The Primary Specificity of Chymotrypsin. Further Evidence for "Wrong-Way" Binding*

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ABSTRACT: A series of acylated L- (and D-) alanine methyl esters has been prepared and the kinetic constants for their steady-state chymotrypsin-catalyzed hydrolysis have been evaluated at 25°, pH 7.9, in the presence of 0.1 M NaCl. The newly synthesized alanine derivatives and their kinetic constants ($K_0 \times 10^{-3}$ mM and k_0 sec^{-1}) are L-furoyl (49 ± 3, 0.51 ± 0.01), L-tetrahydrofuroyl (132 ± 15, 0.40 ± 0.03), L-theophenoyl (15 ± 4, 0.46 ± 0.06), L-nicotinyl (37 ± 2, 0.58 ± 0.02), L-isonicotinyl (29 ± 3, 0.42 ± 0.03), L-picolinyl (18 ± 1, 0.071 ± 0.003), L-*o*-aminobenzoyl (4.7 ± 7, 0.24 ± 0.02), L-quinolinoyl (0.22 ± 0.17, 0.004 ± 0.0007), D-furoyl (49 ± 3, 0.51 ± 0.01), D-tetrahydrofuroyl (61 ± 10, 0.017 ± 0.002), D-theophenoyl (7.4 ± 0.6, 0.0159 ± 0.0006), D-nicotinyl (6 ± 1, 0.0046 ± 0.0002), D-isonicotinyl (27 ± 9, 0.003 ± 0.001), D-picolinyl (17 ± 1, 0.168 ± 0.006), *o*-aminobenzoyl (1.6 ± 0.5, 0.0050 ± 0.0005), and D-quinolinoyl (. . ., 0.001). In both series K_0 decreases with increasing size and total polarizability of the acyl group. For the L-alanine derivatives k_0 also decreases with increasing size and polarizability of the acyl group. This trend, which is contrary to the behavior of a series of acetylated L-amino acid methyl esters, is interpreted as supporting the concept of "wrong-way" binding for alanine derivatives to the enzyme.

For almost two decades the steady-state kinetics of the interaction of chymotrypsin with a large number of acylated amino acid derivatives and related compounds has been quantitatively studied (Niemann, 1964). This work has led to a general correlation between substrate structure and enzyme specificity (Hein and Niemann, 1961, 1962b); especially the *primary specificity* of the enzyme, *i.e.*, its catalytic behavior toward compounds containing only a single amino acid residue. The steady-state behavior of substrates can, in most cases, be successfully described by the familiar Michaelis–Menten equation (eq 1) in which the experimental parameters, K_0 and k_0, are presumably related to the ability of the substrate

$$-d[S]/dt = d[P]/dt = k_0[E][S]/K_0 + [S] \qquad (1)$$

to bind to the enzyme (K_0) and to be hydrolyzed by its catalytic action (k_0). It has formally been recognized since 1925 (Briggs and Haldane) that even in the simple, one-substrate one ES complex formulation of the Michaelis–Menten equation, the parameter K_0 includes both binding and catalysis terms, *i.e.*, it represents a steady state and not an equilibrium constant.

For a variety of reasons, K_0 values have often been considered to represent equilibrium values or, at least to be proportional to them. Within a limited series of compounds such as a series of L-acylated esters of a single amino acid, or a series of L-acetylated esters of different amino acids, the assumption of a monotonic correlation between substrate binding and K_0 values may be justified. At the least, it is possible to compare *trends* in K_0 values for parallel series and to note whether similar structural changes in different series result in the same types of changes in both K_0 and k_0 values.

Recently, it has become apparent that the simple Michaelis–Menten mechanism is not sufficient to explain the kinetic data. A number of experiments, first with *p*-nitrophenyl acetate (Hartley and Kilby, 1954) and most recently with specific substrates (Brandt and Hess, 1966) as well as a number of theoretical arguments (Zerner and Bender, 1964) suggest that the minimum enzymatic mechanism involves the formation of an acyl–enzyme intermediate as well as an initial noncovalently bonded complex. The expansion of the mechanism makes the interpretation of the observed steady-state constants, K_0 and k_0, even more ambiguous.

A further complication arises from observations by Hess and his associates (Moon *et al.*, 1965) and by Lumry and co-workers (Biltonen *et al.*, 1965) that chymotrypsin undergoes profound conformational changes on interaction with substrates and inhibitors. Studies on the temperature dependence of the free-

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TABLE I: Physical Constants of Acylated Alanine Methyl Esters, $RCONHCH(CH_3)CO_2CH_3$.

Compound	Mp (°C)	$[\alpha]_D^{25}$, deg (*c*)		C (%)	H (%)	N (%)
			Calcd for $C_{10}H_{12}N_2O_3$ (208)	57.7	5.8	13.5
L-Isonicotinyl	85.5–86.5	−29.4 (5% in H_2O)	Found	57.8	5.9	13.5
D-Isonicotinyl	85.5–86.5	29.6 (5% in H_2O)	Found	57.8	5.9	13.4
L-Nicotinyl	75.5–76.5	−30.6 (5% in H_2O)	Found	57.1	6.0	13.6
D-Nicotinyl	75.5–76.5	30.0 (5% in H_2O)	Found	57.5	6.0	13.6
L-Picolinyl	59–60	−15.3 (3% in H_2O)	Found	57.6	6.1	13.4
D-Picolinyl	59–60	15.3 (3% in H_2O)	Found	57.8	5.9	13.3
			Calcd for $C_9H_{11}NO_4$ (197)	54.8	5.6	7.1
L-Furoyl	41–42	−2.8 (6% in H_2O)	Found	54.6	5.9	7.3
D-Furoyl	41–42	2.7 (6% in H_2O)	Found	54.9	5.9	7.2
			Calcd for $C_9H_{15}NO_4$ (201)	53.7	7.5	6.9
L-Tetrahydrofuroyl	Bp 86–88° (200 μ)[a]	−29.3 (6% in MeOH)		53.6	7.3	6.7
D-Tetrahydrofuroyl	Bp 95–96° (300 μ)[a]	29.2 (6% in MeOH)		53.8	7.6	6.9
			Calcd for $C_9H_{11}O_3S$[b]	50.7	5.2	6.6
L-2-Theophenoyl	91–92	−16.0 (4% in MeOH)	Found[b]	50.8	5.2	6.6
D-2-Theophenoyl	91–92	15.5 (4% in MeOH)	Found[b]	50.8	5.2	6.3
			Calcd for $C_{11}H_{14}N_2O_3$	59.5	6.3	12.6
L-*o*-Aminobenzoyl	92.5–93.5	−70.0 (4% in MeOH)	Found	59.5	6.3	12.7
D-*o*-Aminobenzoyl	92.5–93.5	70.0 (4% in MeOH)	Found	59.3	6.2	12.6
			Calcd for $C_{14}H_{14}N_2O_2$	65.1	5.5	10.8
L-2-Quinolinyl	60.5–61.5	83.0 (4% in chloroform)	Found	65.1	5.5	10.7
D-2-Quinolinyl	60.5–61.5	−83.3 (4% in chloroform)	Found	64.7	5.4	10.8

[a] n_D^{55} for L and D isomers 1.4671 and 1.4675, respectively. [b] Sulfur analyses: Calcd 15.0, found 14.9 and 15.0 for L and D isomers, respectively.

energy changes during these interactions suggest that the modest free-energy changes observed result from the compensation of large negative enthalpy and entropy changes.

In the light of these complications, the results of steady-state experiments must be reviewed carefully. However, the data are still useful for certain correlations. As indicated above, comparison of K_0 and k_0 values for a series of compounds or for pairs of series, can suggest trends of specificity. Thus, Knowles (1965) has postulated "an inherent tendency toward "better binding–better reaction" on the basis of an inverse relationship between k_0 and K_0 for a series of acetylated amino acid esters. More generally, Bender and Kezdy (1965) have argued that the ratio k_0/K_0, which does not reflect the complications introduced by the acyl–enzyme hypothesis because the additional kinetic terms cancel in the ratio, can be used to define a specificity constant. This constant represents the essentially invariant effect of a side-chain or acyl-substituent modification on the activity of any specific amino acid substrate.

In a similar manner Niemann and co-workers (Huang and Niemann, 1952; Foster and Niemann, 1955; Hein and Niemann, 1962b; Rapp, 1964) have argued that certain trends in K_0 and k_0 values with structural changes support the concept of "wrong-way" binding. Similar arguments have been advanced independently by Cohen and his co-workers (1962, 1963, 1964). The concept of "wrong-way" binding suggests that certain compounds may combine most favorably with the enzyme in a conformation, or orientation, which does not permit enzyme-catalyzed hydrolysis. The kinetic consequences of this type of interaction are such that the *form* of the observed steady-state kinetics is not altered, but the interpretation of the K_0 and k_0 values is modified. Self-inhibition or "wrong-way" binding should result in decreasing k_0 values as K_0 values decrease within a single structural series. If this result is observed it would necessarily provide an exception of Knowles generalization concerning "better binding–better reaction."

The original postulate of "wrong-way" binding (Huang and Niemann, 1952) was based on the mixed inhibition of methyl hippurate hydrolysis by indole. It was later supported by theoretical arguments (Foster and Niemann, 1955) and the results with only two pairs of compounds (Hein and Niemann, 1962a).

TABLE II: α-Chymotrypsin-Catalyzed Hydrolysis of Some α-N-Acyl-L- (and D-) alanine Methyl Esters.[a]

Substrate, RCO in RCONHCH-$(CH_3)CO_2CH_3$	Molar Refraction of R, CC[b]	No. of Expt[c]	[E],[d] mM	[S], mM	K_0[e] mM	k_0,[e] sec^{-1}	k_0/K_0, M^{-1} sec^{-1}
L Series							
Acetyl[f]	5.0				739	1.27	
Furoyl	14.8	8–1	34	12–99	49 ± 3	0.51 ± 0.01	10.4
Tetrahydrofuroyl	18.7	14–0	43	20–201	132 ± 15	0.40 ± 0.03	3.03
Theophenoyl	21.2	11–0	45	2.2–18	15 ± 4	0.46 ± 0.06	30.6
Nicotinyl	22.8	15–0	37	2.6–21	37 ± 2	0.58 ± 0.02	15.7
Isonicotinyl	22.8	11–0	29	5.6–45	29 ± 3	0.42 ± 0.03	14.5
Picolinyl	22.8	10–0	22	2.3–18	18 ± 1	0.071 ± 0.003	3.94
Benzoyl[g]	24.5				9.6	0.23	
o-Aminobenzoyl	27.9	16–0	37	0.68–5.5	4.7 ± 0.7	0.24 ± 0.02	51.1
Quinolinoyl	39.6	12–0	48	0.16–1.3	0.22 ± 0.17	0.0040 ± 0.0007	18.2
D Series							
Acetyl		Not a substrate				0.001	
Furoyl		11–0	34	12–99	49 ± 3	0.51 ± 0.01	10.4
Tetrahydrofuroyl		10–0	56	10–80	61 ± 10	0.017 ± 0.002	0.28
Theophenoyl		9–1	45	1.9–15	7.4 ± 0.6	0.0159 ± 0.0006	2.15
Nicotinyl		13–0	71	2.7–22	6 ± 1	0.0046 ± 0.0002	0.767
Isonicotinyl		8–1	57	4.2–33	27 ± 9	0.003 ± 0.001	14.5
Picolinyl		8–0	63	1.6–13	17 ± 1	0.168 ± 0.006	9.88
Benzoyl[g]					3.3	0.01	
o-Aminobenzoyl		12–(0)	44	0.66–5.3	1.6 ± 0.5	0.005 ± 0.0005	3.12
Quinolinoyl			48	0.80		0.001	

[a] In aqueous solution at 25.0°, pH 7.90, and 0.10 M in sodium chloride. [b] Calculated from bond refractions (Vogel *et al.*, 1952). [c] First number refers to number of experiments performed for evaluation of K_0 and k_0; second number refers to number of experiments rejected by statistical reiterative procedure (Abrash *et al.*, 1960). [d] Based on molecular weight of 25,000 and a nitrogen content of 16.5%. [e] Evaluated by a least-squares fit to the equation $([E][S]/v_0) = (K_0/k_0) + ([S]/k_0)$. [f] Jones *et al.* (1965a). [g] Hein and Niemann (1962a).

Since that time the concept has received support from the results of Cohen (1962, 1963, 1964) and from inhibition studies on valine and norvaline derivatives (Jones, 1965b). In this communication we wish to report the results of an investigation of the steady-state kinetic behavior or the chymotrypsin-catalyzed hydrolysis of a series of D- (and L-) acylated alanine methyl esters. The results strongly support the wrong-way binding concept and provide a significant exception to the better binding–better reaction principle. They also raise questions concerning the general applicability of substrate independent specificity constants, k_0/K_0.

Experimental Section

Synthesis. The acylated amino acid methyl esters were prepared from D- (or L-) alanine methyl ester hydrochloride and the appropriate acid or acid chloride, by means of the mixed anhydride method (Greenstein and Winitz, 1961; Albertson, 1961). The condensing agent was isobutyl chlorocarbonate in chloroform solution with sufficient triethylamine added to take up all the acid formed during the reaction. The products were recrystallized from isopropyl ether to constant melting point. Physical constants for all the compounds are given in Table I.

Kinetic Studies. The procedure was identical with that described previously (Applewhite *et al.*, 1958). All experiments were conducted in aqueous solutions at 25.0°, pH 7.90 ± 0.10, and 0.10 M with respect to sodium chloride. The enzyme preparation was crystalline, bovine, salt-free α-chymotrypsin, Armour Lot No. T-97207 or Worthington C.D.I. 6066-67. Nitrogen determinations on the enzyme preparations, by the micro-Kjeldahl procedure of Redmann (1939) gave values of 13.97 ± 0.11 and 14.91 ± 0.06, respectively. The primary data were analyzed with the aid of a Datatron 220 digital computer, programmed as described earlier (Abrash *et al.*, 1960).

Results

Table II lists the kinetic constants and pertinent experimental data for the compounds reported in this study as well as results previously obtained on

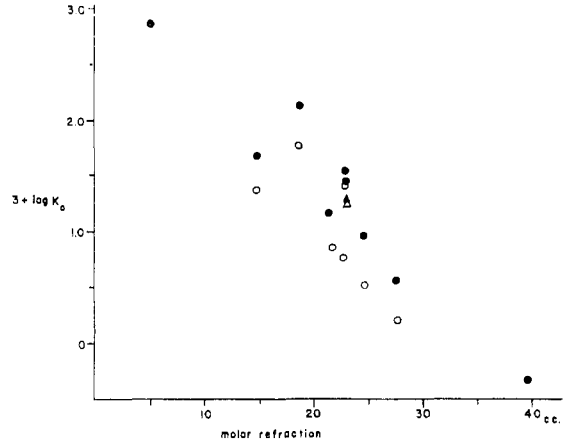

FIGURE 1: Log K_0 values for a series of acylated alanine methyl esters $RCONHCH(CH_3)CO_2CH_3$, plotted against the molar refraction of R. ●, L isomers; ○, D isomers; ▲ and △, L- (and D-) picolinyl derivatives.

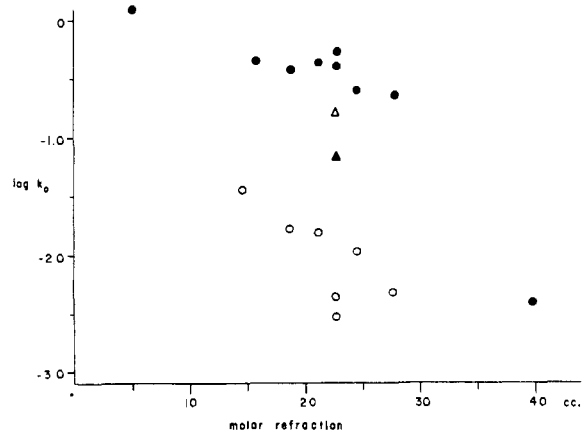

FIGURE 2: Catalytic constants, k_0, for a series of acylated alanine methyl esters, $RCHONCH(CH_3)CO_2CH_3$, plotted against the molar refraction of R. ●, L isomers; ○, D isomers; ▲ and △, L- (and D-) picolinyl derivatives.

the related acetyl and benzoyl derivatives. The compounds have been arranged in order of increasing molar refraction of the hydrocarbon or heterocyclic acyl group. The total molar refraction (Fajans, 1960) a constitutive and additive property of molecules, can be taken as a rough measure of molecular size when calculated for atoms of approximately equal polarizability. In the present case, owing to the electronegative substituents on some of the alicyclic components, this requirement is not strictly met. Nevertheless, the total group refraction provides a convenient method for ordering both substrates and inhibitors of chymotrypsin according to effective size (G. E. Hein, unpublished manuscript).

Discussion

The use of steady-state kinetic constants as the "activity" measure for a structure–activity correlation is difficult, as indicated previously. The decision on the choice of a relevant "structure" parameter also presents problems. It is generally recognized (Niemann, 1964; Jones *et al.*, 1965b; Bender and Kezdy, 1965; Knowles, 1965), that the specificity of chymotrypsin is primarily size specificity, rather than group specificity. In general, neither charged nor polar side chains contribute to the specificity.

This type of specificity can be rationalized by assuming that interaction between substrates and chymotrypsin is controlled largely by factors which effect substrate–solvent interaction and enzyme–solvent interaction rather than through the involvement of specific intermolecular attractive forces, such as coulombic attraction or hydrogen bonds, between substrate and enzyme.

This type of "nonspecific" specificity presents problems in any attempt to generalize a structure–activity correlation. To a first approximation, specificity appears to depend on the size of the substrate, or varying group on the substrate. But the size of an organic residue in solution is not a clearly defined property. One general approach to a structural parameter, is to compare biological activity to another empirical measure such as a partition coefficient or differential solubility. This approach has been used successfully since the Meyer–Overton theory was suggested around 1900 (Overton, 1901).

Another approach is to use as a structure parameter a physical property which is more clearly related to effective molecular size. We have observed (G. E. Hein, unpublished manuscript) that chymotrypsin specificity can be described for any series of substrates to a first approximation, as a simple function of the molar refraction of the group whose structures is varied. The present series illustrates this relation.

In Figure 1, the logarithm of observed Michaelis constants, K_0, for both D- (and L-) acylated alanine methyl esters are plotted against the molar refraction of the acyl group. The values fall in a narrow band, generally decreasing K_0 values as molar refraction increases. This type of result is characteristic for chymotrypsin substrates and inhibitors (Wallace *et al.*, 1963).

Equally characteristic for typical chymotrypsin substrates is the observation that k_0 values increase with increasing molar refraction of the side chain. This is the basis for Knowles (1965) generalization "better binding–better catalysis" and has been discussed in a comparison of the antiparallel variation in K_0 and k_0 for a series of acylated amino acid esters with normal side chains as substrates of chymotrypsin (Jones *et al.*, 1965a). The present series provides a

striking exception to this rule. In Figure 2, the observed k_0 values are plotted against the molar refraction of the acyl group for both D- (and L-) alanine residues. Although the data do not distribute as neatly as might be desired, it is clear that k_0 values generally *decrease* with increasing molar refraction of the side chain. This trend is particularly noticeable in the L series. The ratio of k_0 values for quinuclidyl-L-alanine methyl ester/acetyl-L-alanine methyl ester is 3.1×10^{-3} while the corresponding ratio of K_0 values is 3.6×10^{-4}. Clearly "better binding" does not lead to "better hydrolysis."

The series of acylated alanine methyl esters thus presents a case of "better binding–*poorer* catalysis." An explanation of this phenomenon is provided by the concept of "wrong-way" binding. The larger the acyl group, the more important become the binding modes for the L compound which permit this group to interact with the side-chain specificity site. This is the same conclusion which was reached previously from a comparison of only acetyl and benzoyl derivatives of alanine (Hein and Niemann, 1962b) and valine (Jones *et al.*, 1965b). It is gratifying to note that a more extended series bears out the previous conclusion.

The "wrong-way" binding concept has received support from other sources as well. Zeller (1963, Zeller *et al.*, 1965) has interpreted the data on the inhibition of monoamine oxidase by substituted aromatic amines in an analogous manner. The concept has also been employed in considering the kinetic of polymer cleavage by enzymes (Hansen, 1962; Thoma, 1965). It was previously applied to the hydrolyses of amylodextrins by α-amylase (Thoma and Koshland, 1960).

The use of a general structure criterion, such as molar refraction, to develop a structure–activity relation has an added advantage in that deviations from a simple relationship are easily noted. In the series of acylated alanine methyl esters, a glance at Figures 1 and 2 indicates that one pair of compounds, *N*-picolinyl (D- and L-) alanine methyl esters, has abnormal k_0 values. The unusually high value for the D isomer along with the unusually low one for the L isomer result in an inversion of the usual stereospecificity of chymotrypsin for the pair of compounds (Rapp and Niemann, 1963). In one sense it can be argued that this pair of compounds represents one of the first rational attempts to obtain inversion of stereospecificity. Certainly the series of D- (and L-) acylated alanine compounds were prepared with this goal in mind (Rapp, 1964). At the same time, we must admit that the observation of inversion of stereospecificity with the picolinyl derivatives rather than with any other pair, remains unexplained.

Figures 1 and 2 also illustrate that although K_0 values are roughly equal for both the L and D series, k_0 for these two classes of compounds differs significantly. From this it follows that a specificity constant, k_0/K_0, as defined by Bender and Kezdy (1965), is dependent at the least on the stereochemistry of the substrate.

References

Abrash, H. I., Kurtz, A. N., and Niemann, C. (1960), *Biochem. Biophys. Acta 45*, 387.

Albertson, N. F. (1961), *Org. Reactions 12*, 1.

Applewhite, T. H., Waite, H., and Niemann, C. (1958), *J. Am. Chem. Soc. 80*, 1465.

Bender, M. L., and Kezdy, F. J. (1965), *Ann. Rev. Biochem. 34*, 49.

Biltonen, R., Lumry, R., Madison, V., and Parker, H. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 1412.

Brandt, K. G., and Hess, G. P. (1966), *Biochem. Biophys. Res. Commun. 22*, 447.

Briggs, G. E., and Haldane, J. B. S. (1925), *Biochem. J.* 338.

Cohen, S. G., Crossley, J., Khedouri, E., and Zand, R. (1962), *J. Am. Chem. Soc. 84*, 4163.

Cohen, S. G., Crossley, J., Khedouri, E., Zand, R., and Klee, L. H. (1963), *J. Am. Chem. Soc. 85*, 1685.

Cohen, S. G., and Weinstein, S. Y. (1964), *J. Am. Chem. Soc. 86*, 5326.

Fajans, K. (1960), *in* Physical Methods in Organic Chemistry, Weissberger, A., Ed., New York, N. Y., Interscience, p 1169.

Foster, R. J., and Niemann, C. (1955), *J. Am. Chem. Soc. 77*, 1886.

Greenstein, J. P., and Winitz, M. (1961), Chemistry of the Amino Acids and Proteins, Vol. II, New York, N. Y., Wiley, p 979.

Hanson, K. R. (1962), *Biochemistry 1*, 723.

Hartley, B. S., and Kilby, B. A. (1954), *Biochem. J. 56*, 288.

Hein, G. E., and Niemann, C. (1961), *Proc. Natl. Acad. Sci. U. S. 47*, 1341.

Hein, G. E., and Niemann, C. (1962a), *J. Am. Chem. Soc. 84*, 4487.

Hein, G. E., and Niemann, C. (1962b), *J. Am. Chem. Soc. 84*, 4495.

Huang, C. T., and Niemann, C. (1952), *J. Am. Chem. Soc. 74*, 59.

Jones, J. B., Kunitake, T., Niemann, C., and Hein, G. E. (1965a), *J. Am. Chem. Soc. 87*, 1777.

Jones, J. B., Niemann, C., and Hein, G. E. (1965b), *Biochemistry 4*, 1735.

Knowles, J. R. (1965), *J. Theoret. Biol. 9*, 213.

Moon, A. Y., Mercouroff, J., and Hess, G. P. (1965), *J. Biol. Chem. 240*, 717, and references cited therein.

Niemann, C. (1964), *Science 143*, 1287.

Overton, E. (1901), Studies uber die Narkose, G. Fischer, Jena.

Rapp, J. R. (1964), Ph.D. Thesis, California Institute of Technology, Pasadena, Calif.

Rapp, J. R., and Niemann, C. (1963), *J. Am. Chem. Soc. 85*, 1896.

Redmann, C. E. (1939), *Ind. Eng. Chem. 11*, 635.

Thoma, J. A. (1965), *Biochemistry 5*, 1365.

Thoma, J. A., and Koshland, D. E., Jr. (1960), *J. Am. Chem. Soc. 82*, 3329.

Vogel, A. I., Cresswell, W. T., Leffrey, G. H., and

Leicester, J. (1952), *J. Chem. Soc.*, 531.
Wallace, R. A., Kurtz, A. N., and Niemann, C. (1963), *Biochemistry 2*, 824.
Zeller, E. A. (1963), *Biochem. Zeit. 339*, 13.
Zeller, E. A., Ramachander, G., Fleisher, G. A., Ishimaru, T., and Zeller, V. (1965), *Biochem. J. 95*, 262.
Zerner, B., and Bender, M. L. (1964), *J. Am. Chem. Soc. 86*, 3669.

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 (0.16×10^{-3} M, 0.86 min^{-1}), *N*-acetyl-L-tyrosyl-L-phenylalanine (2.0×10^{-3} M, 0.3 min^{-1}), *N*-acetyl-L-phenylalanyl-L-tyrosine (2.2×10^{-3} M, 5.1 min^{-1}), and *N*-acetyl-L-tyrosyl-L-tyrosine (6.1×10^{-3} M, 0.88 min^{-1}) were determined as were values of K_I for *N*-acetyl-L-phenylalanine (23×10^{-3} M) and *N*-acetyl-L-tyrosine (41×10^{-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; α, the factor that relates the dissociation constant of EIS with that of EI (Webb, 1963).