The α-Chymotrypsin-Catalyzed Hydrolysis of a Series of Analogs of Acetylglycine Methyl Ester*

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Eighteen compounds, many of them analogs of acetylglycine methyl ester, have been examined with respect to their hydrolysis by α-chymotrypsin in aqueous solutions at 25°, *p*H 7.90 and 0.50 M with respect to sodium chloride. While it was possible to evaluate individual kinetic constants in only seven cases, in others the lack of reactivity provided useful information concerning the relation between structure and substrate activity. These observations have been interpreted in terms of a theory proposed earlier to explain the structural and stereochemical specificity of α-chymotrypsin.

In a previous communication (Wolf and Niemann, 1963) an account was given of the kinetics of the α-chymotrypsin-catalyzed hydrolysis of twenty-two acylated glycine methyl esters, in aqueous solutions at 25.0°, *p*H 7.90 and 0.50 M with respect to sodium chloride. Furthermore, these data were interpreted in terms of a theory developed by Hein and Niemann (1961, 1962) to explain the structural and stereochemical specificity of α-chymotrypsin for trifunctional substrates of this enzyme. With the development of an understanding of the behavior of both bi- and trifunctional substrates it became evident that further tests of the theory were desirable as well as its extension into areas not probed by previously investigated substrates. In this communication we wish to describe the behavior of eighteen compounds which, for the most part, may be regarded as analogs of acetylglycine methyl ester and which extend our knowledge of the mechanism of action of α-chymotrypsin.

EXPERIMENTAL

All melting points are corrected. Microanalyses were performed by Dr. A. Elek.

Formylglycine Methyl Ester.—A slight excess of anhydrous ammonia in cold chloroform was added to a suspension of glycine methyl ester hydrochloride in the same solvent, the ammonium chloride was removed by filtration, and the filtrate was evaporated to near dryness *in vacuo* at the lowest temperature compatible with removal of solvent. The residual amino acid ester was then acylated with formic acid and acetic anhydride (du Vigneaud *et al.*, 1932) and the crude product distilled at 88° and 0.15 mm.

Anal. Calcd. for $C_4H_7O_3N$ (117): C, 41.0; H, 6.0; N, 12.0. Found: C, 41.2; H, 6.2; N, 11.8.

Acetylglycine Ethyl Ester.—To an ice-cold solution of 30 g of glycine ethyl ester hydrochloride in 50 ml of water was added 25 ml of an ice-cold solution of 17.2 g of sodium hydroxide in 50 ml of water. To the solution of the ester, contained in an ice-salt bath, was added 22 ml of acetic anhydride in four equal portions. In each instance one-fourth of the remaining sodium hydroxide solution was added immediately after the addition of each portion of the anhydride. The final reaction mixture was adjusted to *p*H 7 with aqueous sodium carbonate and extracted with four 100-ml portions of ethyl acetate, the ethyl acetate extracts were combined and dried, the solvent was evaporated *in vacuo*, and the oily residue was distilled at 90° and 0.3 mm to give, in 70% yield, a product which crystallized on standing. Recrystallization from diisopropyl ether gave acetylglycine ethyl ester, m.p. 47.0–48.5°. Radenhausen (1895) reports m.p. 48°.

Benzoylglycine Glycolamide Ester.—Benzoylglycine cyanomethyl ester, m.p. 102.6–103.6°, 9 g, was prepared from 10.7 g of hippuric acid (Schwyzer *et al.*, 1955). To convert the cyanomethyl ester to the glycolamide ester (Kerr and Niemann, 1958), 7.5 g of the cyanomethyl ester was dissolved in an anhydrous mixture of 75 ml of dioxane, 25 ml of ethyl ether, and 2.0 ml of ethanol, and the solution was cooled in an ice-salt bath and then saturated with dry hydrogen chloride. The reaction mixture, contained in a flask with a wired-on stopper, was stored at 4° for 18 hours, the ether was removed by distillation *in vacuo*, and the residual solution was heated on a steam bath for 20 minutes. The dioxane was removed *in vacuo*, and the solid residue was recrystallized twice from water, with decolorization with Nuchar, to give 4.6 g (57%) of the glycolamide ester, m.p. 124.1–125.0°.

Anal. Calcd. for $C_{11}H_{12}O_4N_2$ (236): C, 55.9; H, 5.1; N, 11.9. Found: C, 56.1; H, 5.2; N, 11.8.

Acetyl - N - methylglycine Methyl Ester.—Sarcosine methyl ester hydrochloride, prepared from sarcosine, methanol, and thionyl chloride (Brenner and Huber, 1953), was acylated with acetyl chloride by the procedure used by Wolf and Niemann (1963) for the preparation of propionylglycine methyl ester and analogous compounds. The crude product, obtained as a light-colored oil, was distilled to give acetyl-*N*, methylglycine methyl ester, b.p. 61° (0.11 mm), n_D^{25} = 1.4490, in 50% yield.

Anal. Calcd. for $C_6H_{11}O_3N$ (145): C, 49.6; H, 7.6; N, 9.6. Found: C, 49.5; H, 7.6; N, 9.6.

Acetyl-L-alanine Methyl Ester.—L-Alanine was esterified with methanol and thionyl chloride (Brenner and Huber, 1953). The ester hydrochloride was acetylated with acetic anhydride and dilute aqueous sodium hydroxide, as described for the preparation of acetylglycine ethyl ester, the reaction product was extracted into ethyl acetate, the extract was dried, and the solvent was removed *in vacuo*, to give a light-colored oil. This oil was distilled to give 55% of acetyl-L-alanine methyl ester, b.p. 76° (0.25 mm), $[\alpha]_D^{23} = -91.7°$ (*c*, 2% in water).

Anal. Calcd. for $C_6H_{11}O_3N$ (145): C, 49.6; H, 7.6; N, 9.6. Found: C, 49.8; H, 7.7; N, 9.6.

Acetyl - D - alanine Methyl Ester.—Acetyl - DL-alanine was esterified by the procedure of Brenner and Huber (1953). Thirty g of *N*-acetyl-DL-alanine methyl

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ester was added to a solution of 100 mg of crystalline α-chymotrypsin, 2.9 g of sodium chloride, and 6.8 g of sodium bicarbonate in 200 ml of distilled water contained in a loosely stoppered flask. The reaction mixture was held at room temperature for 48 hours and then extracted three times with 150-ml portions of ethyl acetate. The extracts were combined and dried, and the solvent was removed *in vacuo*. The residue, obtained in 60% yield, was recrystallized from diisopropyl ether. The product melted too close to room temperature for the m.p. to be determined in a conventional apparatus. The crystals recovered from the cold suspension were collected on a chilled filter and washed twice with hexane to give a product, $[\alpha]_D^{25} = 89.4°$ (*c*, 2% in water) which melted on standing at room temperature.

Methyl α-Acetamidoacrylate.—This compound was prepared in 50–60% yield from diethyl acetamidomalonate essentially as described by Hellmann *et al.* (1958).

Anal. Calcd. for $C_6H_9O_3N$ (143): C, 50.4; H, 6.3; N, 9.8. Found: C, 50.3; H, 6.3; N, 9.5.

Methyl α-Acetamidoisobutyrate.—α - Aminoisobutyric acid was esterified with methanol and thionyl chloride (Brenner and Huber, 1953). The ester hydrochloride, obtained in nearly quantitative yield, was acylated with acetic anhydride in chloroform containing two equivalents of triethylamine to give the crude product in 90% yield. Two recrystallizations from diisopropyl ether gave methyl α-acetamidoisobutyrate, m.p. 100.0–100.5°.

Anal. Calcd. for $C_7H_{13}O_3N$ (159): C, 52.8; H, 8.2; N, 8.8. Found: C, 52.7; H, 8.2; N, 8.8

Methyl β-Acetamidopropionate.—β-Alanine was esterified with methanol and thionyl chloride (Brenner and Huber, 1953), and the ester hydrochloride was acylated with acetyl chloride in toluene by the procedure used earlier for the preparation of propionylglycine methyl ester (Wolf and Niemann, 1963). The crude product, obtained in 85% yield, was distilled to give the final product, b.p. 93.6° (0.30 mm), $n_D^{25} = 1.4537$.

Anal. Calcd. for $C_6H_{11}O_3N$ (145); C, 49.6; H, 7.6; N, 9.6. Found: C, 49.5; H, 7.6; N, 9.6.

Methyl Levulinate.—A Harshaw preparation was redistilled prior to use.

Methyl N-Phenylglycinate.—*N*-Phenylglycine was esterified with methanol and thionyl chloride (Brenner and Huber, 1953), the ester hydrochloride was taken up in cold water saturated with sodium chloride and sodium carbonate, the solution was extracted several times with benzene, the benzene extracts were combined and dried over anhydrous sodium sulfate, and the solvent was removed to give a crude oily product. The latter material was taken up in aqueous ethanol to give a crystalline product which was recrystallized twice from the same solvent to give the desired ester, m.p. 48.1–49.0°, in 60% yield. Meyer (1875) reports m.p. 48°.

O-Acetyl-N-methylolbenzamide.—To a solution of 0.6 g of potassium carbonate in 20 ml of water was added 14 ml of 37% formalin solution. The mixture was brought to 35°, and 21 g of benzamide was added in 3-g portions. When all solid had dissolved the solution was cooled to room temperature. Crystallization was induced by scratching and allowed to proceed with occasional stirring. When crystallization appeared to be complete the suspension was placed in an ice-salt bath, 50 ml of ice-cold distilled water was added, and the solid was collected, washed three times with cold distilled water, and dried *in vacuo*. To a solution of 5 g of the preceding *N*-methylolamide in 50 ml of chloroform was added 10 ml of pyridine, and the mixture was cooled in an ice-salt bath. To the cold solution was added slowly 2 ml of acetyl chloride, and the reaction mixture was allowed to come to room temperature. After standing for one-half hour the reaction mixture was washed three times with cold distilled water. The solvent was removed from the chloroform phase and the residue was recrystallized twice from diisopropyl ether to give *O*-acetyl-*N*-methylolbenzamide, m.p. 67.8–68.8°.

Anal. Calcd. for $C_{10}H_{11}O_3N$ (193): C, 62.1; H, 5.7; N, 7.3: Found: C, 62.2; H, 5.7; N, 7.3.

L-Pyrrolidonecarboxylic Acid Methyl Ester.—L-Glutamic acid was heated at 165–170° and 0.20 mm for 90 minutes. The product was cooled, powdered, and esterified with methanol and thionyl chloride (Brenner and Huber, 1953). The crude product was distilled to give L-pyrrolidonecarboxylic acid methyl ester, b.p. 113° (0.2 mm), $[\alpha]_D^{25} = 0.91°$ (*c*, 2% in water).

Anal. Calcd. for $C_6H_9O_3N$ (143): C, 50.3; H, 6.3; N, 9.8. Found: C, 50.4; H, 6.3; N, 9.8.

dl-Malic Acid Dimethyl Ester.—Esterification of *dl*-malic acid with methanol and thionyl chloride gave the crude diester which was then distilled to give the final product, b.p. 89° (1.6 mm).

Anal. Calcd. for $C_6H_{10}O_5$ (162): C, 44.4; H, 6.2. Found: C, 44.5; H, 6.4.

l-Malic Acid Dimethyl Ester.—This ester, $[\alpha]_D^{25} = -8.33°$ (*c*, 3% in methanol) was prepared as described for the *dl*-ester.

meso-Tartaric Acid Dimethyl Ester.—Esterification of *meso*-tartaric acid with methanol and thionyl chloride gave the diester, m.p. 112.5–113.1°, after recrystallization from a mixture of methanol and diisopropyl ether.

d-Tartaric Acid Dimethyl Ester.—This ester was prepared as described for the *meso*-diester except that the product was distilled at 95–96° and 0.20 mm prior to recrystallization from a mixture of methanol and diisopropyl ether. The final product, m.p. 48.9–49.7°, $[\alpha]_D^{25} = 4.0°$ (*c*, 3% in methanol) was hygroscopic.

l-Tartaric Acid Dimethyl Ester.—This ester, m.p. 49.0–49.9°, $[\alpha]_D^{25} = -4.5°$ (*c*, 3% in methanol) was prepared as described for the *d*-ester.

Kinetic Studies.—All experiments were conducted in aqueous solutions at 25.0 ± 0.1°, *p*H 7.90 ± 0.02 and 0.50 M with respect to sodium chloride. The procedure, which involved use of a *p*H-stat, was identical in all respects to that used previously for evaluation of the kinetic constants of the series of acylated glycine methyl esters (Wolf and Niemann, 1963). As before, where it was possible to determine the reaction kinetics the rates of all enzyme-catalyzed reactions could be described by equation (1). In no case was the maximum extent of reaction more than 10%.

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

Results

All results are summarized in Table I. In a number of cases it was not possible to evaluate the kinetic constants. There are several reasons why this situation arose. We shall consider them individually.

The base-catalyzed hydrolysis of formylglycine methyl ester is much more rapid then that of acetylglycine methyl ester. When an attempt was made to examine the enzyme-catalyzed hydrolysis of the former compound it became apparent that at *p*H 7.90 the contribution of the base-catalyzed hydrolysis was so overwhelming relative to the enzyme-catalyzed hydrolysis that the latter could not be evaluated. We can only conclude that the rate of the enzyme-catalyzed hydrolysis is probably less than that of acetylglycine methyl ester, a conclusion consistent

TABLE I

α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF A SERIES OF ANALOGS OF ACETYLGLYCINE METHYL ESTER[a]

Substrate	$[S]_0$[b] (mM)	K_0 (mM)	k_0[c] (sec.$^{-1}$)	k_0/K_0 (M^{-1} sec.$^{-1}$)	k_B[d] (M^{-1} sec.$^{-1}$)
Formylglycine methyl ester	5.0– 30.1	Too unreactive to evaluate[e]			
Acetylglycine ethyl ester	15.0– 50.0	96 ± 5	0.013 ± 0.001	0.135	1.13
Benzoylglycine glycolamide ester	10.0– 25.0	Too reactive to evaluate[f]			19.88
Acetyl-*N*-methylglycine methyl ester	10.5– 52.7	Too unreactive to evaluate			1.12
Acetyl-L-alanine methyl ester	5.7–330.8	611 ± 10	1.29 ± 0.02	2.11	1.47
Acetyl-D-alanine methyl ester	5.4– 27.1	Too unreactive to evaluate			—
Methyl α-acetamidoacrylate	10.0– 25.0	Too unreactive to evaluate			1.73
Methyl α-acetamidoisobutyrate	5.0	Too unreactive to evaluate			—[g]
Methyl β-acetamidopropionate	5.6– 33.9	Too unreactive to evaluate			0.43
Methyl levulinate	10.4– 26.0	31.3 ± 6.3	0.015 ± 0.001	0.479	0.87
N-Phenylglycine methyl ester	0.3– 1.82	0.30 ± 0.04	0.0076 ± 0.0002	28.00	1.27
O-Acetyl-*N*-methylolbenzamide	—	Too unstable to evaluate[h]			—
L-Pyrrolidonecarboxylic acid methyl ester	5.6– 33.7	First order in [*S*]		1.09	24.0
dl-Malic acid dimethyl ester	1.4– 8.4	First order in [*S*]		7.36	7.00
l-Malic acid dimethyl ester	1.2– 7.2	First order in [*S*]		11.62	—
meso-Tartaric acid dimethyl ester	5.0– 30.0	42.8 ± 4.3	0.031 ± 0.003	0.724	1.10
d-Tartaric acid dimethyl ester	5.0– 30.0	54.8 ± 5.5	0.035 ± 0.003	0.639	1.75
l-Tartaric acid dimethyl ester	5.0– 30.0	102 ± 10	0.677 ± 0.034	6.64	—

[a] In aqueous solutions at 25.0 ± 0.1°, *p*H 7.90 ± 0.02 and 0.50 M with respect to sodium chloride, with $[E] = 3.85–4.17 \times 10^{-5}$ M unless otherwise noted. [b] A minimum of 6 values of [*S*] within the limits indicated were employed to evaluate K_0 and k_0. [c] Based upon a molecular weight of 25,000 and a nitrogen content of 16.5% for α-chymotrypsin. [d] The constant k_B is the second-order rate constant for the equation $v = k_B[S][OH]^*$ where $[OH]^*$ is the activity rather than the concentration of hydroxide ion. [e] At *p*H 7.90 the base-catalyzed hydrolysis is too rapid to permit detection of an enyzme-catalyzed reaction. [f] Reaction too fast to evaluate constants even when enzyme concentration was reduced to *ca.* 4×10^{-6} M. [g] Base-catalyzed hydrolysis too slow for evaluation of k_B under conditions used for evaluation of other substrates. [h] Compound rapidly decomposed at *p*H 7.90.

with the trend noted previously for a series of acylated glycine methyl esters of the type $RCONHCH_2CO_2CH_3$ where R is an alkyl group (Wolf and Niemann, 1963).

Acetyl-L-phenylalanine glycolamide ester is known to be a very reactive substrate of α-chymotrypsin (Kerr and Niemann, 1958; Applewhite *et al.*, 1958). Thus, in a search for reactive substrates within the glycine series attention was directed to benzoylglycine glycolamide ester. However, two factors, a rapid base-catalyzed hydrolysis and an exceptionally fast enzyme-catalyzed reaction for a glycine derivative, prevented evaluation of the kinetic constants of the latter substrate under the conditions employed in this study. Since it is clear that benzoylglycine glycolamide ester is a very reactive substrate and a readily accessible prototype for others of the general formula $R_1'CONHCHR_2CO_2CHR_2'CONH_2$, further study of the former compound is contemplated.

Acetyl-*N*-methylglycine methyl ester, acetyl-D-alanine methyl ester, methyl α-acetamidoacrylate, methyl α-acetamidoisobutyrate, and methyl β-acetamidopropionate were too unreactive to evaluate as substrates under the conditions specified in Table I. It is probable that their values of k_0/K_0 are less than 0.01 M^{-1} sec.$^{-1}$, a value indicative of a very low order of reactivity.

The constant for the base-catalyzed hydrolysis of L-pyrrolidonecarboxylic acid methyl ester is *ca.* nine times that of acetylglycine methyl ester and probably arises because of a neighboring group stabilization of an intermediate in the reaction. This factor and the relatively low reactivity of L-pyrrolidonecarboxylic acid methyl ester as a substrate in the enzyme-catalyzed reaction, as judged by the magnitude of the ratio k_0/K_0, led us to abandon further work with this compound. With the dimethyl esters of *dl*- and *l*-malic acid it appears that large values of K_0 will make it difficult to evaluate K_0 and k_0 for these substrates because of a relatively rapid non-enzyme-catalyzed hydrolysis and the need for high substrate concentrations.

DISCUSSION

The behavior of acetylglycine ethyl ester, $K_0 = 96$ mM, $k_0 = 0.013$ sec.$^{-1}$, is best compared with that of acetylglycine methyl ester, $K_0 = 30.7$ mM, $k_0 = 0.013$ sec.$^{-1}$ (Wolf and Niemann, 1963). The observation that the k_0 values of these two substrates are identical whereas the K_0 value of the former is three times that of the latter suggests that the K_0 values are dominated by dissociation constants of the nonproductive enzyme-substrate complexes and that those of the productive complexes are substantially greater than the determined values of K_0. The preceding data are also compatible with the supposition that with these two substrates an acyl-enzyme intermediate is involved and that, with both, the rate-determining step is deacylation of the enzyme (Bender, 1962; Zerner and Bender, 1963).

In a recent communication Nelson *et al.* (1962) described the kinetics of the α-chymotrypsin-catalyzed hydrolysis of methyl, ethyl, propyl, isopropyl, and *n*-butyl benzoylglycinate in aqueous solutions at 25°, *p*H 7.05 ± 0.05, and 4 mM with respect to a tris(hydroxymethyl)aminomethane–hydrochloric acid buffer. The only point of reference to other work is found in comparison of the values reported by Nelson *et al.* (1962) for methyl benzoylglycinate, $K_0 = 3.23$ mM, $k_0 = 0.125$ sec.$^{-1}$, with those noted by Gordon (1959) for the same substrate in aqueous solutions at 25.0°, *p*H 7.00 and 0.02 M with respect to sodium chloride, $K_0 = 4.76$ mM, $k_0 = 0.122$ sec.$^{-1}$.

Nelson *et al.* (1962) report values of K_0 for methyl, ethyl, propyl, isopropyl, and *n*-butyl benzoylglycinate of 3.23, 2.31, 1.90, 2.05, and 1.11 mM, respectively, and of k_0 of 0.125, 0.098, 0.127, 0.048, and 0.173 sec.$^{-1}$, respectively.

The behavior of the propyl and isopropyl esters is

consistent with the view that, when these compounds are at the active site of the enzyme, attack on the carboalkoxy group by a nucleo- or electrophilic group at the site is blocked to a greater degree by an isopropyl than by a propyl group (Hein and Niemann, 1961, 1962; Waite and Niemann, 1962), hence the lower value of k_0 for the isopropyl derivative. It is tempting to regard the lower value of K_0 for the propyl ester as confirmation of the idea that the dissociation constants of the productive enzyme-substrate complexes are being determined in part by a COR_{β}–ρ_3 interaction (Hein and Niemann, 1961, 1962). However, a more realistic appraisal leads to the conclusion that the values of K_0 for the propyl and isopropyl esters are identical within the limits of experimental error and that the overriding interaction is that of the benzamido group with the ρ_2 locus leading to predominantly nonproductive combination.

In the same vein we do not believe the difference in the values of k_0 for methyl, ethyl, and propyl benzoylglycinate to be significant. Thus, these compounds parallel the behavior of methyl and ethyl acetylglycinate noted above in that within each series the value of k_0 is substantially invariant. However, the value of K_0 of ethyl acetylglycinate is 3.1 times that of methyl acetylglycinate, whereas that of methyl benzoylglycinate is 1.4 times that of ethyl benzoylglycinate. Thus, with the benzoyglycine esters the trend is toward decreasing values of K_0 with increasing chain length of the ester component, whereas with acetylglycine esters it is in the opposite direction, at least for the first two members of the series.

Application of the criterion developed by Bender and his co-workers (Bender, 1962; Zerner and Bender, 1963[1]), and used above for identification of deacylation of an acyl-enzyme intermediate as the rate-determining step in the hydrolysis of the methyl and ethyl esters of acetylglycine, would require the strained conclusion that with the esters of benzoylglycine deacylation of an acyl-enzyme intermediate is rate limiting for the methyl, ethyl, and *n*-propyl esters but not for the isopropyl and *n*-butyl esters.

Methylation of the amido nitrogen atom of α-*N*-acetyl-L-tyrosine methyl ester converts a very reactive substrate into one with a very low order of reactivity (Peterson, R., 1962, unpublished results obtained in these laboratories). Although acetylglycine methyl ester is less reactive than α-*N*-acetyl-L-tyrosine methyl ester by many orders of magnitude, methylation of the amido nitrogen atom of the former substrate again leads to a diminution in substrate activity, this time to a point where it is essentially indeterminable.

The kinetics of the α-chymotrypsin-catalyzed hydrolysis of acetyl-D- and L-alanine methyl ester was investigated because of the close relationship of these compounds to acetylglycine methyl ester. A striking feature of the behavior of acetyl-L-alanine methyl ester, K_0 = 611 mM, k_0 = 1.29 sec.$^{-1}$, relative to that of acetylglycine methyl ester, K_0 = 30.7 mM, k_0 = 0.013 sec.$^{-1}$, is that a hundredfold increase in the value of k_0 for the former substrate is associated with a twenty-fold increase in the value of K_0. It is this observation that leads us to the conclusion that the value of K_0 for acetylglycine methyl ester is dominated by the dissociation constants of nonproductive enzyme-substrate complexes. It also provides support for the view that, with substrates containing a common hydrolyzable group, values of k_0 are largely determined by the effectiveness of orientation of the substrate at the active site and that in passing from a bi- to a trifunctional substrate replacement of one of the α-hydrogen atoms of acetylglycine methyl ester by a group as small as a methyl group greatly enhances orientation of the substrate in the productive enzyme-substrate complex.

The observation that acetyl-D-alanine methyl ester is too unreactive to evaluate as a substrate whereas this is not the case for benzoyl-D-alanine methyl ester (Hein and Niemann, 1961, 1962) is readily explicable. With the latter substrate and its L-antipode, a dominant R_1–ρ_2 interaction leads to a low order of reactivity for D- and L-antipodes and, for both, enzyme-substrate complexes with relatively low dissociation constants. The R_1–ρ_2 interaction, which is an important feature of the benzoyl derivatives, is relatively unimportant with the acetyl derivatives. Hence, with these latter compounds, the dissociation constants of the enzyme-substrate complexes are, or are expected to be, very large and the D-antipode rarely finds itself in an orientation that leads to products. With an anticipated high value of K_0, or K_I, and a very low value of k_0, it is not surprising that acetyl-D-alanine methyl ester is too unreactive to evaluate as a substrate under the conditions used.

Methyl α-acetamidoacrylate was examined because of its relationship to acetyl-D- and L-alanine methyl ester and acetylglycine methyl ester. We have noted previously that the reactivity of acetyl-L-alanine methyl ester is largely a consequence of the orienting influence of the α-methyl group. It will also be recalled that the dissociation constant of the enzyme-substrate complex appears to be very large. With methyl α-acetamidoacrylate we again expect a very large dissociation constant. However, with this compound the tetrahedral disposition of groups about the α-carbon atom found in acetyl-L-alanine methyl ester is replaced by a planar array and with methyl α-acetamidoacrylate the carbomethoxy group is unable to approach the ρ_3 locus at the active site and consequently is not hydrolyzed.

The lack of reactivity of methyl α-acetamidoisobutyrate establishes the fact that the decrease in values of k_0 associated with the placing of an alkyl group in the ρ_H position, previously observed with α-*N*-acyl-α-methyl aromatic α-amino acid esters (Almond *et al.*, 1962), also obtains in the case of α-*N*-acyl-α-methyl aliphatic α-amino acid esters. There is little doubt that the inertness of methyl α-acetamidoisobutyrate as a substrate arises from a very high K_0 value and a very low k_0 value.

The observation that acetyl-*N*-methylglycine methyl ester and methyl β-acetamidopropionate are too unreactive to evaluate as substrates suggests that with substrates of the type $R_1'CONHCH_2CO_2CH_3$ the group $R_1'CONH$ in the α-position is a critical structural feature. The behavior of the next compound listed in Table I destroys this illusion. The kinetic constants of methyl levulinate, K_0 = 31.3 mM, k_0 = 0.015 sec.$^{-1}$, are indistinguishable from those of acetylglycine methyl ester, K_0 = 30.7 mM, k_0 = 0.013 sec.$^{-1}$, despite replacement of the amido NH group in the latter compound by a methylene group in the former. Since the behavior of the methyl esters of other γ-keto acids is currently under investigation, discussion of this class of compounds will be deferred until these studies are completed.

Methyl *N*-phenylglycinate may be viewed as an analog of benzoylglycine methyl ester from which the α-acylamino carbonyl group has been abstracted. This structural change leads to about a 25-fold decrease in the value of both K_0 and k_0 and reduces the latter value to a point where it is comparable to that of phenylacetylglycine methyl ester or one-half of that of

[1] The authors are indebted to Drs. Zerner and Bender for a prepublication copy of this manuscript.

acetylglycine methyl ester (Wolf and Niemann, 1963). It was concluded earlier (Hein and Niemann, 1962; Wolf and Niemann, 1963) that combination of benzoylglycine methyl ester with the active site of α-chymotrypsin is dominated by the formation of nonproductive enzyme-substrate complexes and that the dissociation constant of the productive complex is so large that it has little or no influence on the magnitude of K_0. This conclusion is applicable, with even more force, to the case of *N*-phenylglycine methyl ester. The trio benzoylglycine methyl ester, phenylacetylglycine methyl ester, and *N*-phenylglycine methyl ester provide an instructive set of results. The values of k_0 for the latter two compounds, 0.006 and 0.0076 sec.$^{-1}$, are nearly equivalent and substantially smaller than the first, 0.200 sec.$^{-1}$, presumably because in the productive complexes the carbomethoxy group of phenylacetylglycine methyl ester finds itself beyond the position of optimum interaction with the ρ_3 locus whereas that of *N*-phenylglycine methyl ester falls short of reaching this position. The interesting point is that the structural change that leads to a less reactive substrate in phenylacetylglycine methyl ester has little or no effect, relative to benzoylglycine methyl ester, on the ability of the former molecule to combine with the active site in a nonproductive mode since the K_0 values of both are essentially identical (Wolf and Niemann, 1963). In contrast, the structural modification that leads to a less reactive substrate in *N*-phenylglycine methyl ester enhances the ability of this molecule to combine with the active site in a nonproductive mode to a point where its value of K_0, 0.30 mM, is one of the lowest observed for a benzenoid compound (Wallace, R. A., and Kurtz, A. N., 1962, unpublished results obtained in these laboratories). It is this property that has led us to examine other α-*N*-aryl-α-amino acid esters in a study that is currently in progress.

O-Acetyl-*N*-methylolbenzamide is a structural isomer of benzoylglycine methyl ester in which the position of the potentially hydrolyzable bond has been transposed. Unfortunately the former compound proved to be so unstable in aqueous solutions at *p*H 7.90 that it was impossible to examine its behavior as a potential substrate of α-chymotrypsin.

The last three compounds listed in Table I are not related to acetylglycine methyl ester. They were of interest because of our desire to obtain a pair of readily available D- and L-enantiomers which were not structurally related to the acylated α-amino acid esters but whose hydrolysis by α-chymotrypsin proceeded asymmetrically. It is evident from the data given in Table I that dimethyl *d*- and *l*-tartrate [dimethyl 2(R),3(R)- and 2(S),3(S)-dihydroxysuccinate] are such a pair. Furthermore, their kinetic properties indicate their usefulness as probes for the further definition of the stereospecificity of α-chymotrypsin. We believe it premature to try to interpret the behavior of these compounds and that of the *meso*-derivative until it can be shown, through inhibition studies, that these substrates are hydrolyzed at the same site involved in the hydrolysis of more representative substrates of this enzyme, however probable this may appear at the present time.

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