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The Mode of Action of Pancreatic Carboxypeptidase. II. The Affinity of Carboxypeptidase for Substrates and Inhibitors[1]

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Dipeptides are generally poor substrates of pancreatic carboxypeptidase. This investigation shows that they are effective competitive inhibitors of carboxypeptidase activity. Competitive inhibition studies have provided unique means for determining the affinity of carboxypeptidase for these dipeptides. Evidence indicates that, in the *p*H range normally used for this enzyme, the inhibitory (and substrate) form of a dipeptide is the ionic species with an uncharged α-amino group. The K_i values for dipeptides were determined by using integrated forms of the Michaelis–Menten equations which correct simultaneously for competitive inhibition by the anionic forms of a dipeptide and one of the hydrolytic products, the C-terminal amino acid of the substrate. The data are interpreted qualitatively in terms of the effects of different N-acyl substituents of dipeptides on affinity and hydrolytic susceptibility.

Introduction

Elucidation of the mechanism of action of proteolytic enzymes through the use of structurally related peptides has been possible because of the wide degree of substrate specificity exhibited by these enzymes. Early studies[3] on CPase[4] were concerned primarily with the hydrolytic susceptibility of tripeptide analogs. Later work[5] revealed considerable information regarding the structural components of a substrate which were involved in the interaction of CPase and the substrate.

Since the hydrolytic rates of dipeptides are one hundred to ten thousand-fold lower than those of their corresponding N-acyl derivatives,[6] there was some doubt as to whether these compounds were split by the same enzyme. A study of the kinetic constants of dipeptide hydrolysis was initiated in order to provide a valid basis for comparing substrates of CPase and to obtain unequivocal evidence that dipeptides are substrates of this enzyme.

Two separate lines of experimental evidence indicate that, in the *p*H range normally used for this enzyme, the ionic form of a dipeptide with an uncharged α-amino group is the substrate and that the dipolar ion has a very low affinity for the enzyme. Therefore, ionization of a dipeptide must be considered in the determination of its kinetic constants.

The finding that D-Leu-L-Tyr had a lower K_m than the typical substrate, Cbz-Gly-L-Phe, suggested that the dipeptides should be competitive inhibitors of CPase activity. Under suitable conditions it was indeed possible to demonstrate that relatively poor substrates (dipeptides) were effective inhibitors of the hydrolysis of a highly susceptible substrate.

The determination of the K_m values for most dipeptides was not feasible because of the low hydrolytic rates; therefore, competitive inhibition studies provided a convenient method for determining the affinity of CPase for dipeptides. Most of these studies were carried out at *p*H 9 so that the predominant ionic species of a dipeptide would be the form with an uncharged α-amino group. Correction for product inhibition is required at this *p*H, since the anionic form of L-phenylalanine is a potent inhibitor of the hydrolysis of Cbz-Gly-L-Phe.[7] Modified forms of the Michaelis–Menten equations were derived in order to determine and test the kinetic constants for dipeptides. Aside from the terms required to correct for the concentrations of the active ionic forms of dipeptide and product at a given *p*H, these equations are the same as those of Huang and Niemann.[8]

The effects of specific substituents on the acyl moiety of dipeptides have been determined from a correlation of the hydrolytic susceptibilities and affinities of these substrates.

Experimental

Materials and Methods.—The sources of the compounds and the enzyme used in this study are listed in the preceding paper.[6] The CPase was a six-times recrystallized preparation which showed no measurable activity toward L-Leu-Gly and benzoylargininеamide. The assay method has been described earlier.[6] In the kinetic studies, 0.2-ml. aliquot of the enzymatic reaction mixtures were pipetted into volumetric flasks containing 1 ml. of 5% trichloroacetic acid. After neutralization and dilution to volume, suitable aliquots, containing ninhydrin-positive materials equivalent to 0.04–0.15 μmole of leucine, were assayed in duplicates.

Conventional designations are used in the steady state treatment of the hydrolysis of peptides by CPase

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (ES) \xrightarrow{k_3} E + P \qquad (1)$$

where E, S, (ES) and P are the concentrations of free enzyme, free substrate, enzyme–substrate complex and product, respectively. The experimentally determined Michaelis–Menten constant K_m is equal to $(k_2 + k_3)/k_1$, whereas the true dissociation constant for the enzyme–substrate complex K_s is equal to k_2/k_1. Methods employed to analyze the kinetic data are described in the text.

Results and Discussion

Studies on the effect of *p*H on the hydrolysis of D-Leu-L-Tyr by CPase provided presumptive evidence that only one ionic species of a dipeptide was

(1) A preliminary report was presented at the 128th meeting of the Am. Chem. Soc., Minneapolis, September, 1955.

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(3) (a) N. M. Green and H. Neurath, "The Proteins," Vol. II, Academic Press, New York, N. Y., 1954, p. 1054; (b) H. Neurath and G. W. Schwert, *Chem. Revs.*, **46**, 69 (1950).

(4) The following abbreviations are used: CPase, pancreatic carboxypeptidase; amino acid residues of peptides, abbreviated according to E. Brand and J. T. Edsall, *Ann. Rev. Biochem.*, **16**, 224 (1947); AMPD, 2-amino-2-methyl-1,3-propanediol.

(5) (a) J. E. Snoke and H. Neurath, *J. Biol. Chem.*, **181**, 789 (1949); (b) E. L. Smith, R. Lumry and W. J. Polglase, *J. Phys. Colloid Chem.*, **55**, 125 (1951).

(6) S. Yanari and M. A. Mitz, THIS JOURNAL, **79**, 1150 (1957).

(7) E. Elkins-Kaufman and H. Neurath, *J. Biol. Chem.*, **175**, 893 (1948).

(8) H. T. Huang and C. Niemann, THIS JOURNAL, **73**, 1541 (1951).

the substrate. When the concentration of D-Leu-L-Tyr is a rate-limiting factor, the *p*H optimum, as shown in Fig. 1, is significantly higher than that of N-acyl dipeptides (*p*H 7.5). Such a result would be expected if the observed rates were dependent not only on the effect of *p*H on the enzyme but also on the concentration of available substrate at a given *p*H. If only the ionic species of a dipeptide with an uncharged α-amino group were the true substrate of CPase, the hydrolytic rate at a higher *p*H would tend to be excessively high in relation to that at a lower *p*H.

For enzyme systems in which the concentration of available substrate varies with *p*H, a closer approximation of the true *p*H optimum of the enzyme can be obtained by a method described by Johnson.[9] The "corrected" curve in Fig. 1 was obtained by dividing the first order reaction rate constant at a given pH by the concentration of the anionic form of D-Leu-L-Tyr at that *p*H.[10] The "corrected *p*H optimum" is closer to the accepted value for CPase. Studies to be described support the assumption that only the anionic form of a dipeptide is the substrate, at least in the lower range of *p*H indicated in Fig. 1.

Ionization is an important factor in the determination of the kinetic constants of dipeptides as substrates of CPase. The following modification of the conventional Michaelis–Menten equation is applicable for the determination of the K_m for a dipeptide

$$\frac{1}{v} = \frac{K_m}{V_{max}} \times \frac{1}{S}\left[1 + \frac{(H^+)}{K'}\right] + \frac{1}{V_{max}} \qquad (2)$$

The term K' is the dissociation constant for the α-amino group of the dipeptide. The ratio H^+/K' is of considerable importance when the experimental *p*H is lower than the $pK'_{NH_3^+}$ value of a dipeptide. For example, by assuming that the anionic form of D-Leu-L-Tyr ($pK'_2 = 8.3$[11]) is the substrate, a K_m of 5×10^{-4} M is obtained at *p*H 7.5. On the other hand a value of 4×10^{-3} M is obtained when the ionization of the substrate is ignored. The specific activation rate, k_3, for D-Leu-L-Tyr (0.025), although high for dipeptides, is considerably lower than that of Cbz-Gly-L-Phe (2.1). The determination of the K_m values for other dipeptides such as Gly-L-Tyr was not feasible because of low hydrolytic rates.

The Inhibitory Form of Dipeptides.—The fact that the K_m for D-Leu-L-Tyr was substantially lower than that of Cbz-Gly-L-Phe ($K_m = 0.03$) suggested that dipeptides should be effective competitive inhibitors of CPase, even though they were poorer substrates than their corresponding N-acyl derivatives. It was possible to study the splitting of Cbz-Gly-L-Phe in the presence of a dipeptide without significant hydrolysis of the latter by using very low enzyme concentrations.

Evidence as to the nature of the ionic form of a dipeptide which is bound by CPase is shown in

(9) M. J. Johnson, "Respiratory Enzymes," Burgess Publ. Co., Minneapolis, Minn., 1949, pp. 51-55.

(10) No quantitative interpretation is attempted, since many factors may affect the data, *e.g.*, simple first-order kinetics may not apply, product inhibition (to be discussed) will increase as the *p*H increases, and the inhibition by phosphate may vary with *p*H.

(11) Unpublished results.

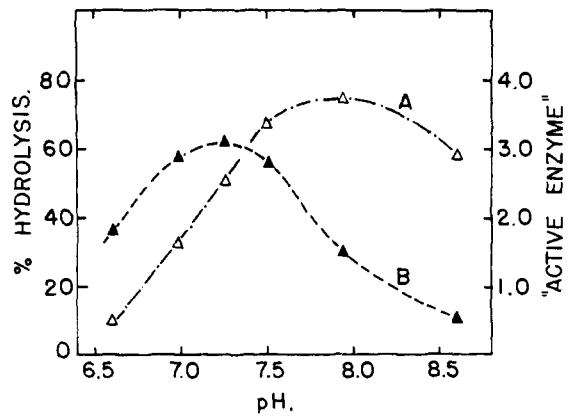

Fig. 1.—The *p*H optimum for the hydrolysis of a "limiting" concentration of D-Leu-L-Tyr by CPase. Curve A, % hydrolysis; curve B, "active enzyme concentration." The substrate concn. was 0.002 M; enzyme concn., 25 μg. protein N per ml.; reaction period, 30 minutes. Tris-(hydroxymethyl)-aminomethane (0.05 M) was the buffer at *p*H 8.6; phosphate (0.02 M) at other *p*H's.

Table I. The *p*H values were selected so that the dipeptides would exist predominantly as dipolar ions at the lower *p*H (6.5) and as ions with uncharged α-amino groups at the higher *p*H (9.0). The dependence of the degrees of inhibition by these dipeptides on *p*H may then be attributed to the nature of the ionic species predominating at each of the *p*H values. The fact that D-Leu-L-Tyr and Gly-L-Tyr are more inhibitory at *p*H 9.0 than at *p*H 6.5 is all the more significant, since the inhibition by the control compound, acetyl-L-Tyr, which does not undergo significant changes in ionization in this *p*H range, is opposite in nature. More detailed studies between *p*H's 6.5 and 7.5 showed that, as the *p*H increased, the inhibition by Gly-L-Tyr increased, whereas that of acetyl-L-Tyr decreased. The reason for the decrease in inhibition by acetyl-L-Tyr with an increase in *p*H is unknown. It is concluded from these data that the anionic forms of dipeptides are the inhibitory species.[12,13] These conclusions may be correlated with the findings of Elkins–Kaufman and Neurath[7] that the anionic form of an L-amino acid, but not the dipolar ion, was a competitive inhibitor of CPase activity.

Analysis of the data by the conventional method of Lineweaver and Burk[14] indicates that the nature of the inhibition of CPase activity by dipeptides is competitive (Fig. 2). The $1/v$ *vs.* $1/S$ plots for the hydrolysis of Cbz-Gly-L-Phe, in the presence and

(12) Unpublished results, to be reported later, indicate that a positively charged group at the active site, presumably responsible for repelling the dipolar ionic form of a dipeptide, may become uncharged as the *p*H is raised, thus permitting the binding of the dipolar ion. Since the mole fractions of the dipolar forms of dipeptides decrease with an increase in *p*H, these experiments do not contradict the assumption in this paper that the dominant ionic species involved in the enzyme–dipeptide interaction is that with an uncharged α-amino group.

(13) Evidence similar to the data in Table I has been used to determine the active ionic species of substrates and inhibitors in other enzyme systems: I. B. Wilson and F. Bergmann, *J. Biol. Chem.*, **185**, 479 (1950); I. B. Wilson, *ibid.*, **208**, 123 (1954); and R. J. Foster, R. R. Jennings and C. Niemann, THIS JOURNAL, **76**, 3142 (1954).

(14) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).

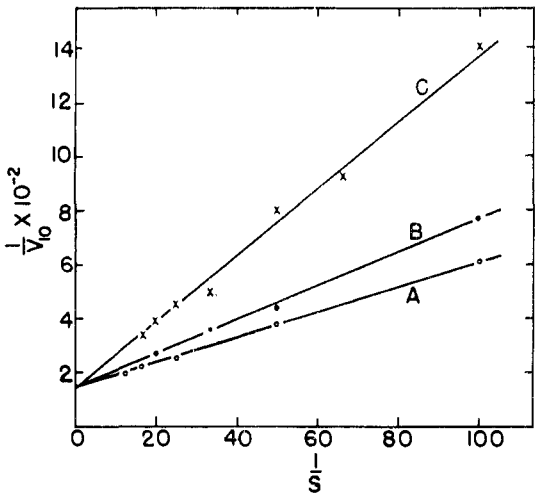

Fig. 2.—Competitive inhibition by dipeptides: A, 0.02 *M* substrate, Cbz-Gly-L-Phe; B, substrate + 0.01 *M* D-Leu-L-Tyr; C, substrate + 0.01 *M* Gly-L-Tyr. The enzyme concn. was 0.5 μg. protein N per ml.; buffer, 0.05 *M* AMPD *p*H 9.0; reaction period, 10 to 25 minutes.

in the absence of dipeptides, yield intercepts which are nearly equal. The fact that Gly-L-Tyr is more inhibitory than D-Leu-L-Tyr is of significance, since its hydrolytic rate is much lower.[6]

TABLE I

THE IONIC STATE OF THE INHIBITORY FORM OF A DIPEPTIDE[a]

Inhibitor	Inhibitor / Substrate	*p*H 9.0 A/D[b]	*p*H 9.0 Inhibition, %	*p*H 6.5 A/D[b]	*p*H 6.5 Inhibition, %
N-Acetyl-L-Tyr	2	..	5	..	34
D-Leu-L-Tyr	2	5/1	30	1/64	5
Gly-L-Tyr	0.5	4/1	56	1/80	10

[a] The concentration of the substrate, Cbz-Gly-L-Phe, was 0.02 *M*; the enzyme concn., 0.5 μg. protein N per ml.; and the reaction period, 12 min. The reaction mixture also contained 0.1 *M* LiCl and 0.05 *M* buffer (imidazole at *p*H 6.5 and AMPD at *p*H 9.0). The degree of hydrolysis of the controls at *p*H's 6.5 and 9.0 were 28 and 21%, respectively. [b] The ratio of the anionic form to the dipolar ionic form calculated from pK'_2 values of 8.3 and 8.4 for D-Leu-L-Tyr[11] and Gly-L-Tyr,[15] respectively.

The fact that dipeptides are competitive inhibitors of CPase activity is indicative that they are substrates.[16] The fact that dipeptides are bound at the active site of CPase, and yet are not hydrolyzed at a measurable rate indicates that the activation of the enzyme–dipeptide complex is the rate-limiting step in the hydrolysis of dipeptides. The formation of the initial enzyme–dipeptide complex is not rate determining.

Correction for Product Inhibition.—Subsequent studies on the competitive inhibition of the hydrolysis of Cbz-Gly-L-Phe by dipeptides were carried out at *p*H 9.0 in order to obtain a high effective concentration of inhibitor and to avoid high ninhydrin blanks resulting from the dipeptide. Unequivocal K_i values for dipeptides at *p*H 9.0 could not be determined from Lineweaver and Burk[14] plots because of the uncertainty of the correction required for product inhibition[7] by the anionic form of L-phenylalanine.

The course of the hydrolysis of Cbz-Gly-L-Phe and other substrates[5a,7] at *p*H 7.5 is described by the integrated form[17] of the Michaelis-Menten equation

$$k_3et = 2.3K_m \log S_0/S + (S_0 - S) \qquad (3)$$

A plot of the term on the right hand side of this equation *vs.* time, *t*, yields a straight line whose slope is equal to k_3e, the maximal velocity. As the *p*H is increased above 7.5, competitive inhibition by the anionic form of L-phenylalanine, resulting from the hydrolysis of Cbz-Gly-L-Phe, becomes an increasingly important factor. Thus, when eq. 3 is applied to experiments at *p*H 9.0, a negative deviation from a straight line results.

In order to correct for product inhibition eq. 4 can be applied.

$$k_3et = 2.3K_m \left[1 + \frac{\alpha(S_0 + P_0)}{K_p}\right] \log S_0/S + (S_0 - S)\left(1 - \frac{\alpha K_m}{K_p}\right) \qquad (4)$$

K_p is the dissociation constant for the complex between the enzyme and the inhibitory form of the product; α, the mole fraction of the inhibitory form of the product; and P_0, the concentration of the product at zero time. In general cases where K_p is much larger than K_m or α is very small eq. 4 reverts to eq. 3. The α value for L-phenylalanine at *p*H 7.5 (0.023) is considerably lower than that at *p*H 9.0 (0.426); therefore, much less correction for product inhibition is required in the case of experiments at *p*H 7.5.

Competitive Inhibition by Product and Dipeptide.—The following is a modification of the conventional Lineweaver-Burk equation for special cases in which the substrate, the anionic form of the product, and the anionic form of a dipeptide all compete for the same active site on the surface of the enzyme.

$$1/v = K_m/V_m \times 1/S\left[1 + \frac{\alpha(S_0 - S)}{K_p} + \frac{\alpha' I}{K_i}\right] + 1/V_m \qquad (5)$$

The term α' represents the mole fraction of the actual inhibitory species of a dipeptide at the experimental *p*H; *I*, the total dipeptide concentration; and K_i, the dissociation constant for the enzyme–dipeptide complex.

Equation 5 was not used for the determination of the K_i values of dipeptides, because of the uncertainty of the term for product inhibition, $\alpha(S_0 - S)/K_p$. Integration of eq. 5 leads to the following relationship

$$k_3et = 2.3K_m \left[1 + \frac{\alpha S_0}{K_p} + \frac{\alpha' I}{K_i}\right] \log S_0/S + (S_0 - S)\left(1 - \frac{\alpha K_m}{K_p}\right) \qquad (6)$$

(15) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publ. Corp., New York, N. Y., 1943, p. 85.

(16) It is, of course, possible that the complex formed by a dipeptide with CPase as a competitive inhibitor may be different from that formed as a substrate. However, known competitive inhibitors (of chymotryptic activity) have been shown to be substrates (M. L. Bender and K. C. Kemp, THIS JOURNAL, **79**, 111 (1957)). Conversely, compounds which were known substrates (of trypsin) were later shown to be competitive inhibitors (S. A. Bernhard, *ibid.*, **77**, 1973 (1955)).

(17) D. D. Van Slyke and G. E. Cullen, *J. Biol. Chem.*, **19**, 141, 211 (1914).

The constants obtained from one series of experiments, employing eq. 4 and 6, were verified by a number of methods, *e.g.*, varying the substrate concentration, adding product at zero time, and varying the substrate/inhibitor ratio. These equations are the same as those derived by Huang and Niemann[8] except for the terms α, α' and P_0, which are absent from the equations of these workers.

K_i Values for Dipeptides.—The dissociation constants for the enzyme–substrate and the enzyme–product complexes must be known before the K_i value for a dipeptide can be determined. The K_m for Cbz-Gly-L-Phe (2×10^{-2} *M*) was calculated by conventional methods[15] from initial velocities so that the effect of product inhibition would be minimal. Equation 4 was used to calculate the K_p (4×10^{-4} *M*) for L-phenylalanine from a series of experiments in which the substrate concentration was varied from 0.01 *M* to 0.05 *M*. Equation 6 was then applied to calculate the K_i values for various dipeptides. Typical data are shown in Figs. 3 and 4. The plots of % hydrolysis *vs.* time (Fig. 3) reveal appreciable decreases in hydrolytic

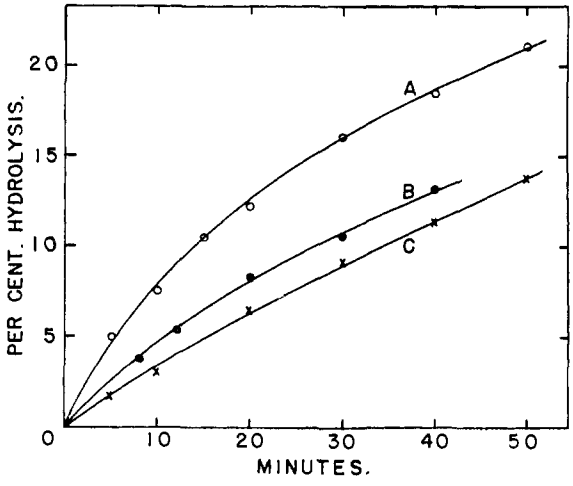

Fig. 3.—Kinetic study of competitive inhibition: A, 0.02 *M* substrate, Cbz-Gly-L-Phe; B, substrate + 0.01 *M* D-Leu-L-Tyr; C, substrate + 0.004 *M* L-phenylalanine. The enzyme concn. was 0.3 μg. protein N per ml.; buffer, 0.05 *M* AMPD, *p*H 9.0.

rates when L-phenylalanine or D-Leu-L-Tyr was added at zero time. When eq. 6 is applied, the experimental points fall along a common straight line (Fig. 4). The values employed for K_m, K_p, K_i, α, α' were 2×10^{-2} *M*, 4×10^{-4}*M*, 4×10^{-3}*M*, 0.426 and 0.83, respectively. The K_i values for L-Ser-L-Tyr, L-Leu-L-Tyr and acetyl-L-Tyr were also determined by this method. These data should be interpreted only qualitatively because of the indirect method used for the determination of these values.

The Effect of Ionic Strength.—The foregoing kinetic studies were carried out in solutions with ionic strengths of 0.2 ± 0.04. Lumry and Smith[18] have shown recently that, although K_m and k_3 for the substrate vary with ionic strength, they become constant at ionic strengths greater than 0.5.

(18) R. Lumry and E. L. Smith, *Disc. Faraday Soc.*, **20**, 105 (1955).

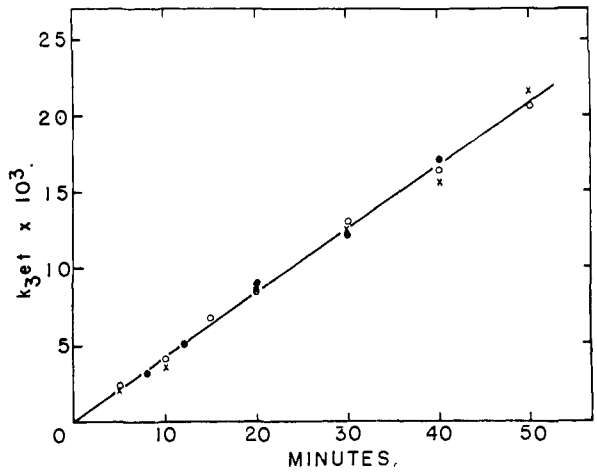

Fig. 4.—Correction of data in Fig. 3 for competitive inhibition by product and dipeptide. Equation 6 was applied.

The K_m for Cbz-Gly-L-Phe was, therefore, redetermined at an ionic strength of 0.5. The value obtained at *p*H 9.0, 0.014 *M*, is almost equal to that determined by Lumry and Smith at *p*H 7.5.[19] Their observation of substrate inhibition[20] when the concentration of the substrate exceeds 0.04 *M* has been confirmed. The dissociation constants for the product and dipeptides are also modified by an increase in ionic strength. For example, values for K_m(Cbz-Gly-L-Phe), K_p(L-phenylalanine) and K_i(D-Leu-L-Tyr) determined at an ionic strength of 0.5 are 0.014, 0.0005, 0.002 *M*, respectively, as compared with values of 0.02, 0.0004 and 0.004 *M*, respectively, obtained with an ionic strength of 0.2. Although the K_i values for dipeptides are different at the two ionic strengths, the relative order remains unchanged.

Correlation of Hydrolytic Susceptibility and Affinity.—In Table II data on the hydrolytic rates of N-acyl derivatives of L-tyrosine[6] are correlated with the dissociation constants for the complexes between these compounds and CPase. Although the hydrolytic rates are not absolute measures of susceptibility and the K_i values may not be simple dissociation constants, these values fall into categories which differ sufficiently so that they may be used as relative measures of hydrolytic susceptibility and affinity. The K_i values for relatively poor substrates, such as those in Table II, are probably closer measures of the true dissociation constants of their complexes with CPase than the K_m values of highly susceptible substrates.[21]

(19) R. Lumry and E. L. Smith (ref. 18) have shown that the K_m for Cbz-Gly-L-Try becomes constant above *p*H 7.5.

(20) R. Lumry, E. L. Smith and R. R. Glantz, THIS JOURNAL, **73**, 4330 (1951).

(21) The K_m for a susceptible substrate is a maximal value for the true dissociation constant, k_2/k_1, because of the contribution of the specific activation rate, k_3, to the experimentally determined constant, $(k_2 + k_3)/k_1$. Lumry and co-workers (ref. 20) have presented evidence for interpreting the Michaelis constant for highly susceptible substrates of CPase as a ratio of the constants for two consecutive reactions, *i.e.*, $K_m = k_3/k_1$. The fact that the K_i (equal to K_m) for a dipeptide was determined under conditions such that no measurable hydrolysis of the dipeptide occurred indicates that the contribution of k_3 to the K_i value is relatively small.

TABLE II

COMPARISON OF CARBOXYPEPTIDASE SUBSTRATES

Substrate	Hydrolytic rate[a]	Affinity index[b]
Gly-L-Tyr	1	7
L-Ser-L-Tyr	3	9
L-Leu-L-Tyr	130	30
D-Leu-L-Tyr	120	40
Acetyl-L-Tyr	18	400
Cbz-Gly-L-Phe	18,000	200
L-Phe	....	4

[a] Data from ref. 6. [b] Dissociation constant $\times 10^4$. Based on K_i values for all compounds except for the K_m for Cbz-Gly-L-Phe.

The compounds in this table fall into three categories with respect to affinity for CPase. Gly-L-Tyr and L-Ser-L-Tyr are almost as effectively bound as the free amino acid, L-phenylalanine. The diastereoisomers of Leu-L-Tyr are somewhat less tightly bound. Acetyl-L-Tyr and Cbz-Gly-L-Phe evidently form even less stable complexes.

The order of hydrolytic susceptibility is quite different from that of affinity. The low hydrolytic rates of dipeptides cannot be ascribed merely to low affinity for the enzyme, since the poorest substrate in the group, Gly-L-Tyr, forms the most stable complex with CPase. The data further indicate that an increase in hydrolytic susceptibility of the unsubstituted dipeptides is accompanied by a decrease in affinity.

The greater affinity of CPase for Gly-L-Tyr as compared with acetyl-L-Tyr may be a resultant of an additional interaction of some substituent of Gly-L-Tyr with the active site of the enzyme, *e.g.*, coördination of the uncharged amino group with the zinc[22] ion of the enzyme. The low hydrolytic susceptibility of Gly-L-Tyr may then be a direct consequence of the nature of its relatively stable complex with CPase. A hydrolytic rate greater than that of acetyl-L-Tyr would be expected of a substrate containing an "electron-withdrawing" group in the acyl moiety, such as the dipolar ionic form of Gly-L-Tyr or chloroacetyl-L-Tyr. Hence, the fact that Gly-L-Tyr is hydrolyzed at a significantly lower rate than acetyl-L-Tyr at *p*H 9.0 as well as at *p*H 7.5[6] further indicates that the dominant ionic species of a dipeptide involved in the calculation of the data in Table II is that with an uncharged α-amino group.[12]

It seems unlikely that the marked differences in the hydrolytic rates and affinities of the leucyl and glycyl derivatives of L-tyrosine are due to differences in inductive effects inherent in the peptides. Strain on the susceptible bond, resulting from the orientation of the side chain of the leucyl residue at the active site, may account for the higher hydrolytic rates as well as lower affinities of the leucyl dipeptides.

The primary specificity of CPase is directed toward the C-terminal residue of a peptide.[23] Specific orientation of the susceptible bond of a substrate at the active site of the enzyme results from the specific binding of the carboxylate group[23] and the sidechain[5b,23] of the C-terminal residue. It follows, therefore, that the adjacent amino acid residue of a substrate will also be specifically oriented, if only one of its substituents, other than the carbonyl group, is bound at a specific site on the enzyme. The fact that acetyl-D-Leu-L-Tyr is neither effectively bound[11] nor activated[6] is an indication of steric restriction on the active site of CPase in the region where the α-carbon atom of the second residue is oriented.[6] Hydrogen bonding of the secondary peptide bond of tripeptide analogs[5a] may account for the specific orientation.

The influence of the secondary peptide bond of N-acyl dipeptides on k_3, the specific activation rate, is responsible for the principal difference between dipeptides and their corresponding N-acyl derivatives as substrates of CPase, which is reflected in differences in hydrolytic rates of 100- to 10,000-fold. The comparable peptide bond in chymotrypsin substrates has a similar effect.[24]

It is concluded that the N-acyl moiety of a dipeptide is oriented at the active site in a manner which is quite different from that of the corresponding residue in a tripeptide analog. This deduction is based on the low K_i values for dipeptides and the equivalence of the diastereoisomers of Leu-L-Tyr with respect to affinity and hydrolytic susceptibility. Furthermore, it is concluded that dipeptides form more stable complexes with CPase. The fact that these complexes are not as readily activated as those of their corresponding N-acyl derivatives indicates that the formation step of the enzyme–dipeptide complex is also distinctly different from the activation step.

Acknowledgment.—The valuable advice on the kinetic aspects of this problem, provided by Dr. Arthur Veis, is gratefully acknowledged.

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(22) B. L. Vallee and H. Neurath, THIS JOURNAL, **76**, 5006 (1954).

(23) E. Elkins-Kaufman and H. Neurath, *J. Biol. Chem.*, **178**, 645 (1949).

(24) M. L. Bender and B. W. Turnquest, THIS JOURNAL, **77** 4271 (1955).