[CONTRIBUTION NO. 2830 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY, PASADENA, CALIF.]

Steric Course and Specificity of α-Chymotrypsin-catalyzed Reactions. I[1]

BY GEORGE E. HEIN[2] AND CARL NIEMANN[3]

RECEIVED APRIL 16, 1962

The kinetics of the α-chymotrypsin-catalyzed hydrolysis of formyl-D- and L-phenylalanine methyl ester, benzoyl-D- and L-alanine methyl ester, D- and L-3-carbomethoxydihydroisocarbostyril and 3-carbomethoxyisocarbostyril have been determined. The inhibition of the hydrolysis of benzoyl-D- and L-alanine methyl ester and of D- and L-3-carbomethoxydihydroisocarbostyril by indole has also been examined. From these data it has been demonstrated: (a) that α-chymotrypsin-catalyzed reactions proceed with relative rather than absolute stereospecificity; (b) that the degree of relative stereospecificity is determined, as a first approximation, by the size of the groups attached to the asymmetric carbon atom; and (c) that with D- and L-3-carbomethoxydihydroisocarbostyril an inversion of antipodal specificity is observed.

Introduction

The stereospecificity encountered in enzyme-catalyzed reactions has long been associated with the formation of a diastereoisomeric enzyme substrate complex.[4-8] In particular cases the greater, if not overwhelming, reactivity of one member of an enantiomorphic pair of substrates has been accounted for by reference to the predicted different molecular properties of the two possible diastereoisomeric enzyme–substrate complexes. Nevertheless, comprehension of reactions catalyzed by enzymes requiring no coenzyme has progressed relatively little beyond the apt, but vague, lock and key analogy proposed by Fischer.[6] This is particularly true for the proteinases, where the almost universal use of conformationally indeterminate model substrates has given practically no information about the conformation of the active site of the enzyme other than to confirm its asymmetry.

In contrast to the above, notable progress has been made, for example, in understanding reactions catalyzed by DPN and TPN dependent dehydrogenases[9] by use of conformationally constrained substrates to elucidate the steric course of these enzyme-catalyzed reactions. The recent discovery of a conformationally constrained substrate of α-chymotrypsin[10] has opened a path to an interpretation of the steric course and specificity of α-chymotrypsin-catalyzed reactions which we shall develop in this and subsequent communications.

Results

The substrates selected for study were three pairs of structurally related compounds, all derivatives of alanine methyl ester: formyl-D- and L-phenylalanine methyl ester (I), benzoyl-D- and L-alanine methyl ester (II) and D- and L-3-carbomethoxydihydroisocarbostyril (III); *cf.* Fig. 1. For each pair the hydrolysis of *both* enantiomers was catalyzed to a measurable extent by α-chymotrypsin; *cf.* Table I.

The hydrolysis of both enantiomers of each of the preceding three pairs confirms and extends previous observations[11-13] that the stereospecificity of α-chymotrypsin-catalyzed reactions is relative rather than absolute. In contrast to the earlier examples, which were esters of α-halo or α-hydroxy acids,[11-13] those described here demonstrate that relative stereospecificity may also be observed with certain α-amino acid derivatives.[14]

Although the stereospecificity may be relative rather than absolute, it is usually assumed that for different pairs of enantiomorphic compounds, a particular enzyme will either show no stereospecificity or will always favor those enantiomers possessing related absolute configurations. In fact, the enzymatic determination of the absolute configuration of α-amino acids depends upon the validity of this assumption.[15]

All previous experience with α-chymotrypsin-catalyzed reactions would lead one to expect a relative stereospecificity in favor of substrates possessing the L- or S[16] configuration. The data summarized in Table I does not support this expectation. For two of the pairs, formyl-D- and L-phenylalanine methyl ester and benzoyl D- and L-alanine methyl ester, the more rapidly hydrolyzed enantiomer belongs to the L-series, the behavior usually observed with α-chymotrypsin. However, for the third pair, D- and L-3-carbomethoxydihydroisocarbostyril, *the more rapidly hydrolyzed compound is the* D-*antipode!*

In order to assert that an inversion of antipodal specificity has occurred, it is necessary to ascertain that the more rapidly hydrolyzed members of the pairs in question are indeed of opposite absolute configuration. Formyl-L-phenylalanine methyl ester and benzoyl-L-alanine methyl ester were both prepared in an unambiguous way from the corresponding L-α-amino acids. These acids have been related and are of the S-series.[16] D-3-Carbomethoxydihydroisocarbostyril was prepared from

(1) Supported in part by a grant from the National Institutes of Health, U. S. Public Health Service.

(2) National Science Foundation Postdoctoral Fellow 1961–1962; present address, Dept. of Chemistry, Boston Univ., Boston 15, Mass.

(3) To whom inquiries regarding this article should be sent.

(4) L. Pasteur, *Compt. rend.*, **46**, 615 (1858).

(5) E. Fischer and H. Thierfelder, *Ber.*, **27**, 2036 (1894).

(6) E. Fischer, *ibid.*, **27**, 2985 (1894).

(7) E. Fischer, *Z. physiol. Chem.*, **26**, 60 (1898).

(8) A. G. Ogston, *Nature*, **162**, 963 (1948).

(9) For a recent review see G. W. Wolstenholme and C. M. O'Connor, "Steric Course of Microbiological Reactions," Ciba Foundation Study Group No. 2, Little, Brown and Co., Boston, Mass., 1959.

(10) G. E. Hein, R. B. McGriff and C. Niemann, *J. Am. Chem. Soc.*, **82**, 1830 (1960).

(11) J. E. Snoke and H. Neurath, *J. Biol. Chem.*, **182**, 577 (1950).

(12) J. E. Snoke and H. Neurath, *Arch. Biochem.*, **21**, 351 (1949).

(13) S. Kaufman and H. Neurath, *ibid.*, **21**, 437 (1949).

(14) In unpublished experiments conducted in these laboratories Dr. R. M. Bock found that acetyl-D-tryptophan methyl ester is also hydrolyzed by α-chymotrypsin.

(15) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," John Wiley and Sons, Inc., New York, N. Y., 1961, Vol. 1, pp. 130–152, 728–750; Vol. 2, pp. 1753–1812.

(16) R. S. Cahn, C. K. Ingold and V. Prelog, *Experientia*, **12**, 81 (1956).

TABLE I

α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF SOME ACYLATED α-AMINO ACID ESTERS[a]

Substrate	Expts.[b]	$[S]_0$, mM	$[E]$,[c] M	K_0,[d] mM	k_0,[d] sec.$^{-1}$	k_0/K_0, M^{-1} sec.$^{-1}$	$\frac{(k_0/K_0)_L}{(k_0/K_0)_D}$
Methyl formyl-L-phenylalaninate		Hydrolyzed too rapidly to evaluate K_0 and k_0				~10[5][g]	~10[4]
Methyl formyl-D-phenylalaninate	19—3	0.189–15.9	3.54×10^{-8}	0.248 ± 0.232	0.00343 ± 0.00006	13.83	
Methyl benzoyl-L-alaninate	20—0	2.50 –15.9	3.54×10^{-8}	9.75 ± 0.86	0.261 ± 0.011	26.77	8.23
Methyl benzoyl-D-alaninate	26—7	0.98 –18.9	3.54×10^{-8}	3.29 ± 0.18	0.0107 ± 0.0002	3.25	
L-3-Carbomethoxydihydroisocarbostyril	17—1	.406– 3.63	2.01×10^{-8}[e]	11.69 ± 1.53	0.124 ± 0.014	10.61	2.46×10^{-4}
D-3-Carbomethoxydihydroisocarbostyril	31—3	.216– 2.14	1.05×10^{-7}[f]	0.527 ± 0.80	22.7 ± 1.2	4.31×10^4	
Carbomethoxyisocarbostyril	14—0	.127– 0.509	1.66×10^{-8}	1.41 ± 0.41	0.134 ± 0.052	95.04	

[a] In aqueous solutions at 25.0°, *p*H 7.90 ± 0.05 and 0.20 M in sodium chloride. [b] Number of experiments performed for evaluation of K_0 and k_0; second number refers to those rejected by statistical reiterative procedure used for evaluation of K_0 and k_0. [c] Based upon a molecular weight of 25,000 and a nitrogen content of 16.5%. [d] Evaluated by a least squares fit to the equation $([E][S]/v_0) = (K_0/k_0) + ([S]/k_0)$ as described in text. [e] Mean value of range from 1.94×10^{-8} to 2.09×10^{-8}. [f] Mean value of range from 0.94×10^{-7} to 1.15×10^{-7}. [g] Estimated from the rate of hydrolysis at low values of $[S]_0$.

D-phenylalanine and, barring any odd number of steps in the synthesis which inverted configuration, should belong to the D-phenylalanine, or R, series. The nature of the synthetic route from D-phenylalanine to D-3-carbomethoxydihydroisocarbostyril makes any inversion improbable, since at no time were any of the bonds between the asymmetric carbon atom and its adjacent four atoms broken, nor was there any valence change in any atom attached to the asymmetric carbon atom. Some racemization was encountered in the conversion of D-phenylalanine to D-3-carboxy-1,2,3,4-tetrahydroisoquinoline. However, the preparation of both enantiomers of this intermediate and of the final product, D- and L-3-carbomethoxydihydroisocarbostyril, with experimentally equal and opposite rotations affords ample evidence for the optical integrity and purity of these substrates. Thus, an inversion of the usual antipodal specificity observed in α-chymotrypsin-catalyzed reactions has been demonstrated.

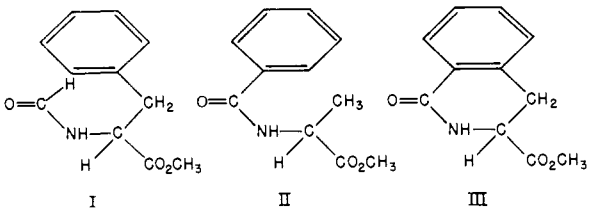

Fig. 1.—Substrates of α-chymotrypsin.

All kinetic experiments were conducted in aqueous solutions at 25.0°, *p*H 7.90 ± 0.05 and 0.20 M in sodium chloride with the rates being determined with the aid of a *p*H-stat.[17,18] The primary data, which consisted of recorder traces of the amount of base added to the reaction system *vs.* time, were first reduced to obtain values of the so-called initial velocities, which in turn were used to compute values of K_0 and k_0 for the rate equation

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

All computations were performed on a Datatron previously.[19] With this procedure it was possible 220 digital computer programmed as described to follow reactions whose initial velocities did not exceed $10^{-4}M$/min. and to evaluate the constants k_0 and K_0 of the rate equation for all substrates except formyl-L-phenylalanine methyl ester. With this very reactive substrate, instrumental limitations were encountered. Any attempt to slow down the reaction by decreasing the concentration of substrate or enzyme resulted in conditions unfavorable for the determination of the kinetic constants in the former instance or in non-reproducible results in the latter. It had been found earlier[20] that reactions conducted at enzyme concentrations of less than 10^{-7} M are complicated by adsorption of enzyme on the surfaces of the glass reaction vessels and electrodes. At these concentrations only a part of the enzyme is molecularly dispersed, the amount being markedly dependent upon the prior history of the glass surfaces. Thus, the lack of reproducibility noted above is understandable. The kinetic properties of the systems containing D-3-carbomethoxydihydroisocarbostyril were uncomfortably close to the limits of experimental capability and consequently a relatively large number of experiments was required to obtain significant values for the two kinetic constants of this substrate. Empirical standardization of the experimental procedure which could have led to more consistent, but not necessarily more accurate, results at low enzyme concentrations was considered and rejected because there is no assurance that molecularly dispersed and surface adsorbed enzyme have the same kinetic properties.

While it was not possible to evaluate k_0 and K_0 for formyl-L-phenylalanine methyl ester, it was possible to arrive at an estimate of k_0/K_0 for this substrate. Since the rate of an enzyme-catalyzed reaction described by eq. 1 is proportional to k_0 and, when $[S]_0 << K_0$, inversely proportional to K_0, the ratio k_0/K_0 is a useful index of the reactivity of a substrate. For enantiomorphic pairs of so-

(17) T. H. Applewhite, R. B. Martin and C. Niemann, *J. Am. Chem. Soc.*, **80**, 1457 (1958).

(18) T. H. Applewhite, H. Waite and C. Niemann, *ibid.*, **80**, 1465 (1958).

(19) H. I. Abrash, A. N. Kurtz and C. Niemann, *Biochem. et Biophys. Acta*, **45**, 378 (1960).

(20) R. L. Bixler and C. Niemann, *J. Am. Chem. Soc.*, **81**, 1412 (1959).

TABLE II

INHIBITION OF THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF SEVERAL ACYLATED α-AMINO ACID ESTERS[a]

System	Expts.[b]	$[S]_0$, mM	$[E]$,[c] M	[I], mM	K_0,[d] mM	k_0,[d] sec.$^{-1}$	K_I,[e] mM
Methyl benzoyl-L-alaninate and indole	20—0	2.50 -15.9	3.54×10^{-5}	0.000	9.75 ± 0.86	0.261 ± 0.011	...
	11—2	3.57 -21.5	2.83×10^{-5}	.197	14.0 ± .4	.279 ± .004	0.57 ± 0.52
	11—0	3.57 -17.9	2.83×10^{-5}	.394	18.0 ± .4	.284 ± .005	.58 + .33
Methyl benzoyl-D-alaninate and indole	26—7	0.98 -18.9	3.54×10^{-5}	.000	3.29 ± .18	.0107 ± .0002	...
	8—1	.98 - 6.83	3.56×10^{-5}	.276	5.33 ± .39	.0118 ± .0006	.57 ± .30
	8—1	.98 - 5.85	3.56×10^{-5}	.552	5.89 ± .89	.0108 ± .0012	.63 ± .31
D-3-Carbomethoxy-dihydroisocarbostyril and indole	31—3	.216- 2.14	1.05×10^{-7}	.000	0.527 ± .80	22.7 ± 1.2	...
	8—1	.238- 1.66	1.17×10^{-7}	.394	0.624 ± .11	21.5 ± 1.7	.77 ± .25
	8—1	.233- 1.11	0.99×10^{-7}	1.340	1.20 ± .50	23.4 ± 4.0	.76 ± .33
L-3-Carbomethoxy-dihydroisocarbostyril and indole	17—1	.406- 3.63	2.01×10^{-5}	0.000	11.69 ± 1.53	0.124 ± 0.014	...
	16—3	.406- 3.63	2.01×10^{-5}[g]	.246	12.0 ± 1.0	.089 ± .007	.61 ± .11[f]
	12—2	.415- 2.93	1.80×10^{-5}	.445	10.4 ± 1.9	.072 ± .011	.60 ± .16[f]
	14—0	.406- 3.63	2.01×10^{-5}[g]	.492	14.7 ± 4.9	.082 ± .025	.94 ± .39[f]
	9—1	.988- 3.46	0.93×10^{-5}	.694	8.3 ± 1.0	.045 ± .005	.39 ± .09[f]
	11—1	.988- 3.46	0.93×10^{-5}	1.386	13.4 ± 8.4	.041 ± .023	.67 ± .22[f]
L-3-Carbomethoxy-dihydroisocarbostyril and acetyl-D-tryptophanamide	17—1	.406- 3.63	2.01×10^{-5}	0.000	11.69 ± 1.53	.124 ± .014	...
	12—0	.418- 2.92	1.80×10^{-5}	2.12	11.10 ± 2.80	.060 ± .013	1.96 ± .56[f]

[a] In aqueous solutions at 25.0°, *p*H 7.90 ± 0.05 and 0.20 *M* in sodium chloride. [b] Number of experiments performed for evaluation of K_0 and k_0; second number refers to those rejected by statistical reiterative procedure used for evaluation of K_0 and k_0. [c] Based upon a molecular weight of 25,000 and a nitrogen content of 16.5%. [d] Evaluated by a least squares fit to the equation $([E][S]/v_0) = (K_0/k_0) + ([S]/k_0)$ as described in text. [e] Evaluated from the relation $K_I = [I]/(A'/(A - 1))$ where A' and A are the intercepts of the least squares computation for inhibited and non-inhibited experiments, respectively. Errors were calculated from the relation $\sigma K_I = K_I(\sigma A/A + \sigma B/B + \sigma A'/A')((A'/A)/(A'/(A - 1)))$ [f] Evaluated from the equation $k_0 = k_0'(1 + [I]/K_I)$, where k_0' is the value observed in absence of inhibitor. [g] Mean value of range from 1.94×10^{-5} to 2.09×10^{-5}.

called "specific substrates," where hydrolysis of the D-antipode is not ordinarily observed, the ratio $(k_0/K_0)_L/(k_0/K_0)_D$ is probably of the order of 10^6 to 10^7. For formyl-L-phenylalanine methyl ester $k_0/K_0 \cong 10^5\ M^{-1}$ sec.$^{-1}$ and the ratio $(k_0/K_0)_L/(k_0/K_0)_D$ for the enantiomorphic pair $\cong 10^4$. For benzoyl-D and L-alanine methyl ester, where the hydrolysis of both isomers could readily be followed in the *p*H-stat, the $(k_0/K_0)_L/(k_0/K_0)_D$ ratio drops to 8.2. The reduced degree of stereospecificity observed for the above two pairs of compounds, while retaining preference for the isomer of the L, or S, series, can readily be correlated with the structures of these compounds (see Discussion).

In the same way that the behavior of systems containing α-chymotrypsin and formyl-D- and L-phenylalanine methyl ester or benzoyl-D- and L-alanine methyl ester leads to the conclusion that the antipodal specificity of this enzyme is relative and, in these cases as well as in others,[21,22] favors the L-antipode, the data given in Table I for D- and L-3-carbomethoxydihydroisocarbostyril leads directly to the second conclusion that the predominant antipodal specificity of systems involving this enzyme is not an invariant property of the enzyme.[10] It is important to recognize that this latter conclusion is not a hypothesis, as alleged by Awad, Neurath and Hartley,[23] but is a straightforward statement of fact.

The results obtained with D- and L-3-carbomethoxydihydroisocarbostyril are sufficiently unusual so that it may be questioned whether these compounds are to be compared with other acylated α-amino acid derivatives which are substrates of α-chymotrypsin. Relative to other substrates of this enzyme D-3-carbomethoxydihydroisocarbostyril is comparable in reactivity to those substrates of the L-configuration which have been classified by Hein and Niemann[24] as approximating the $S^3E_{R_2R_4}$ limit type. Also, Fig. 2 illustrates that the dihydroisocarbostyril derivatives exhibit *p*H *vs.*

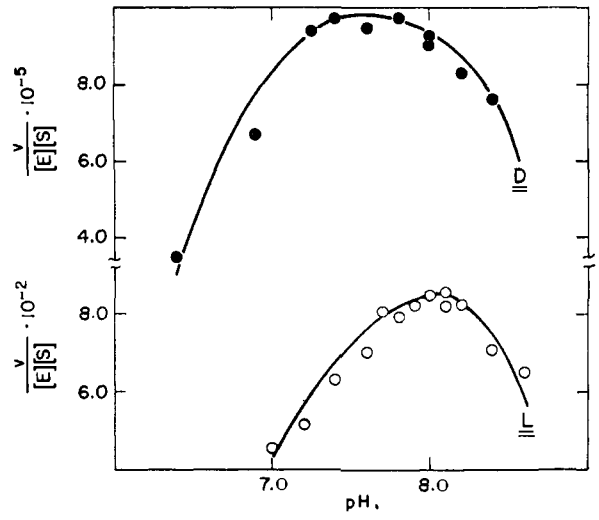

Fig. 2.—*p*H-activity relationships for α-chymotrypsin-catalyzed hydrolysis of D- and L-3-carbomethoxydihydroisocarbostyril.

activity curves similar to those observed for other neutral ester type substrates.[17,18] While the pre-

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

(22) R. J. Foster, H. J. Shine and C. Niemann, *ibid.*, **77**, 2378 (1955).

(23) E. S. Awad, H. Neurath and B. S. Hartley, *J. Biol. Chem.*, **235**, PC 35 (1960).

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

ceding characteristics suggest that the dihydroisocarbostyril derivatives and the more conventional substrates interact with α-chymotrypsin at a common site, the evidence is not rigorous. Therefore, a series of inhibition studies capable of more direct interpretation was performed. These studies are summarized in Table II.

The inhibition of the α-chymotrypsin-catalyzed hydrolysis of benzoyl-D- and L-alanine methyl ester by indole was examined because earlier studies[17,25] had shown that this inhibitor could be used to distinguish between so-called bi- and trifunctional substrates of α-chymotrypsin.[25] With the former, represented by benzoylglycine methyl ester, fully competitive inhibition is not observed, but instead simultaneous combination of enzyme, substrate and inhibitor to give a ternary complex capable of yielding reaction products. With the latter, represented by α-nicotinyl-L-tryptophanamide, fully competitive inhibition results, *i.e.*, only binary complexes of enzyme and substrate or enzyme and inhibitor are formed. The low order of relative stereospecificity, in favor of the L-antipode, observed for benzoyl-D- and L-alanine methyl ester raises the question whether these substrates function as bi- or trifunctional substrates in their interaction with α-chymotrypsin.

The data given in Table II clearly demonstrate that inhibition of the α-chymotrypsin-catalyzed hydrolysis of *both* benzoyl-D- and L-alanine methyl ester by indole is fully competitive. The values of K_I obtained with the D- and L-antipodes, *i.e.*, 0.60 ± 0.30 and 0.58 ± 0.43 mM, respectively, are in reasonable agreement with the earlier value of 0.80 ± 0.30 mM.[25,26] The inhibition of the α-chymotrypsin-catalyzed hydrolysis of D-3-carbomethoxydihydroisocarbostyril by indole is also fully competitive. The value of K_I so determined, 0.76 ± 0.35 mM, is consistent with the values noted above. The conclusion that D-3-carbomethoxydihydroisocarbostyril and the more conventional substrates of α-chymotrypsin are hydrolyzed at a common active site is inescapable.

In contrast to the behavior observed with the D-antipode, inhibition of the α-chymotrypsin-catalyzed hydrolysis of L-3-carbomethoxydihydroisocarbostyril by indole is not fully competitive. In fact, it was possible to calculate values of K_I in good agreement with those obtained previously for cases of fully competitive inhibition by assuming in this instance fully non-competitive inhibition. The data summarized in Table II demonstrates the essential independence of K_0 and the dependence of k_0 upon [I] required for fully non-competitive inhibition.

The types of inhibition exhibited by indole in the preceding studies, along with the previously observed mixed type for this inhibitor,[17,25] demonstrate that the type of inhibition observed in an enzyme–substrate–inhibitor system is a function of the substrate as well as of the enzyme and inhibitor. For indole and α-chymotrypsin there are now available examples of fully competitive, fully non-competitive and mixed, depending upon the structure of the substrate.

In earlier studies[22,27] α-N-acetyl-D-tryptophanamide was found to be a fully competitive inhibitor of the α-chymotrypsin-catalyzed hydrolysis of eight representative substrates of this enzyme including two α-N-acylated-L-tryptophanamides, four α-N-acylated-L-tyrosinamides, benzoyl-L-valine methyl ester and benzoylglycine methyl ester.[22,27] A mean value of K_I = 2.3 ± 0.4 mM was obtained for the amide type substrates[22] and values of 2.6 ± 0.4 and 2.1 ± 1.2 mM for the two ester type substrates. When the same inhibitor was evaluated against L-3-carbomethoxydihydroisocarbostyril the inhibition was fully non-competitive with a value of K_I = 1.96 ± 0.56 mM; *cf.* Table II. These results confirm and extend those obtained with indole and demonstrate that L-3-carbomethoxydihydroisocarbostyril and α-N-acetyl-D-tryptophanamide can form a ternary complex with α-chymotrypsin. However, this ternary complex, in contrast to that formed from α-chymotrypsin, benzoylglycine methyl ester and indole,[17,25] apparently is incapable of decomposing to give reaction products.

Discussion

Substrates represented by the formula R_1-CHR_2COR_3, where $R_2 \neq H$, may combine with α-chymotrypsin through interaction of $R_1 = R_1'$-CONH, R_2 and COR_3 with their complementary loci, ρ_1, ρ_2 and ρ_3, present at the asymmetric active site of the enzyme. All three interactions are presumed to be important for orientation of the substrate, but all three are not necessarily involved in determining the magnitude of the enzyme–substrate dissociation constant.[24] When any one of the three groups responsible for orientation of the substrate in the complex fails to function efficiently, because it is too small or is lacking in critical structural features,[24] then the compound may assume alternate orientations which for the L-antipode are either non-productive or less reactive than the single optimum orientation, but for the D-antipode may be productive. Thus, for formyl-D- and l-phenylalanine methyl ester, with $R_1' = H$, alternative orientations become more favored than when $R_1' = CH_3$ or a group of greater steric requirement, with the result that with substrates of this type, where the enzyme–substrate dissociation constant is largely determined by R_2–ρ_2 and COR_3–ρ_3 interactions,[24] the rate of formation of products from the L-antipode is depressed and from the D-antipode enhanced relative to the situation where R_1 is more effective in its orienting role, as for example in acetyl-D- and L-phenylalanine methyl ester.

Although benzoyl-L-alanine methyl ester probably approximates the $S^{3E}_{R_1R_2}$ limit type, where the enzyme–substrate dissociation constant is largely determined by R_1–ρ_1 or R_1–ρ_2 and COR_3–ρ_3 interactions,[24] the expected behavior of the enantiomorphic pair is the same as that noted above and in this instance arises from the inability of $R_2 = CH_3$ to function effectively in its orienting role, presumably because of its small size. That the

(25) H. T. Huang and C. Niemann, *J. Am. Chem. Soc.*, **75**, 1395 (1953).

(26) R. J. Foster and C. Niemann, *ibid.*, **77**, 3370 (1955).

(27) T. H. Applewhite and C. Niemann, *ibid.*, **81**, 2208 (1959).

ratio of $(k_0/K_0)_L/(k_0/K_0)_D$ for benzoyl-D- and L-alanine methyl ester is near one is not due to a surprisingly rapid rate of hydrolysis of the D-antipode, but rather because the L-antipode is not a particularly good substrate. The data given in Table I for benzoyl-D- and L-alanine methyl ester provide impressive support for the proposition that *a large decrease in relative stereospecificity arising from loss of orienting function is clearly associated with a low order of reactivity for both* D- *and* L-*antipodes.*

The results of the inhibition study further reveal that although the side chain methyl group in benzoyl-D- and L-alanine methyl ester does not effectively participate in either the orienting or binding processes it is so disposed in the enzyme–substrate complexes formed from either antipode and is of sufficient size to prevent simultaneous combination of both substrate and inhibitor at the active site. It is significant that this situation should obtain for a pair of antipodal substrates whose hydrolyses proceed with a relatively modest stereospecificity in favor of the L-antipode and where one might have expected a behavior approximating that of the analogous bifunctional substrate benzoylglycine methyl ester.

The decreased stereospecificity observed for benzoyl-D- and L-alanine methyl ester emphasizes that assumptions concerning the degree of relative stereospecificity are dangerous, especially when relatively poor substrates of an enzyme are investigated. The use of D,L-mixtures, assuming that only one isomer will be hydrolyzed, as did Hearon, Bernhard, Friess, Botts and Morales[28] for benzoyl-D- and L-alanine methyl ester, can only lead to ambiguous results and questionable conclusions.

A loss in relative stereospecificity may be accounted for in terms of the failure of a structural component to function effectively in orientation of the substrate at the active site of the enzyme. An inversion of antipodal specificity, as observed for the dihydroisocarbostyril derivatives, requires more detailed consideration.

Before proceeding with our argument we wish to comment upon two explanations of the observations reported by Hein, McGriff and Niemann[10] remembering that these interpretations[23,29] were advanced without benefit of the quantitative observations reported in this communication and prior to our comprehensive analysis of the kinetic behavior of model substrates of α-chymotrypsin.[24]

Wilson and Erlanger[29] claim that the inversion of antipodal specificity observed with D- and L-3-carbomethoxydihydroisocarbostyril "can be explained simply and plausibly within the framework of the polyaffinity concept without the necessity of invoking new principles." Their explanation ignores the fact that L-3-carbomethoxydihydroisocarbostyril is a substrate of α-chymotrypsin and errs in their assumption that the reactivity of a given substrate is necessarily explained in terms of simple additive contributions of its various structural components to an over-all binding energy and that *in all cases* there is a direct relation between values of K_0 and the reactivity of the substrate. Superimposed upon these dubious assumptions are those of an active site of invariant conformation and a unique mode of combination of substrate. Unfortunately, the validity and generality of these assumptions, which are inherent in their application of the so-called polyaffinity concept, are not supported by available evidence.[24]

The argument of Wilson and Erlanger[29] necessarily assigns to D-3-carbomethoxydihydroisocarbostyril a reactivity comparable to that of benzoyl-L-alanine methyl ester and predicts that L-3-carbomethoxydihydroisocarbostyril would not function as a substrate. In fact, it is the L-isomer ($k_0/K_0 = 10.6\ M^{-1}$ sec.$^{-1}$) which has a reactivity comparable to benzoyl-L-alanine methyl ester ($k_0/K_0 = 26.8\ M^{-1}$ sec.$^{-1}$) while the D-isomer ($k_0/K_0 = 4.31 \times 10^4\ M^{-1}$ sec.$^{-1}$) is approximately 10^3 times more reactive. In summary, the explanation of Wilson and Erlanger[29] fails to meet a reasonable quantitative test and in fact does not explain the observed inversion of antipodal specificity. As indicated above, the reasons for this failure are to be found in their basic premises as well as in their assertion that the benzoyl group in benzoyl-L-phenylalanine methyl ester makes a significant positive contribution to the binding of this substrate to the active site of the enzyme and inferentially to its reactivity. We are unaware of any experiments which provide support for this latter assertion and in fact have noted evidence which would deny that it has an important function, in the compound considered, in determining the enzyme–substrate dissociation constant.[24]

A more ambitious explanation was advanced by Awad, Neurath and Hartley.[23] However, the argument of these authors contains several questionable assertions. Their statement that "specific substrates of α-chymotrypsin are derivatives of amino acids of the L-configuration" is unfortunate in that it implies that only amino acid derivatives of the L-configuration are specific substrates.

Awad, Neurath and Hartley[23] reject one of the three explanations offered by Hein, McGriff and Niemann,[10] "that the antipodal specificity may arise during the act of combination of enzyme and substrate by a change in the conformation of the active site of the enzyme that is mediated by the structure of the substrate," on the grounds that this is "unlikely because it implies a disruption of the tertiary structure of the enzyme and a reassembly of the peptide chains in an antipodal manner." The suggestion[10] that the conformation of the active site of the enzyme is mediated by the structure of the substrate requires no drastic rearrangement of entire peptide chains but only the assumption of a certain degree of flexibility of the interacting groups which make up the active site. Similar conformational variations are envisioned by Koshland in his concept of an induced fit.[30,31]

Awad, Neurath and Hartley[23] reject a second alternative explanation of Hein, McGriff and Niemann,[10] namely, "that enzyme–substrate com-

(28) J. F. Hearon, S. A. Bernhard, S. C. Friess, D. J. Botts and M. F. Morales in "The Enzymes," Vol. 1, P. D. Boyer, H. Lardy and K. Myrbäck, Ed., Academic Press, Inc., New York, N. Y., 1959, p. 49.

(29) I. B. Wilson and B. F. Erlanger, *J. Am. Chem. Soc.*, **82**, 6122 (1960).

(30) D. E. Koshland, Jr., *Proc. Natl. Acad. Sci.*, **44**, 98 (1958).

(31) D. E. Koshland, Jr., *Adv. Enzymology*, **22**, 45 (1960).

plexes, although formed at only one active site of essentially invariant conformation, can decompose to give products through the intermediacy of more than one type of complex" on the grounds that this is unlikely "since all previous evidence implies a single catalytic site and an unique mechanism for chymotrypsin." While the question of uniqueness of mechanism of α-chymotrypsin can hardly be regarded as resolved,[32,33] the question whether the D- and L-antipodes form more than one type of complex is answered directly and affirmatively by our observation that one antipode, *i.e.*, the L, is fully non-competitively inhibited by indole whereas the other exhibits fully competitive inhibition.

Perhaps recognizing the frailty of their argument against "different pathways," Awad, Neurath and Hartley[23] go on to state that, "If. . .different pathways of hydrolysis were possible, one would expect that [L-3-carbomethoxydihydroisocarbostyril] would be hydrolyzed by the pathway for [acetyl-L-phenylalanine methyl ester], and that [acetyl-D-phenylalanine methyl ester] would be hydrolyzed by means of the [D-3-carbomethoxydihydroisocarbostyril] pathway." It is difficult to reconcile this bold assertion with their later attempt to explain the observed inversion of antipodal specificity without invoking any new principles.

Finally, the interpretation offered by Awad, Neurath and Hartley,[23] which attempts to explain the observations of Hein, McGriff and Niemann[10] by reference to only four compounds, cannot be considered satisfactory for the following reasons: 1. No provision is made for degrees of reactivity. At best, Awad, Neurath and Hartley's model[23] can predict reactivity or lack of it; it cannot predict relative rates or binding constants. For example, their model, like Wilson and Erlanger's,[29] predicts that L-3-carbomethoxydihydroisocarbostyril will be unreactive, similar to acetyl-D-phenylalanine methyl ester, whereas it is at least 10^2 times more reactive.

2. From a very tenuous argument, which considers optical isomers as "hardly distinguishable from each other" in a particular conformation, they conclude that only a conformation with the carbomethoxy group out of the plane of the dihydroisocarbostyril ring system will result in significant substrate activity. Our observation that 3-carbomethoxyisocarbostyril, *cf.* Table I, a rigidly planar compound, is a substrate of α-chymotrypsin invalidates this conclusion.

3. The proposed model[23] assigns only a minor function to the α-acylamino side chain which is not in accord with considerable data available.[24] In fact, their model, which ascribes the lack of reactivity of acetyl-D-phenylalanine methyl ester solely to "steric interference between the phenyl ring and the α-acylamino group" predicts that formyl-D-phenylalanine methyl ester, for which models show no such interference in the conformation preferred by Awad, Neurath and Hartley,[23] should react at a rate comparable to that of formyl-L-phenylalanine methyl ester. However, the difference in reactivity between these two compounds is of the same order of magnitude as the difference in reactivity between D- and L-3-carbomethoxydihydroisocarbostyril.

In our examination of the proposals of Wilson and Erlanger[29] and of Awad, Neurath and Hartley[23] and in the development of our own interpretation of our experimental observations[24] it became evident that a reasonable explanation of the observed inversion of antipodal specificity was dependent upon the development of a general theory of the steric course and specificity of α-chymotrypsin-catalyzed reactions. Therefore, in this communication we have presented our experimental observations and have restricted discussion to the question of relative stereochemistry and to the shortcomings of explanations of the inversion of antipodal specificity based upon limited and essentially qualitative information. The general theory and its attendant explanation of the observed inversion of antipodal specificity is developed in the accompanying communication designated as the second of a series bearing the same title.

Experimental[34]

α-Bromo-*o*-tolunitrile.—To 100 g. of *o*-tolunitrile, heated in an oil-bath at 140°, was added 140 g. of bromine over a period of 4 hours. The bromine was introduced below the surface of the stirred liquid which was illuminated with a No. 2 photoflood lamp. The dark colored reaction product was distilled *in vacuo* to give the bromide, b.p. 140–150° (5 mm.), which solidified on cooling. One recrystallization from 95% ethanol gave 65% of α-bromo-*o*-tolunitrile, m.p. 70–71°, reported[35] m.p. 71–72°.

Ethyl α-Carbethoxy-α-acetamido-β-(*o*-cyanophenyl)-propionate.—Approximately 500 ml. of anhydrous ethanol was distilled from sodium ethoxide into a 1-liter flame-dried flask fitted with a reflux condenser, stirrer and drying tube. Sixteen grams of sodium was dissolved in the ethanol; 152 g. of diethyl acetamidomalonate was added to the stirred solution which was then heated to 50° for 1 hour prior to the dropwise addition of 127 g. of α-bromo-*o*-tolunitrile, dissolved in the minimum amount of absolute ethanol. The reaction mixture was stirred for 5 hours at 50°, chilled in an ice-bath, neutralized with dry gaseous hydrogen chloride and evaporated *in vacuo* to remove the solvent. The yellow semi-solid residue was partitioned between chloroform and water, the chloroform phase successively washed with water, aqueous sodium bicarbonate and water, dried over anhydrous magnesium sulfate and the solvent removed to give a residue, m.p. 103–105°. Recrystallization of the residue from a mixture of chloroform and hexane gave 93–95% of product, m.p. 104–105°. Further recrystallization from water gave ethyl α-carbethoxy-α-acetamido-β-(*o*-cyanophenyl)-propionate, m.p. 105°.

Anal. Calcd. for $C_{17}H_{20}O_5N_2$(332): C, 61.4; H, 6.1; N, 8.4. Found: C, 61.6; H, 6.2; N, 8.4.

DL-3-Carboxy-1,2,3,4-tetrahydroisoquinoline.—DL-Phenylalanine, 37.5 g., was condensed with formaldehyde in concd. hydrochloric acid as described by Julian, Karper, Magnani and Meyer[36] to give 58–80% of crude product. Recrystallization of the crude product from aqueous ethanol gave DL-3-carboxy-1,2,3,4-tetrahydroisoquinoline, decomp. point in an evacuated sealed tube, 331°; Julian, *et al.*,[36] report a decomp. point of 335°.

Anal. Calcd. for $C_{10}H_{11}O_2N$ (177): C, 67.7; H, 6.3; N, 7.9. Found: C, 67.9; H, 6.2; N, 8.0.

(32) M. L. Bender and W. A. Glasson, *J. Am. Chem. Soc.*, **82**, 3336 (1960).

(33) S. A. Bernhard, W. C. Coles and J. F. Nowell, *ibid.*, **82**, 3013 (1960).

(34) All analyses by Spang Microanalytical Laboratory, Ann Arbor, Mich. Melting points are corrected. Rotations were determined at 25 ± 3° and have an uncertainty of approximately ±3%.

(35) R. C. Fuson, *J. Am. Chem. Soc.*, **48**, 830 (1926).

(36) P. L. Julian, W. J. Karper, A. Magnani and E. Meyer, *ibid.*, **70**, 180 (1948).

D-3-Carboxy-1,2,3,4-tetrahydroisoquinoline.—Condensation of D-phenylalanine with formaldehyde, as described for the DL-mixture, gave a partially racemized product which was digested with boiling 25% aqueous ethanol until the residue after recrystallization from aqueous ethanol exhibited a constant rotation and gave D-3-carboxy-1,2,3,4-tetrahydroisoquinoline, decomp. point in an evacuated sealed tube 328.5°, $[\alpha]_D$ 173.6° (*c* 2.2% in 1.4 *N* aqueous sodium hydroxide).

Anal. Calcd. for $C_{10}H_{11}O_2N$ (177): C, 67.7; H, 6.3; N, 7.9. Found: C, 67.8; H, 6.2; N, 7.7.

L-3-Carboxy-1,2,3,4-tetrahydroisoquinoline.—This compound, decomp. point in an evacuated sealed tube 327.5°, $[\alpha]_D$ − 176.1° (*c* 1.8% in 1.4 *N* aqueous sodium hydroxide), was prepared from L-phenylalanine as described for the D-antipode.

Anal. Calcd. for $C_{10}H_{11}O_2N$ (177): C, 67.7; H, 6.3; N, 7.9. Found: C, 67.7; H, 6.3; N, 8.0.

N-Benzoyl-DL-3-carboxy-1,2,3,4-tetrahydroisoquinoline.—The acylation procedure was patterned after one described by Steiger.[37] A solution of the sodium salt of DL-3-carboxy-1,2,3,4-tetrahydroisoquinoline, prepared from 64.7 ml. of 2.04 *N* aqueous sodium hydroxide and 23.4 g. of the acid in 150 ml. of water, was cooled in a methanol–ice-salt-bath to give a suspension of the salt. To the stirred suspension, maintained below 2°, was added dropwise in the course of 90 min., 18.6 g. of benzoyl chloride and 64.7 ml. of 2.04 *N* aqueous sodium hydroxide. The cold reaction mixture was stirred for an additional 15 min., filtered through a layer of Hiflo filter aid and the filtrate rapidly acidified with 6 *N* hydrochloric acid. The resulting colorless semi-solid was collected by decantation and recrystallized three times from aqueous acetone to give 25.9 g. (63%) of N-benzoyl-DL-3-carboxy-1,2,3,4-tetrahydroisoquinoline, m.p. 160–163° dec.

Anal. Calcd. for $C_{17}H_{15}O_3N$ (281): C, 72.6; H, 5.4; N, 5.0. Found: C, 72.3; H, 5.4; N, 4.91; neut. equiv., 282.3.

N-Benzoyl-D-3-carboxy-1,2,3,4-tetrahydroisoquinoline.—Benzoylation of D-3-carboxy-1,2,3,4-tetrahydroisoquinoline, as described for the DL-mixture, gave 62% of product, m.p. 152–157° dec., after three recrystallizations from aqueous ethanol. This product was recrystallized an additional three times from a mixture of hexane and ethyl acetate to give N-benzoyl-D-3-carboxy-1,2,3,4-tetrahydroisoquinoline, m.p. 146–150° dec., $[\alpha]_D$ 33.5° (*c* 1.8% in methanol).

Anal. Calcd. for $C_{17}H_{15}O_3N$ (281): C, 72.6; H, 5.4; N, 5.0. Found: C, 72.4; H, 5.3; N, 5.0; neut. equiv., 281.5.

N-Benzoyl-L-3-carboxy-1,2,3,4-tetrahydroisoquinoline, m.p. 152–153° dec., $[\alpha]_D$ − 33.7° (*c* 2.1% in methanol), was prepared as described for the D-antipode.

Anal. Calcd. for $C_{17}H_{15}O_3N$ (281): C, 72.6; H, 5.4; N, 5.0. Found: C, 72.6; H, 5.5; N, 5.2.

N-Benzoyl-*o*-carboxy-DL-phenylalanine.—To a vigorously stirred solution of 17.7 g. of potassium carbonate in 2 liters of water was added 36.0 g. of N-benzoyl-DL-3-carboxy-1,2,3,4-tetrahydroisoquinoline prior to the addition, over a 3-hour period, of 40.4 g. of potassium permanganate. The suspension was stirred for an additional hour, the residual permanganate reduced with sodium bisulfite and the reaction mixture filtered through a layer of Hiflo filter aid. The filtrate was acidified, concentrated *in vacuo*, the precipitate collected and recrystallized seven times from aqueous acetone to give 15.7 g. (39%) of N-benzoyl-*o*-carboxy DL-phenylalanine, m.p. 202–203.5°.

Anal. Calcd. for $C_{17}H_{15}O_5N$ (313): C, 65.2; H, 4.8; N, 4.5. Found: C, 65.0; H, 4.9; N, 4.5; neut. equiv., 156.4.

N-Benzoyl-*o*-carboxy-D-phenylalanine was prepared from N-benzoyl-D-3-carboxy-1,2,3,4-tetrahydroisoquinoline as described for the DL-mixture. The product, obtained in 43% yield after two recrystallizations from aqueous acetone, was recrystallized two additional times from the same solvent to give N-benzoyl-*o*-carboxy-D-phenylalanine, m.p. 193–193.5°, $[\alpha]_D$ 119.2° (*c* 2.2% in methanol).

Anal. Calcd. for $C_{17}H_{15}O_5N$ (313): C, 65.2; H, 4.8; N, 4.5. Found: C, 65.0; H, 4.8; N, 4.5; neut. equiv., 157.8.

(37) R. E. Steiger, *J. Org. Chem.*, **9**, 396 (1944).

N-Benzoyl-*o*-carboxy-L-phenylalanine.—Oxidation of N-benzoyl-L-3-carboxy-1,2,3,4-tetrahydroisoquinoline as described for the DL-mixture and D-antipode gave N-benzoyl-*o*-carboxy-L-phenylalanine, m.p. 193–193.5°, $[\alpha]_D$ − 119.9° (*c* 1.5% in methanol).

Anal. Calcd. for $C_{17}H_{15}O_5N$ (313): C, 65.2; H, 4.8; N, 4.5. Found: C, 65.3; H, 4.8; N, 4.5; neut. equiv., 157.9.

DL-3-Carboxydihydroisocarbostyril. A.—A suspension of 15.7 g. of N-benzoyl-*o*-carboxy-DL-phenylalanine in 225 ml. of 6 *N* hydrochloric acid was heated under refluxing conditions for 18 hours. Sufficient hot 6 *N* hydrochloric acid (*ca.* 150 ml.) was added to the hot reaction mixture to effect solution of all components, the solution cooled to 4°, the precipitate collected and recrystallized twice from water to give 7.04 g. (73.5%) of product, m.p. 231–243°. This product was recrystallized three times from water to give DL-3-carboxydihydroisocarbostyril, m.p. 238.5–240.5°, dec.

Anal. Calcd. for $C_{10}H_9O_3N$ (191): C, 62.8; H, 4.7; N, 7.3. Found: C, 62.9; H, 4.9; N, 7.5; neut. equiv., 191.6.

B.—Ethyl α-carbethoxy-α-acetamido-β-(*o*-cyanophenyl)-propionate, 100 g., in 150 ml. of 6 *N* aqueous sodium hydroxide was heated under refluxing conditions in a copper flask until the evolution of ammonia had ceased. The solution was acidified to *p*H 2 and again heated until the evolution of carbon dioxide had ceased. The slurry was transferred to a glass flask, evaporated to dryness *in vacuo* and the residue heated under refluxing conditions with 200 ml. of 48% hydrobromic acid. The resulting solution was poured onto ice, the precipitated acid collected and recrystallized three times from water to give 20 g. (35%) of DL-3-carboxydihydroisocarbostyril, m.p. 235–237°, dec.

Anal. Calcd. for $C_{10}H_9O_3N$ (191): C, 62.8; H, 4.7; N, 7.3. Found: C, 63.0; H, 4.8; N, 7.1; neut. equiv., 192.

The infrared spectra of the acids obtained by methods A and B were identical.

D-3-Carboxydihydroisocarbostyril. A.—This acid, m.p. 232.5–234° dec., $[\alpha]_D$ − 45.2° (*c* 1.5% in methanol), was obtained from N-benzoyl-*o*-carboxy-D-phenylalanine as described for the analogous DL-mixture.

Anal. Calcd. for $C_{10}H_9O_3N$ (191): C, 62.8; H, 4.7; N, 7.3. Found: C, 62.9; H, 4.9; N, 7.2; neut. equiv., 189.5.

B.—To a solution of 2.0 g. of DL-3-carbomethoxydihydroisocarbostyril, *vide post*, in 200 ml. of 30% aqueous methanol was added 66.7 mg. of α-chymotrypsin and the *p*H of the solution, as determined by a Beckman model G *p*H meter, maintained between *p*H 7.0 and 7.5 by the manual addition of 0.535 *N* aqueous sodium hydroxide to the reaction mixture. After approximately 1 hour, the reaction abated, 10.1 ml. of base having been added. The cloudy reaction mixture was filtered, the filtrate shaken with 100 ml. of chloroform, both phases filtered through a layer of Celite, the two phases separated, the aqueous phase extracted twice with 50 ml. of chloroform, the chloroform phases combined and dried over anhydrous magnesium sulfate. The aqueous phase was acidified with 6*N* hydrochloric acid, the precipitated acid collected and recrystallized twice from water to give 360 mg. (39%) of D-3-carboxydihydroisocarbostyril, m.p. 233–234° dec., $[\alpha]_D$ − 45.2° (*c* 2% in methanol).

L-3-Carboxydihydroisocarbostyril, m.p. 236–238° dec., $[\alpha]_D$ 46.4° (*c* 2.5% in methanol), was prepared from N-benzoyl-*o*-carboxy-L-phenylalanine as described for the analogous DL-mixture.

Anal. Calcd. for $C_{10}H_9O_3N$ (191): C, 62.8; H, 4.7; N, 7.3. Found: C, 62.6; H, 4.8; N, 7.2; neut. equiv., 193.8.

DL-3-Carbomethoxydihydroisocarbostyril.—The DL-acid was esterified, with methanol and thionyl chloride, by a procedure similar to that described by Brenner and Huber.[38] To 50 ml. of methanol, in a flask equipped with an addition tube, stirrer and drying tube and contained in an ice-salt-bath, was added dropwise and with stirring 4.3 ml. of thionyl chloride. To this solution was added 10 g. of DL-3-carboxydihydroisocarbostyril, the stirred slurry allowed to slowly come to room temperature and the

(38) M. Brenner and W. Huber, *Helv. Chim. Acta*, **36**, 1109 (1953).

clear solution heated to 40° for 1 hour. Removal of the solvent by evaporation *in vacuo* gave an oil which was taken up in chloroform, the chloroform solution washed successively with water, aqueous sodium bicarbonate and water, dried over anhydrous sodium sulfate, the solvent removed by evaporation *in vacuo* and the solid residue recrystallized from a mixture of hexane and chloroform to give 80–90% of ester. Two to three additional recrystallizations from the same solvent gave DL-3-carbomethoxydihydroisocarbostyril, m.p. 115–116.5°.

Anal. Calcd. for $C_{11}H_{11}O_3N$ (205): C, 64.4; H, 5.4; N, 6.8. Found: C, 64.6; H, 5.8; N, 6.9.

D-3-Carbomethoxydihydroisocarbostyril.—Esterification of the D-acid as described for the DL-mixture gave D-3-carbomethoxydihydroisocarbostyril, m.p. 88–89.5°, $[\alpha]_D$ − 83.7° (*c* 2% in methanol).

Anal. Calcd. for $C_{11}H_{11}O_3N$ (205): C, 64.4; H, 5.4; N, 6.8. Found: C, 64.6; H, 5.5; N, 7.0.

L-3-Carbomethoxydihydroisocarbostyril. A.—Esterification of the L-acid as described for the DL-mixture gave L-3-carbomethoxydihydroisocarbostyril, m.p. 88–89.5°, $[\alpha]_D$ 83.5° (*c* 2% in methanol).

Anal. Calcd. for $C_{11}H_{11}O_3N$ (205): C, 64.4; H, 5.4; N, 6.8. Found: C, 64.7; H, 5.4; N, 6.8.

B.—To the dried chloroform solution obtained from the α-chymotrypsin-catalyzed hydrolysis of DL-3-carbomethoxydihydroisocarbostyril, *vide ante*, was added sufficient hexane to precipitate the crude ester. The product was recrystallized several times from a mixture of hexane and chloroform to give 630 mg. (63%) of L-3-carbomethoxydihydrocarbostyril, m.p. 88–89°, $[\alpha]_D$ 81.9° (*c* 2% in methanol).

3-Carbomethoxyisocarbostyril was prepared as directed by Bain, Perkin and Robinson[39] from *o*-carbomethoxybenzaldehyde *via* an azlactone synthesis followed by an acid-catalyzed solvolysis. The reaction product was recrystallized from aqueous ethanol to give 3-carbomethoxyisocarbostyril, m.p. 160–161.5°; Perkin and Robinson[39] report m.p. 161–162°.

Anal. Calcd. for $C_{11}H_9O_3N$ (203): C, 65.0; H, 4.5; N, 6.9. Found: C, 65.1; H, 4.4; N, 7.0.

Methyl Benzoyl-D-alaninate.—Esterification of 20 g. of D-alanine with methanol and thionyl chloride[38] followed by benzoylation of the crude ester hydrochloride with one equivalent of benzoyl chloride and two equivalents of cold, aqueous sodium hydroxide gave a crude product which was recrystallized twice from a mixture of chloroform and hexane to give 13.6 g. (29%) of methyl benzoyl-D-alaninate, m.p. 56–58°, $[\alpha]_D$ − 30.8° (*c* 5% in *sym*-tetrachloroethane).

Anal. Calcd. for $C_{11}H_{13}O_3N$ (207): C, 63.7; H, 6.4; N, 6.8. Found: C, 63.4; H, 6.4; N, 6.9.

Methyl benzoyl-L-alaninate, m.p. 56.5–57.5°, $[\alpha]_D$ 30.6° (*c* 4.3% in *sym*-tetrachloroethane), was prepared as described for the D-antipode.

Anal. Calcd. for $C_{11}H_{13}O_3N$ (207): C, 63.7; H, 6.4; N, 6.8. Found: C, 63.8; H, 6.5; N, 6.8.

Methyl formyl-DL-phenylalaninate was prepared by the procedure of du Vigneaud and Meyer[40] from DL-phenylalanine methyl ester hydrochloride, formic acid and acetic anhydride. Fractional distillation of the crude product gave a center fraction, b.p. 163° (2 mm.).

Anal. Calcd. for $C_{11}H_{13}O_3N$ (207): C, 63.7; H, 6.3; N, 6.8. Found: C, 63.7; H, 6.3; N, 6.8.

Methyl Formyl-D-phenylalaninate.—To a solution of 4.10 g. (0.02 mole) of methyl formyl-DL-phenylalaninate in 150 ml. of water was added 30 mg. of Armour, bovine, salt-free α-chymotrypsin, the *p*H adjusted to *ca.* 7 and maintained at this value by the manual addition of 0.535 *N* aqueous sodium hydroxide. After approximately 30 minutes the reaction abated, 0.0103 mole of base having been added. The solution was extracted with chloroform, the chloroform phase dried briefly over anhydrous sodium sulfate, filtered through a layer of anhydrous magnesium sulfate and the solvent removed to give methyl formyl-D-phenylalaninate as a colorless oil, $[\alpha]_D$ − 27.4° (*c* 2.3% in methanol). Hydrolysis of 0.50 g. of the preceding ester with 15 ml. of 5% aqueous sodium hydroxide followed by acidification gave 0.31 g. (83%) of formyl-D-phenylalanine, m.p. 168–170°, $[\alpha]_D$ − 70° (*c* 2.0% in methanol). When a system containing the preceding D-ester and α-chymotrypsin was examined in a *p*H-stat, it was observed that a rapidly hydrolyzed impurity was present, equivalent to 1.3% of the L-antipode. Kinetic data were corrected for the presence of this impurity.

The aqueous layer from the enzymatic resolution was acidified, concentrated and filtered to give 1.9 g. (92%) of formyl-L-phenylalanine. Three recrystallizations of this product from water gave the L-acid, m.p. 168.5–170.5°, $[\alpha]_D$ 72° (*c* 3.1% in methanol).

Kinetic Studies.—All kinetic studies were performed as described previously.[17,18] The standard reaction system consisted of 10 ml. of solution: 1.0 ml. of enzyme stock solution added to 1.0 ml. of 2.0 *M* aqueous sodium chloride and 1.0–8.0 ml. of substrate stock solution with inhibitor stock solution and water added where appropriate. Solutions were prepared from pre-boiled, CO_2-free distilled water and a steam of nitrogen was passed over the reaction vessel during titration. The α-chymotrypsin used, bovine, salt-free, Armour lot No. T-97207, was analyzed by a micro Kjeldahl procedure and found to contain 14.6% nitrogen on an as-is basis. The *p*H-activity relationships given in Fig. 2 were evaluated at 25.0° in aqueous systems 0.20 *M* in sodium chloride and in which $[E] = 1.90 \times 10^{-7}$ *M* and $[S] = 9.586 \times 10^{-4}$ *M* for the D-antipode and $[E] = 3.54 \times 10^{-5}$ *M* and $[S] = 1.152 \times 10^{-3}$ *M* for the L-antipode.

(39) D. Bain, W. H. Perkin, Jr., and R. Robinson, *J. Chem. Soc.*, **105**, 2392 (1914).

(40) V. du Vigneaud and C. E. Meyer, *J. Biol. Chem.*, **98**, 295 (1932).