ethanol-soluble product which weighed 37 mg. after one recrystallization from aqueous ethanol and which melted at 150–152° after three additional recrystallizations from the same solvent. The m.p. of phenylcarbamyl-D-phenylalanine is 171°.[27] However, it was noted that when an earlier preparation of this acid which melted at 167–168° was recrystallized from hot water a product was obtained which melted at 150–152° and whose m.p. was unchanged upon further recrystallization from the same solvent.

Anal. Calcd. for $C_{11}H_{11}O_2N$ (189): C, 69.9; H, 5.9; N, 7.4. Found: C, 69.6; H, 5.9; N, 7.5.

Since the above analysis clearly indicated that the product, m.p. 150–152°, could arise from one of the starting materials it was concluded that there was no evidence for the formation of phenylcarbamyl-D-phenylalanylphenylhydrazide.

Miscellaneous Enzyme Experiments.—In the attempted synthesis of *sym*-bis-(benzoyl-L-phenylalanyl)-hydrazine, benzoyl-L-phenylalanine (0.5 millimole, 12.5 millimoles per liter), was incubated at 35°, with hydrazine (100 millimoles per liter) in the presence of α-chymotrypsin (1 mg. per ml. of reaction mixture) and a 0.5 *M* citrate buffer adjusted to *p*H 6.0. No precipitation was observed even after 15 days.

In the attempted synthesis of benzoyl-L-alanylphenylhydrazide, benzoyl-DL-alanine (1.0 millimole, 33.3 millimoles per liter) was incubated at 35° with phenylhydrazine (66.6 millimoles per liter) in the presence of the usual quantity of α-chymotrypsin and a series of 0.5 *M* citrate buffers adjusted to *p*H 3.5, 4.1, 4.5, 5.0, 5.5, 6.0 and 6.5, respectively. After 10 days only a small amount of a black tar was formed in all cases.

From the incubation of benzoyl-L-phenylalanine (1.0 millimole, 8.7 millimoles per liter) with *p*-toluidine (17.4 millimoles per liter) at 35° in the presence of the usual amount of α-chymotrypsin and a 0.5 *M* citrate buffer adjusted to *p*H 6.0, there was obtained, after 114 hours, 16.5% of benzoyl-L-phenylalanin-*p*-toluide, m.p. 219.5–220° after two recrystallizations from aqueous ethanol, $[\alpha]^{25}D$ +30.3° (*c* 1.06 in pyridine).

Anal. Calcd. for $C_{23}H_{22}N_2O_2$ (233): C, 77.1; H, 6.2; N, 7.8. Found: C, 77.2; H, 6.2; N, 7.7.

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[CONTRIBUTION NO. 1688 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The Kinetics of the α-Chymotrypsin Catalyzed Hydrolysis of Methyl Hippurate in Aqueous Solutions at 25° and *p*H 7.9[1]

BY H. T. HUANG AND CARL NIEMANN[2]

RECEIVED APRIL 28, 1952

It has been found that the α-chymotrypsin catalyzed hydrolysis of methyl hippurate in aqueous media proceeds at a sufficiently rapid rate at 25° and *p*H 7.9 to allow a study to be made of the kinetics of this reaction. From inhibition experiments with the above specific substrate and several trifunctional competitive inhibitors whose enzyme–inhibitor dissociation constants had been evaluated previously it appears that the catalytically active site involved in the hydrolysis of methyl hippurate is the same as that responsible for the hydrolysis of a number of acylated amides derived from L-tryptophan, L-tyrosine and L-phenylalanine. It has also been found that trypsin will catalyze the hydrolysis of methyl hippurate but at a much lower rate than is observed with α-chymotrypsin.

It has been suggested in a previous communication[3] that specific substrates and competitive inhibitors of α-chymotrypsin, derived from α-amino acids and described by the general formula R_1CHR_2-R_3,[4] may combine with the enzyme through interaction of the groups R_1, R_2 and R_3 with their respective complementary centers ρ_1, ρ_2 and ρ_3 which are assumed to be a characteristic feature of the catalytically active site of the enzyme. The well defined antipodal specificity of α-chymotrypsin which distinguishes the behavior of an L-specific substrate from that of the corresponding enantiomorphic D-competitive inhibitor, may then be interpreted in terms of steric limitations which the configuration about the asymmetric α-carbon atom must necessarily impose upon the three major interactions, *i.e.*, R_1–ρ_1, R_2–ρ_2 and R_3–ρ_3, assumed to be responsible for the formation of the intermediate complexes ES or EI.[5] Thus, in the case where the configuration about the asymmetric α-carbon atom is L it is possible that the formation of ES results in a strain which is centered in the R_3 group and which facilitates the transformation of ES into enzyme and reaction products either directly or by subsequent attack of ES by other reactants present in the reaction medium. Formally these two routes would correspond to the unimolecular and bimolecular mechanisms which have been proposed for the acid or base catalyzed solvolysis of esters.[6,7] In contrast, when the configuration about the asymmetric α-carbon atom is D two factors may be operative in contributing to the stability of R_3, one, the formation of EI by the interaction of all three R groups with their respective ρ centers with little or no strain, and two, an unfavorable orientation of the R_3 group in EI with respect to subsequent attack.

It is important, in a consideration of the mode of action of α-chymotrypsin, to inquire to what extent bifunctional compounds of the type $R_1CH_2R_3$ and $R_2CH_2R_3$, in which the asymmetry of the α-carbon atom is eliminated but where the optimum distance between R_3 and the remaining R group is

(1) Supported in part by a grant from Eli Lilly and Co.

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

(3) H. T. Huang and C. Niemann, THIS JOURNAL, **73**, 3223 (1951).

(4) Where R_1 = an amino or acylamino group, R_2 = the side chain of the α-amino acid, and R_3 = the carboxyl group or a functional derivative thereof.

(5) For definition of symbols, *cf.*, H. T. Huang and C. Niemann, THIS JOURNAL, **73**, 1541 (1951).

(6) *Cf.*, E. A. Braude, *Annual Reports*, **46**, 119 (1950).

(7) I. B. Wilson and D. Nachmansohn, in F. F. Nord, *Advances in Enzymology*, **12**, 302 (1951), have proposed a detailed unimolecular mechanism of hydrolysis for the system acetylcholine–acetylcholine esterase which appears to accommodate the available data on the effect of *p*H on the activity of this enzyme toward a number of its specific substrates and competitive inhibitors. For the hydroperoxidases B. Chance, *cf.*, Sumner and Myrbäck, "The Enzymes," Vol. II, Part I, Academic Press, New York, N. Y., 1951, p. 428, has definitely established the bimolecular nature of the reaction between the enzyme–substrate complexes and the donor molecules. See also C. G. Swain, *Record Chem. Prog.*, **12**, 21 (1951).

retained, can function as specific substrates of this enzyme. It has been observed that a compound of the type $R_2CH_2R_3$, *i.e.*, methyl β-phenylpropionate, where R_2 = benzyl and R_3 = carbomethoxy, can function as a specific substrate for α-chymotrypsin[8] although it also has been found that the corresponding amide, *i.e.*, β-phenylpropionamide, is resistant to hydrolysis and functions as a competitive inhibitor.[9]

Compounds of the type $R_1CH_2R_3$ are derivatives of glycine and it has been reported[10] that the glycinamide bond in glycylglycinamide and L-tyrosylglycinamide is hydrolyzed in the presence of α-chymotrypsin. However, these reactions were observed to proceed at such a low rate, in spite of the very high enzyme concentrations employed, that the above results may be questioned. We have accordingly examined, with careful controls, the effect of α-chymotrypsin on glycylglycinamide and several other derivatives of glycine and a summary of these experiments is given in Table I.

TABLE I

EFFECT OF α-CHYMOTRYPSIN ON SEVERAL GLYCINE DERIVATIVES[a]

Compound	Hydrolyzed in 24 hr., %
Glycylglycinamide	0
Aceturamide	0
Nicotinuramide	0
Hippuramide	0
Methyl aceturate	2[b]
Methyl hippurate	17 in 10 min.

[a] In aqueous media at 25° and *p*H 7.9 ± 0.1, enzyme concentration equivalent to 0.208 mg. protein nitrogen per ml., substrate concentration 20 × 10^{-3} *M*, 0.02 *M* tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer, extent of hydrolysis determined by formol titration. [b] Corrected for 13% hydrolysis in the absence of enzyme.

Of the six compounds listed in Table I only one, *i.e.*, methyl hippurate, is clearly a specific substrate of α-chymotrypsin. Furthermore, the rate of hydrolysis of this substrate is, in fact, comparable to that observed for the more rapidly hydrolyzed acylated α-amino acid amides such as nicotinyl-L-tryptophanamide[5] and nicotinyl-L-tyrosinamide.[11] Since methyl hippurate, in contrast to methyl β-phenylpropionate, is relatively soluble in aqueous media it was possible to determine the kinetics of hydrolysis of the former substrate under the same conditions used previously in our studies on the acylated α-amino acid amides.[5,11–14] However, with methyl hippurate when the 0.02 *M* tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer[5,11–14] was employed it was noted that the *p*H of the system dropped appreciably during the course of the reaction. Rather than to increase the buffer concentration and to thereby decrease the accuracy of the formol titration it was decided to study only the initial stages of the reaction, *i.e.*, less than 20% hydrolysis, since with this restriction the decrease in *p*H was never more than 0.2 of a *p*H unit. As an added precaution in the determination of initial velocities the initial *p*H of the reaction medium was adjusted to *p*H 8.0 so that the reaction *p*H could be regarded as being 7.9 ± 0.1. It should also be noted that with our experimental conditions the initial stages of the reaction were approximately zero order.

A determination of the dependency of the initial velocity upon *p*H, at a given substrate concentration, *cf.*, Fig. 1, indicated that the uncertainty caused by the slight change in *p*H during the course of the reaction was not great since the observed initial velocity was nearly constant from *p*H 7.2 to 8.3. The curve given in Fig. 1 is reminiscent of, though not identical with, those recorded previously for a number of acylated α-amino acid amides[5,11–14] in that maximum activity is observed in the region between *p*H 7.2 and 8.5, in contrast to the α-amino acid esters where the region of maximum activity is between *p*H 5.5 and 7.5.[15–17]

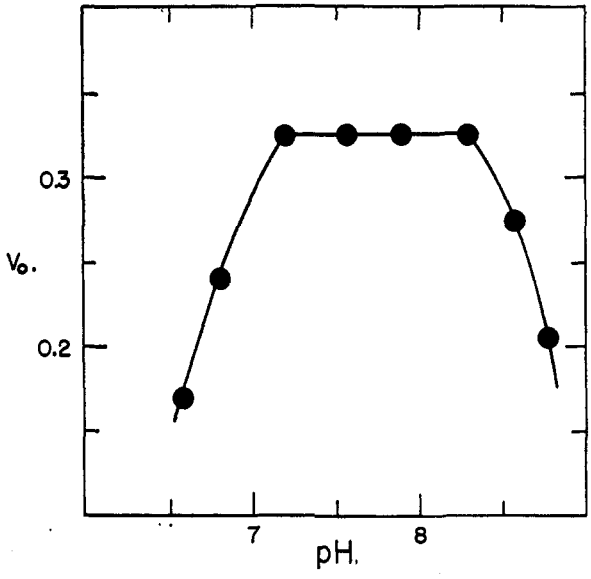

Fig. 1.—*p*H-activity relationship for α-chymotrypsin and methyl hippurate; $[S]_0$ = 20 × 10^{-3} *M*; v_0 in 10^{-3} *M* hydrolyzed per min.; [E] = 0.208 mg. protein nitrogen per ml.; all experiments conducted at 25° in aqueous solutions 0.02 *M* with respect to the amine component of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer; controls gave no indication of non-enzymatic hydrolysis at *p*H values less than 8.8.

The results of our studies on the α-chymotrypsin catalyzed hydrolysis of methyl hippurate are summarized in Fig. 2. In view of the possibility that the benzamido moiety of methyl hippurate may interact with both centers ρ_1 and ρ_2, for reasons discussed in detail elsewhere,[9] it would appear advisable to consider that methyl hippurate can

(8) J. E. Snoke and H. Neurath, *Arch. Biochem.*, **21**, 351 (1949).
(9) H. T. Huang and C. Niemann, THIS JOURNAL, **74**, Oct. (1952).
(10) J. S. Fruton and M. Bergmann, *J. Biol. Chem.*, **145**, 253 (1942).
(11) H. T. Huang, R. V. MacAllister, D. W. Thomas and C. Niemann, THIS JOURNAL, **73**, 3231 (1951).
(12) D. W. Thomas, R. V. MacAllister and C. Niemann, *ibid.*, **73**, 1548 (1951).
(13) H. J. Shine and C. Niemann, *ibid.*, **74**, 97 (1952).
(14) H. T. Huang, R. J. Foster and C. Niemann, *ibid.*, **74**, 105 (1952).
(15) E. F. Jansen, M. D. Fellow-Nutting, R. Jang and A. K. Balls, *J. Biol. Chem.*, **185**, 209 (1950).
(16) H. Goldenburg and V. Goldenburg, *Arch. Biochem.*, **29**, 154 (1950).
(17) H. Goldenburg, V. Goldenburg and A. D. McLaren, *Biochim. Biophys. Acta*, **7**, 110 (1950).

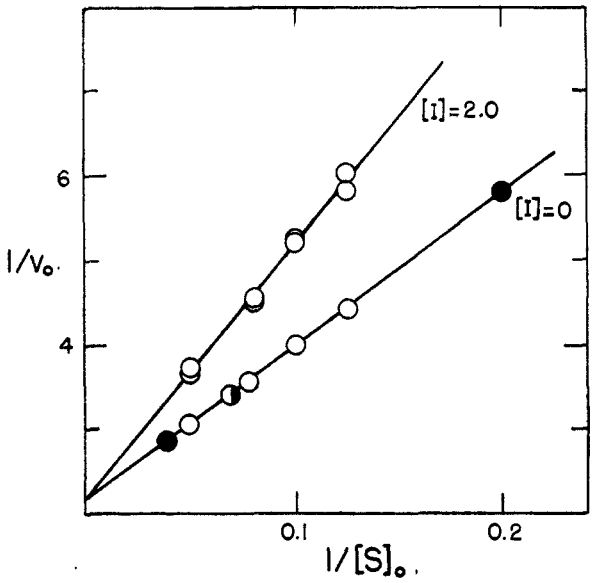

Fig. 2.—α-Chymotrypsin catalyzed hydrolysis of methyl hippurate and its competitive inhibition by acetyl-D-tryptophanamide; v_0 in 10^{-3} M per min.; $[S]_0$ and [I] in units of 10^{-3} M; [E] = 0.208 mg. protein nitrogen per ml.; 0.02 M; tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer: ●, single experiment; ◑, mean of duplicates; ○, mean of triplicates.

react with the catalytically active site of the enzyme in two distinct ways, giving rise to the complex ES when R_1 interacts with ρ_1 and R_3 with ρ_3, and ES′ when R_1 interacts with ρ_2 and R_3 does not participate in an interaction. Clearly, ES alone can undergo hydrolysis. The reaction system may therefore be formulated in terms of equations (1) and (2) where $K_S = (k_2 + k_3)/k_1$ and $K\sigma = k_3/k_4$.

$$E_f + S_f \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E_f + P_{1f} + P_{2f} \quad (1)$$

$$E_f + S_f \underset{k_5}{\overset{k_4}{\rightleftharpoons}} ES' \quad (2)$$

Assuming essentially zone A conditions[5,18,19] and that $d[S]/dt \gg d[ES]/dt$ it follows that

$$[ES] = \frac{[E][S]}{K_S + [S](1 + K_S/K\sigma)} \quad (3)$$

and

$$\frac{1}{v} = \frac{K_S}{V'} \times \frac{1}{[S]} + \frac{1}{V'}\left(1 + \frac{K_S}{K\sigma}\right) \quad (4)$$

For the $1/v_0$ *versus* $1/[S]_0$ plot[20] given in Fig. 2 the apparent intercept $1/V' = (1 + K_S/K\sigma)/V$ and the apparent Michaelis constant $K_S' = K_S K\sigma/(K_S + K\sigma)$. In the limiting case where $K_S \gg K\sigma$, then $K_S' \rightarrow K\sigma$, and where $K_S \ll K\sigma$ then $K_S' \rightarrow K_S$. From the $1/v_0$ *versus* $1/[S]_0$ plot, *cf.*, Fig. 2, it was estimated that for the system α-chymotrypsin–methyl hippurate under the conditions previously specified $K_S' = 8.5 \times 10^{-3}$ M and $k_3' = 2.2 \times 10^{-3}$ M per min. per mg. protein nitrogen per ml. It is beyond the scope of our present methods to provide any direct evidence of the existence of the complex ES′ or any notion of the relative contributions of K_S and $K\sigma$ to the above value of K_S'.

The value of K_S' of 8.5×10^{-3} M for methyl hippurate is very close to that found for the apparent enzyme–inhibitor dissociation constant K_I' of hippuramide, 11×10^{-3} M when this latter compound was studied in competition with nicotinyl-L-tryptophanamide, *cf.*, Fig. 3. The designation K_I' used above appears to be justified, since like methyl hippurate, hippuramide should also be capable of combining with the enzyme in two ways giving two dissociation constants K_I and K_i related to the two possible complexes EI, as in ES and EI′, as in ES′. K_I' is then a function K_I and K_i and is equal to $K_I K_i/(K_I + K_i)$.

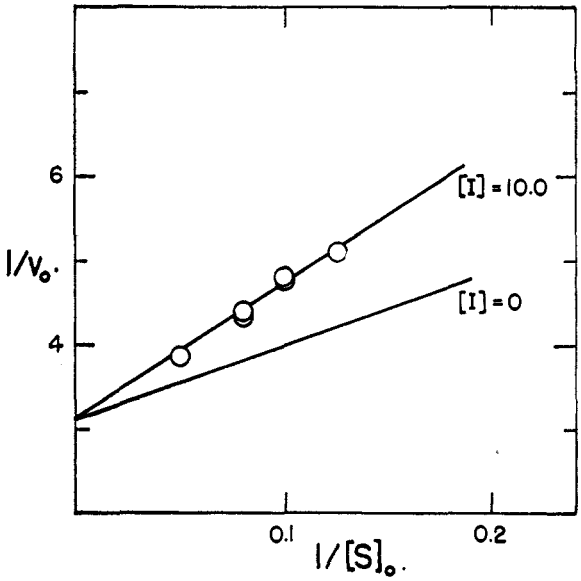

Fig. 3.—Evaluation of K_I for α-chymotrypsin and hippuramide; v_0 in 10^{-3} M per min.; $[S]_0$ in units of 10^{-3} M nicotinyl-L-tryptophanamide; [I] in units of 10^{-3} M hippuramide; [E] = 0.208 mg. protein nitrogen per ml.; 0.02 M tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer.

Aceturamide and nicotinuramide were also examined for inhibitory activity but the degree of inhibition observed, with nicotinyl-L-tryptophanamide as the specific substrate, was too small to permit a reliable determination of the respective enzyme–inhibitor dissociation constants.

The possible presence of trypsin in the α-chymotrypsin preparation used in this study led us to examine the behavior of methyl hippurate in aqueous systems containing the former enzyme. At *p*H 7.9 and 25° and with two different crystalline trypsin preparations present in a concentration equivalent to *ca.* 0.025 mg. of protein nitrogen per ml., methyl hippurate, at $[S] = 20 \times 10^{-3}$ M was hydrolyzed to the extent of 5% in one hour. In view of this result it is most unlikely that the hydrolytic reaction which is the subject of this communication can be ascribed to the presence of trypsin in the α-chymotrypsin preparation.

In order to determine whether the enzymatic

(18) O. H. Straus and A. Goldstein, *J. Gen. Physiol.*, **26**, 559 (1943).
(19) A. Goldstein, *ibid.*, **27**, 527 (1944).
(20) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).

site responsible for the hydrolysis of methyl hippurate is the same as that involved in the hydrolysis of the trifunctional acylated-L-α-amino acid amides previously investigated in these laboratories[5,11-14] the enzyme–inhibitor dissociation constants of two competitive inhibitors, *i.e.*, acetyl-D-tryptophanamide and acetyl-D-phenylalanine methyl ester, which had been determined earlier with a trifunctional acylated-L-α-amino acid amide type specific substrate,[9,14] were reëvaluated using methyl hippurate as the specific substrate.

For this latter reaction system in addition to equations (1) and (2) we have

$$E_f + I_f \underset{k_7}{\overset{k_6}{\rightleftharpoons}} EI \qquad (5)$$

which leads to equation (6)

$$\frac{1}{v} = \frac{K_S}{V}\left(1 + \frac{[I]}{K_I}\right)\frac{1}{[S]} + \frac{1}{V}\left(1 + \frac{K_S}{K\sigma}\right) \qquad (6)$$

for competitive inhibition where $K_I = k_7/k_6$. Thus for the customary $1/v_0$ *versus* $1/[S]_0$ plot the intercept is unchanged and the slope is increased by the quantity $K_S[I]/VK_I$. Substituting K_S' for $K_SK\sigma/(K_S + K\sigma)$ and V' for $V(1 + K_S/K\sigma)$ equation (6) is reduced to the conventional Lineweaver–Burk equation for competitive inhibition from which the K_I values of the trifunctional competitive inhibitors can be evaluated. The results of the two series of inhibition experiments are summarized in Figs. 2 and 4 and the K_I values obtained from these plots, *i.e.*, acetyl-D-tryptophanamide, $K_I = 2.9 \times 10^{-3}$ *M*, and acetyl-D-phenylalanine methyl ester, $K_I = 2.8 \times 10^{-3}$ *M*, are in good agreement with the previously determined values of 2.7×10^{-3} *M*[5] and 2.5×10^{-3} *M*,[14] respectively. It can therefore be concluded that one and the same catalytically active site is responsible for the hydrolysis of methyl hippurate and the previously investigated acylated-L-α-amino acid amides.[5,11-14]

If it is assumed that the effect of methanol on the kinetic constants of the system α-chymotrypsin–methyl β-phenylpropionate[8] is similar to that observed for the system α-chymotrypsin–acetyl-L-tyrosinamide[21] it is estimated that the K_S' and k_3' values for the former system in aqueous media are approximately 2×10^{-3} *M* and 2×10^{-3} *M* per min. per mg. protein nitrogen per ml., respectively. Despite the crudeness of the above extrapolation it appears that the K_S' and k_3' values for the system α-chymotrypsin–methyl β-phenylpropionate are comparable in magnitude to those observed for the system α-chymotrypsin–methyl hippurate. Furthermore, the difference in behavior of the pair methyl hippurate–hippuramide parallels that observed for the pair methyl β-phenylpropionate–β-phenylpropionamide.[8,22]

When a trifunctional specific substrate $R_1CHR_2R_3$ is compared with a bifunctional derivative thereof, whether it be $R_1CH_2R_3$ or $R_2CH_2R_3$, it is at once apparent that the susceptibility to hydrolysis of the R_3 group in the intermediate complex is greatly diminished in the case of the bifunctional specific substrate and that hydrolysis is only

(21) S. Kaufman and H. Neurath, *J. Biol. Chem.*, **180**, 181 (1949).
(22) S. Kaufman and H. Neurath, *ibid.*, **181**, 623 (1949).

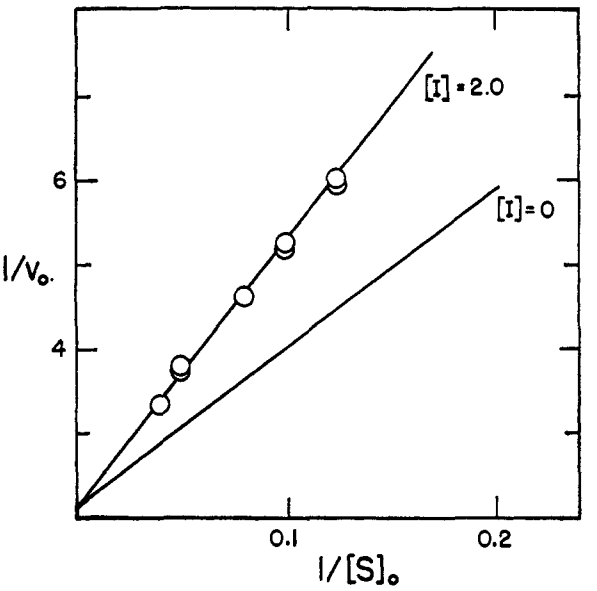

Fig. 4.—Competitive inhibition of the α-chymotrypsin catalyzed hydrolysis of methyl hippurate by acetyl-D-phenylalanine methyl ester; v_0 in 10^{-3} *M* per min.; $[S]_0$ and [I] in units of 10^{-3} *M*; [E] = 0.208 mg. protein nitrogen per ml.; 0.02 *M* tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer.

observed when the two contributary factors, *i.e.*, an inherent high reactivity of the R_3 group and a high affinity of the enzyme for the remaining R group, are both operative at favorable levels. That the susceptibility to hydrolysis of the above types of bifunctional specific substrates is not suppressed to the point of apparent non-reactivity as is the case with the intermediate complexes of the trifunctional D-competitive inhibitors, which are enantiomorphs of the trifunctional L-specific substrates, seems to us to be an observation of sufficient importance to warrant closer examination.

From purely steric considerations it appears reasonable to expect that the two R groups of a bifunctional specific substrate should be capable of interacting with their respective complementary centers present at the catalytically active site of the enzyme with greater ease than would be possible for the same two R groups present in a trifunctional specific substrate where the spatial orientation associated with the interaction of the remaining R group must be simultaneously satisfied. Thus, the reduced susceptibility to hydrolysis of the intermediate complexes formed from bifunctional specific substrates as compared with those formed from the analogous trifunctional specific substrates is consistent with the concept expressed earlier[3,9] that a strain centered in the R_3–ρ_3 interaction is in part responsible for the subsequent transformation of the enzyme–substrate complex into enzyme and reaction products. However, similar considerations indicate that the R_3–ρ_3 interaction in a complex formed from a trifunctional-D-competitive inhibitor must necessarily be in a state of strain greater than, or in the limiting case, equal to, the same interaction present in a complex formed from the analogous bifunctional specific substrate or competitive inhibitor. Therefore, the stability of

the complexes formed from the trifunctional-D-competitive inhibitors, in spite of their frequent higher free energy of formation than those derived from the corresponding enantiomorphic L-specific substrates,[3,12,14] is probably largely due to an inaccessibility of the R_3 group present in the former complexes to subsequent attack. Conversely, the above argument can be extended to imply that in the actual mechanism of hydrolysis, a bimolecular reaction between the intermediate complex of an L-specific substrate and non-specific reactants such as water molecules or hydroxyl ion, rather than a unimolecular breakdown of the complex, is to be preferred.

The fact that methyl hippurate is hydrolyzed in the presence of α-chymotrypsin may be taken as an indication that suitable derivatives of other α-amino acids, which do not possess the side chains commonly associated with specific substrates of this enzyme,[23] may also exhibit specific substrate activity. Thus it is entirely within reason that the ethyl esters of benzoyl-L-norleucine[24] and benzoyl-L-norvaline[24] and the methyl ester of benzoyl-L-arginine[25] are slowly hydrolyzed in the presence of α-chymotrypsin.[26] Furthermore, in the light of the present discussion, it is conceivable that peptide bonds present in a protein molecule, which are not associated with the carboxyl groups of tryptophan, tyrosine, phenylalanine or methionine residues, but which are in a sense activated by a favorable acyl moiety, may undergo an α-chymotrypsin catalyzed hydrolysis. Thus the observed hydrolysis, in the presence of α-chymotrypsin, of polylysine,[27] certain malonylated proteins,[28] and salmine[29] are readily comprehensible in terms of a proper understanding of the role of both the R_1 and R_2 groups in influencing the susceptibility of a peptide bond to an α-chymotrypsin catalyzed hydrolysis.

(23) H. Neurath and G. W. Schwert, *Chem. Revs.*, **46**, 69 (1950).

(24) Unpublished experiments of J. E. Snoke, cited in ref. 23.

(25) G. W. Schwert, H. Neurath, S. Kaufman and J. E. Snoke, *J. Biol. Chem.*, **172**, 221 (1948).

(26) It should be noted that these observations refer to systems containing methanol. In an aqueous system the rates would be expected to be considerably higher.

(27) E. Brand and E. Katchalski, cited by E. Katchalski in M. L. Anson and J. T. Edsall, "Advances in Protein Chemistry," Academic Press, Inc., New York, N. Y., 1951, pp. 6, 162.

(28) W. F. Ross and A. H. Tracey, *J. Biol. Chem.*, **145**, 19 (1942).

Experimental[30,31]

Substrates and Inhibitors.—Those specific substrates and competitive inhibitors whose preparation had not been described in earlier communications from these laboratories were prepared by standard methods. Their properties are given in Table II.

TABLE II

PROPERTIES OF GLYCINE DERIVATIVES

Compound	M.p., °C.	Analyses, % Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found
Glycylglycinamide acetate	130–134	54.3	54.4	5.7	5.8
Aceturamide	129–130	41.4	41.5	6.9	6.9
Nicotinuramide	192–193	53.7	53.8	5.1	5.1
Hippuramide	182–183	60.7	60.6	5.7	5.7
Methyl aceturate	57–58	45.8	45.9	6.9	7.0
Methyl hippurate	82–83	62.2	62.3	5.7	5.7

Enzyme Experiments.—The methods used in this study were similar to those described previously[5] and were modified only to accommodate the very much shorter reaction period, *i.e.*, 10–12 min., employed in this investigation. The initial 1 min. aliquot was collected as described[5] and subsequent aliquots were withdrawn at 2-min. intervals. The aliquots were then titrated in the order in which they were collected. The *p*H of the reaction system was checked at the beginning and end of each run and in the experiments summarized in Table I suitable controls were provided. The α-chymotrypsin used, lot no. 90402, was an Armour preparation. The K_S value for nicotinyl-L-tryptophanamide, which was used in several of the inhibition experiments was taken to be 2.7×10^{-3} *M*. It will be noted that the specific enzyme concentrations $[E_S']$ and $[E_T']$ for all experiments herein reported were such as to provide essentially zone A conditions in all cases. The crystalline trypsin preparations were obtained from Armour and from Kuster.

PASADENA 4, CALIFORNIA

(29) R. A. Portis and K. I. Altman, *ibid.*, **169**, 203 (1947).

(30) All melting points are corrected.

(31) Microanalyses by Dr. A. Elek.