely approximates the line for first-order kinetics (Figure 3, curve 4) up to 50% hydrolysis, in agreement with both Baker (1954) and Silver *et al.* (1965). It deviates from this line above 50% hydrolysis but the deviation is small. If we assume as Baker did that inhibition by Ac-Phe is competitive, a small change in the assumed $K_I$ from 1- to 1.8-$K_M$ would suffice to produce the observed deviation. In essence then we confirm the apparent first-order kinetics for the hydrolysis of Ac-Phe-Tyr but the value of $K_I$ of approximately $10K_M$, established by direct measurement, clearly indicates that competitive inhibition with $K_I$ equal to $K_M$ cannot be the kinetic mechanism. It is equally clear that the explanation does not lie in a system of mixed inhibition by Ac-Phe alone using the experimentally determined values of $K_I$, $23 \times 10^{-3}$ M, and $\alpha$, 1.9 (Figure 3, curve 2). A system without product inhibition (Figure 3, curve 1) is included for reference. These observations suggest that the other product of the reaction, L-tyrosine, is also an inhibitor. Unfortunately, studies on L-tyrosine inhibition can not be carried out by our method since the levels of L-tyrosine needed to observe inhibition would be so high that the amount of ninhydrin-reacting material produced during the initial velocity period would be very small compared with the ninhydrin-reacting material present at the start of the reaction.

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